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ORIGINAL ARTICLE

β-Catenin is essential for survival of leukemic stem cells insensitive to kinase inhibition in mice with BCR-ABL-induced chronic myeloid leukemia

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Philadelphia chromosome-positive (Ph$^+$) chronic myeloid leukemia (CML) induced by the BCR-ABL oncogene is believed to be developed from leukemic stem cells (LSCs), and we have previously shown in mice that LSCs for CML express the same cell surface markers that are also expressed on normal hematopoietic stem cells (HSCs). Although the inhibition of BCR-ABL kinase activity by imatinib is highly effective in treating human Ph$^+$ CML in chronic phase, it is difficult to achieve molecular remission of the disease, suggesting that LSCs remain in patients. In this study, we find that following imatinib treatment, LSCs not only remained but also accumulated increasingly in bone marrow of CML mice. This insensitivity of LSCs to imatinib was not because of the lack of BCR-ABL kinase inhibition by imatinib, and proliferating leukemic cells derived from LSCs were still sensitive to growth inhibition by imatinib. These results identify an LSC survival pathway that is not inhibited by imatinib. Furthermore, we show that β-catenin in the Wnt signaling pathway is essential for survival and self-renewal of LSCs, providing a new strategy for targeting these cells.
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Introduction

The BCR-ABL tyrosine kinase inhibitor, imatinib, is the standard of care for Ph$^+$ leukemia, and has been shown to induce a complete hematologic response in interferon-resistant chronic phase chronic myeloid leukemia (CML) patients.[1] However, molecular remission is difficult to be achieved in imatinib-treated CML patients,[2–5] suggesting the existence of some residual leukemic cells, likely leukemic stem cells (LSCs), which are insensitive to imatinib treatment. We and others have shown that imatinib prolongs survival of mice with BCR-ABL-induced CML,[6,7] but does not cure the disease,[6] consistent with the observation that imatinib is unable to completely eliminate BCR-ABL-expressing leukemic cells from CML patients.[8,9] The inability of imatinib to cure CML mice is not because of the development of imatinib resistance.[10] These findings suggest that the inhibition of BCR-ABL kinase activity alone is insufficient to eradicate LSCs, and that an unknown BCR-ABL kinase activity-independent pathway in LSCs plays a crucial role in the maintenance of these cells. This idea is supported by the insensitivity of LSCs to inhibition by BCR-ABL kinase inhibitors in CML mice.[10] It is critical for understanding key pathways that regulate survival and self-renewal of LSCs.

We have shown that LSCs in CML mice express the cell surface markers that are also present on normal hematopoietic stem cells (HSCs),[10] suggesting that LSCs and normal HSCs may share similar biological properties such as survival and self-renewal. The Wnt/β-catenin pathway plays an important role in regulation of self-renewal of HSCs, and has been linked with many cancers. Activated β-catenin translocates to the nucleus, where it interacts with lymphoid enhancer/T-cell transcription factors and regulates the expression of genes, including c-myc and cyclin D1.[11] Activating β-catenin mutations are frequently detected in many epithelial cancers.[12–16] In addition, deregulation of self-renewal pathways, which are normally tightly regulated in HSCs,[17–24] has recently been recognized as an important step in leukemia progression.[24,25] Also, β-catenin has been shown to be involved in BCR-ABL leukemogenesis. BCR-ABL stabilizes β-catenin in myeloid cells through induction of tyrosine phosphorylation of β-catenin.[26] Activation of β-catenin in BCR-ABL-positive granulocute-macrophage progenitors from blastic phase CML patients facilitates the acquisition by these cells of properties of LSCs.[27] In this study, we used CML mouse model to provide further evidence that there is an LSC survival pathway that is not inhibited by imatinib, and β-catenin is essential for survival and self-renewal of LSCs.

Materials and methods

BM transduction/transplantation

The retroviral vector MSCV-*BCR-ABL(p210)*-IRES-*eGFP*, MSCV-*Cre*-IRES-*eGFP*, MSCV-*BCR-ABL(p210)*-IRES-*Cre*, and MINV-*Cre*-IRES-*BCR-ABL(p210)* were used to generate virus stocks as described previously.[28] Four- to ten-week-old wild type C57BL/6J and B6.129–*Ctnnb1*^{tm2Kem}/KnwJ mice (obtained from The Jackson Laboratory)[29,30] were used for induction of CML.[28,29] Briefly, donor mice were pretreated with 5-fluorouracil (5-FU; 200 mg/kg) through intravenous tail injection. Four days later, bone marrow cells were harvested, prestimulated with interleukin-3, interleukin-6 and stem cell factor, and subjected to two rounds of cosedimentation with retroviral stock. The transduced marrow cells were transplanted by lateral tail vein injection into recipient mice (0.5×10^6/mouse) of the same genetic background as the donor mice. Before transplantation, all recipient mice received two doses of 550-cGy γ-irradiation separated by 3 h. Recipient mice were evaluated daily for symptoms of CML, including weight and heat loss, splenomegaly and overall signs of morbidity (decreased level of activity). Hematopoietic tissues and leukemic cells from bone marrow and peripheral blood (PB) were collected from the diseased mice for further pathological and FACS analyses.

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Purification of Scal-1$^+$ cells
Bone marrow cells from CML mice were collected by flushing the bones with DMEM containing 2% FBS, and were passed through 30-mm nylon filters to remove the cell clumps. Following the manufacturer's instructions, cells were labeled with PE-conjugated Scal-1$^+$ antibody and subsequently with anti-PE MicroBeads using the MACS Column and Separator (Miltengi Biotech., Munchen, Germany).

Flow cytometry
Hematopoietic cells were collected from the diseased mice and analyzed by FACS analysis as described previously.[6]

Homing experiment
The homing ability of wild type and β-catenin-deficient bone marrow cells to the bones of recipients was compared. A green fluorescent protein (GFP) strain (C57BL/6-Tg(CAG-EGFP), obtained from The Jackson Laboratory) was used as wild-type mouse, in which all blood cell lineages express GFP. *CTNNB1loxp* mouse was crossed with *Mx-1-Cre* mouse, and pIpC was used to induce the deletion of the β-catenin gene in *CTNNB1loxp-Mx-1-Cre* mouse. Both GFP mouse and *CTNNB1loxp-Mx-1-Cre* mouse are positive for CD45.2. Bone marrow cells from the GFP mouse and those from pIpC-treated *CTNNB1loxp-Mx-1-Cre* mouse were 1:1 mixed and then injected into CD45.1 mice. Percentages of CD45.2$^+$GFP$^+$ (wild type) and CD45.2$^+$GFP$^-$ (β-catenin deficient) cells in PB of CD45.1 recipient mice were compared.

Drug treatment
Imatinib was dissolved in water directly at a concentration of 10 mg/ml. The drug was administered orally in a volume of less than 0.5 ml by gavage twice a day at a dose of 150 mg per kg of body weight, beginning at 14 days after bone marrow transplantation.

Western blot analysis and antibodies
Antibodies against phosphotyrosine, c-ABL, β-actin and β-catenin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein lysates were prepared by lysing cells in RIPA buffer (150 mM NaCl, 1.0% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) and western blotting was carried out as described previously.[31]

Results

LSCs in CML mice increase during imatinib treatment
We have previously demonstrated that reatment of CML mice with the BCR-ABL kinase inhibitors imatinib and dasatinib prolonged survival, but did not eradicate LSCs (GFP$^+$Lin$^-$ c-Kit$^+$Sca-1$^+$), and all mice eventually died of CML.[10] Because proliferating leukemic cells in CML mice are still sensitive to inhibition by these kinase inhibitors, we hypothesize that the death of the treated mice might be caused by continuous accumulation of imatinib-insensitive LSCs in bone marrow, leading to gradually increased accumulation of proliferating leukemic cells in the bone marrow and lungs. To test this hypothesis, we analyzed levels of LSCs in bone marrow of imatinib-treated CML mice at multiple time points. We first induced CML in C57BL/6 (B6) mice using BCR-ABL-transduced total donor bone marrow cells, followed by purification of Sca-1$^+$ cells from bone marrow of these primary CML mice, and then transferred these cells into secondary B6 recipient mice. Fluorescence-activated cell sorting (FACS) analysis showed that these primary CML mice contained similar percentage of Sca-1$^+$ cells in bone marrow, with an average of 2.78%. After 14 days, these CML mice were treated with imatinib (150 mg/kg, twice a day) up to 55 days, and placebo-treated CML mice were used as control. Treated mice were analyzed by FACS at 14, 17, 27, and 55 days after CML induction. The percentage of Sca-1$^+$ cells in each CML mouse was similar with an average of 3.06% at day 14 in placebo-treated group, 3.03% at day 17 in placebo-treated group, 1.93% at day 17 in imatinib-treated group, 1.60% at day 27 in imatinib-treated group and 1.75% at day 55 in imatinib-treated group. Bone marrow cells from CML mice were also analyzed by FACS for the presence of LSCs (GFP$^+$Lin$^-$ c-Kit$^+$ Sca-1$^+$; Figure 1a). We should mentioned that these LSCs contained both long-term and short-term HSCs that expressed BCR-ABL, and both types of BCR-ABL-expressing HSCs could function as LSCs in CML mice. From days 14 to 17, the number of LSCs increased in placebo-treated CML mice (Figure 1b), suggesting that BCR-ABL promotes self-renewal of LSCs. This BCR-ABL-driven increase in LSCs was inhibited to a low level by imatinib treatment (Figure 1b), confirming that BCR-ABL promotes self-renewal of LSCs through its kinase activity. To observe whether inhibition of BCR-ABL kinase activity by imatinib could lead to a complete eradication of LSCs, we analyzed imatinib-treated CML mice at later time points. Strikingly, total numbers and percentages of LSCs in bone marrow of the treated mice continued to increase during imatinib treatment (Figure 1b). This result suggests that an unknown pathway that could not be inhibited by imatinib contributes to the maintenance of the survival and self-renewal of LSCs, although inhibition of BCR-ABL kinase activity by imatinib prevents the increase of LSCs stimulated by BCR-ABL (Figure 1b). The increased numbers and percentages of LSCs were correlated with increased levels of GFP$^+$ leukemic cells in PB (Figure 1c) and bone marrow (Figure 1d) of imatinib-treated CML mice. This LSC increase during imatinib treatment was also correlated with an increased infiltration of leukemic cells into the lungs and progressively more severe destruction of lung architecture of imatinib-treated CML mice (Figure 1e). At this point, we could not tell whether the failure of imatinib to cure CML mice is related to gradually increased LSCs during imatinib treatment (Figure 1b). To investigate whether normal HSCs, which comprise a small fraction of Lin$^-$Sca-1$^+$c-Kit$^+$ cells, in imatinib-treated CML mice also increase during imatinib treatment, we analyzed the GFP$^-$ Lin$^-$Sca-1$^+$c-Kit$^+$ cells in the bone marrow by an FACS. We found that the percentages and total numbers of normal HSCs also gradually increased during the imatinib treatment (Supplementary Figure S1).

LSCs contribute to the failure of imatinib to cure CML mice
We have previously shown that imatinib treatment prolonged the survival of CML mice, but all mice eventually died of CML.[10] We reasoned that this could be because of gradually increased accumulation, in the lungs and bone marrow, of leukemic cells derived from LSCs, which percentage and total number increased in CML mice during imatinib treatment (Figure 1b). If this is the case, disease latency of recipient mice receiving bone marrow cells from imatinib-treated CML mice should differ depending on the duration of imatinib treatment. To test this idea, bone marrow cells from primary CML mice were enriched for Sca-1$^+$ cells using a Sca-1$^+$ cell purification column, and

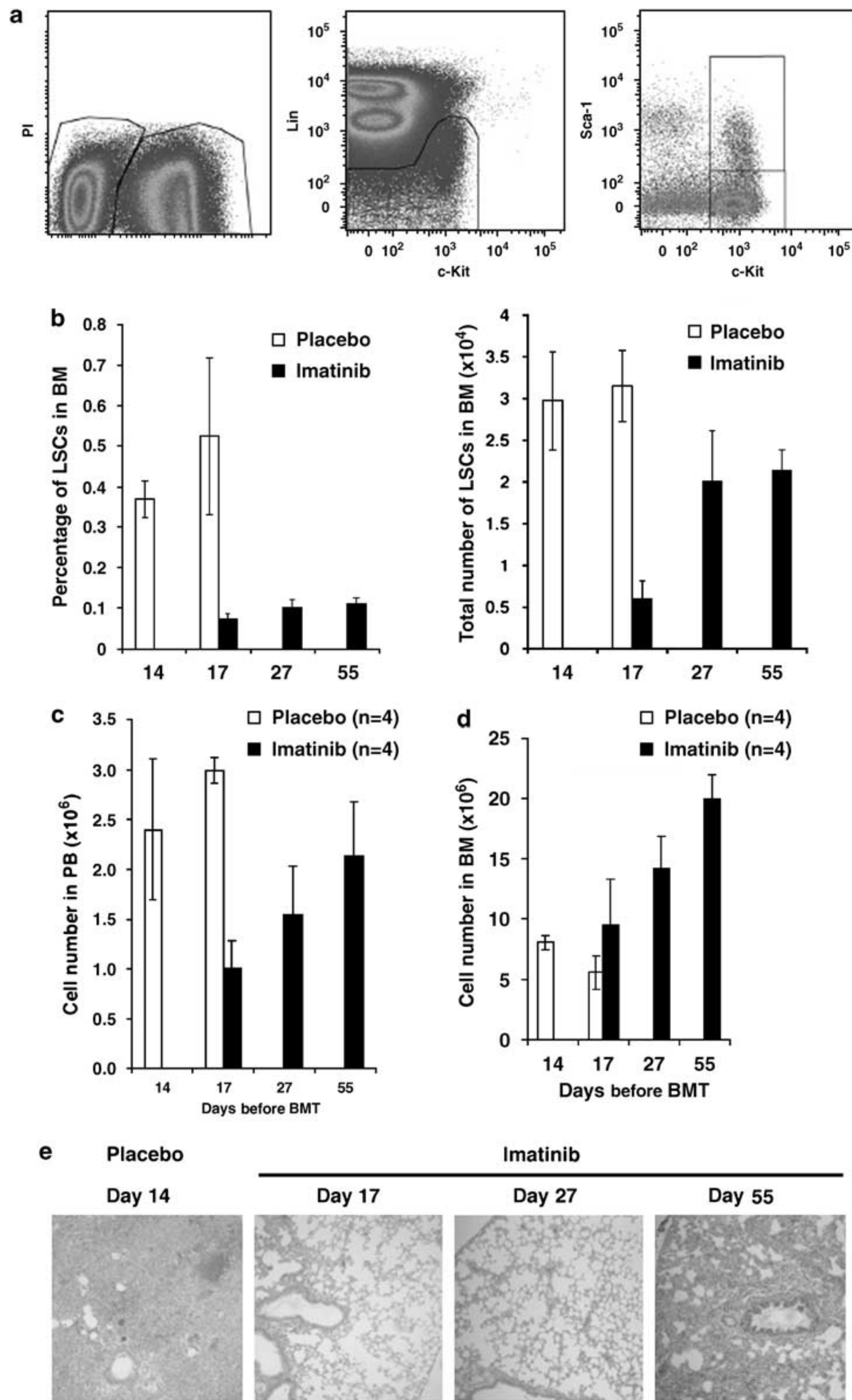

Figure 1 LSCs in CML mice increase during imatinib treatment. (**a**) Bone marrow cells were isolated from CML mice, and gated GFP^+ cells were further analyzed by FACS to identify the $Lin^-c\text{-}Kit^+$ population, followed by the identification of $c\text{-}Kit^+Sca\text{-}1^+$ LSCs ($GFP^+Lin^-Sca1^+c\text{-}Kit^+$). (**b**) CML mice treated with imatinib were analyzed by FACS, as shown in panel a, at 14, 17, 27 and 55 days before BMT. Percentages (left panel) and total numbers (right panel) of LSCs ($GFP^+Lin^-Sca1^+c\text{-}Kit^+$) were indicated. Total numbers of LSCs were calculated by the percentage of LSCs × total number of bone marrow cells. (**c**) Total leukemic cells in PB (percentage of GFP^+ cells × white blood cell count) of CML mice treated with imatinib. (**d**) Total leukemic cells in bone marrow (percentage of GFP^+ cells × total number of bone marrow cells) of CML mice treated with imatinib. (**e**) Pathological analysis of the lungs of CML mice treated with a placebo at 14 days before BMT or with imatinib at 17, 27 and 55 days before BMT. Placebo-treated CML mice showed complete infiltration of the lungs with myeloid leukemic cells, whereas leukemic cell infiltration of the lungs in imatinib-treated CML mice was much less severe. BMT, bone marrow transplantation; CML, chronic myeloid leukemia; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; LSC, leukemic stem cell; PB, peripheral blood.

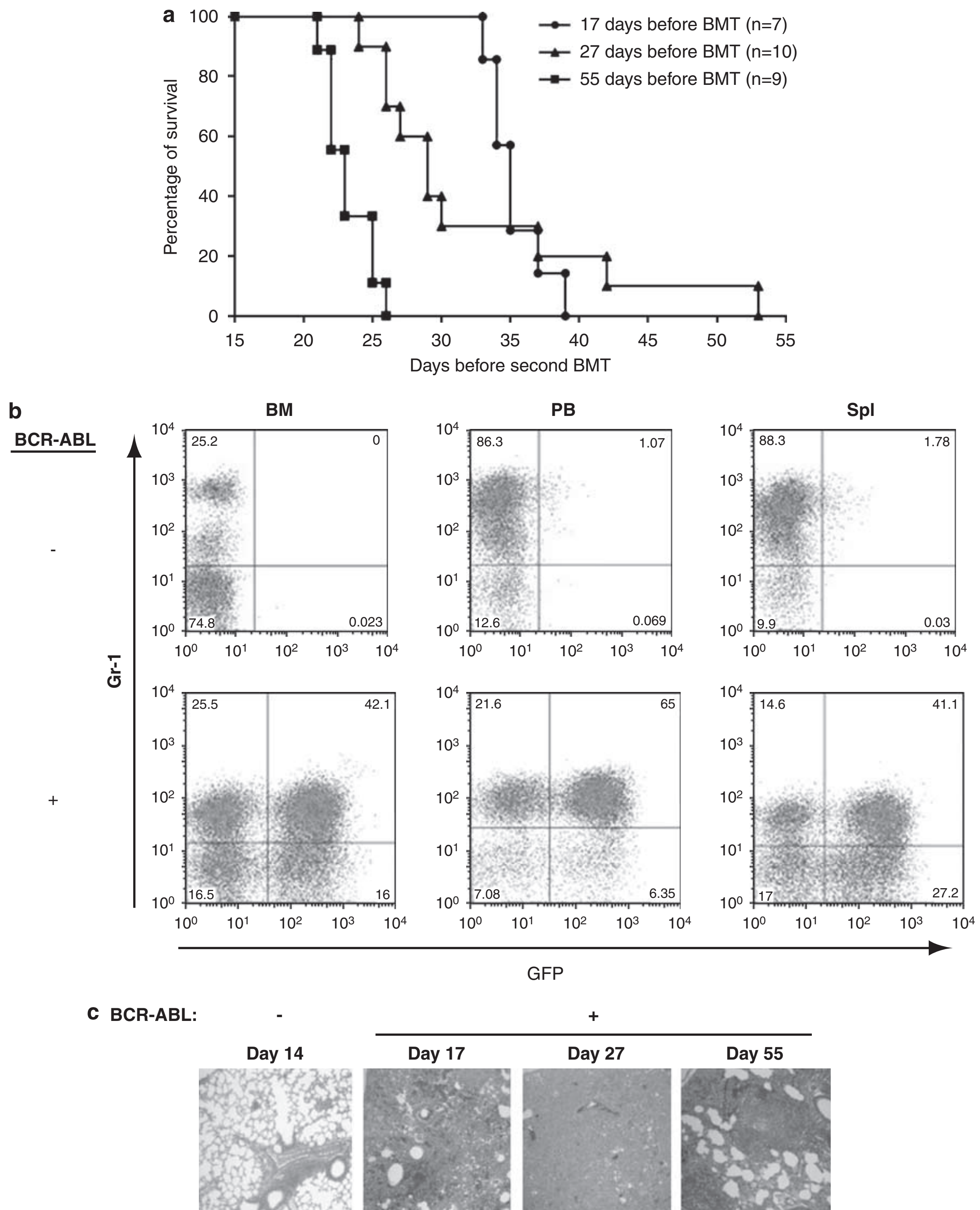

Figure 2 LSCs contribute to the failure of imatinib to cure CML mice. (**a**) Bone marrow cells from 30 primary CML mice were enriched for Sca-1$^+$ cells using a Sca-1$^+$ cell purification column, and the purified Sca-1$^+$ cells (3×10^5 per mouse) were transplanted into secondary recipient mice to induce CML. The same number of bone marrow cells (1.5×10^6 per mouse) from the secondary CML mice treated with imatinib at different times (up to 17, 27 and 55 days before BMT, respectively) into corresponding groups of tertiary recipient mice. Kaplan–Meier-style survival curves of tertiary recipients showed that all diseased mice developed CML exclusively. The differences in survival among the groups are statistically significant (*P*-values are 0.039, 0.000 and 0.004, respectively, for the groups). (**b**) At day 14, before BMT, FACS analysis of leukemic cells (GFP$^+$Gr-1$^+$) in BM PB and Spl of tertiary recipients of BM cells from secondary CML mice treated with imatinib showed the development of CML in these tertiary recipient mice. FACS analysis of mice receiving empty vector-transduced BM cells from normal C57BL/6 mice at day 14 before BMT was used as control. (**c**) Pathological analysis of the lungs of tertiary recipients of BM cells from secondary CML mice treated with imatinib at 17, 27 and 55 days before BMT. Complete infiltration of the lungs with myeloid leukemic cells was observed at all three time points. The lungs of recipients of empty vector-transduced BM cells from normal C57BL/6 mice at day 14 before BMT were used as control. BM, bone marrow; BMT, BM transplantation; CML, chronic myeloid leukemia; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; LSC, leukemic stem cell; PB, peripheral blood; Spl, spleen.

the purified Sca-1$^+$ cells were transplanted into secondary recipient mice to induce secondary CML. We then transferred the same number of bone marrow cells from the secondary CML mice treated with imatinib at 17, 27, and 55 days into three groups of tertiary recipient mice, and compared disease latencies among these different groups of recipient mice. We found that the longer the secondary CML mice were treated with imatinib, the faster the bone marrow cells from these secondary CML mice induced tertiary CML in recipient mice (Figure 2a). FACS analysis of these mice showed the presence of GFP$^+$ Gr-1$^+$ cells in bone marrow, peripheral blood and the spleen (Figure 2b), and pathological analysis showed lung hemorrhages caused by the infiltration of myeloid leukemic cells (Figure 2c). These results indicate that these mice developed typical CML, and that LSCs contributed to the failure of imatinib to cure CML mice.

Resistance of LSCs to imatinib is not caused by the loss of response of BCR-ABL to imatinib

Mutations in the BCR-ABL kinase domain result in resistance of BCR-ABL to imatinib,[32] and BCR-ABL mutations occur more often than do mutations to its downstream signaling molecules.[33] To rule out the possibility that the resistance of LSCs to inhibition by imatinib in CML mice (Figure 1a) is caused by the lost response of BCR-ABL to imatinib treatment, spleen cells isolated from recipients of bone marrow cells from imatinib-treated CML mice were treated with imatinib *in vitro*. Protein lysates were analyzed by western blotting to test whether BCR-ABL phosphorylation was inhibited by imatinib. We found that BCR-ABL phosphorylation was greatly reduced after imatinib treatment (Figure 3a). In addition, there were no detectable BCR-ABL kinase domain mutations in CML mice treated with imatinib for 2 months (data not shown). We used the spleen cells that contained more mature leukemic cells to test whether imatinib-treated CML mice developed BCR-ABL kinase domain mutations, and it is possible that the insensitivity of LSCs to imatinib is caused by the failure of imatinib to inhibit BCR-ABL kinase activity in LSCs but not by kinase domain mutations. However, we have previously shown that imatinib inhibits BCR-ABL kinase activity in LSCs.[10] Thus, the observed LSC resistance to imatinib is not because of resistance of BCR-ABL to imatinib.

To rule out the possibility that the insensitivity of LSCs to imatinib results from the activation of BCR-ABL downstream signaling pathways independently of BCR-ABL kinase activity, we treated the mice receiving bone marrow cells from CML mice with imatinib or a placebo for 55 days, and compared white blood cell counts and spleen weights between placebo- and imatinib-treated groups at the 55-day time point of imatinib treatment, and found that both white blood cell counts and spleen weights of imatinib-treated CML mice were significantly lower than those of placebo-treated mice (Figures 3b and c), indicating that BCR-ABL-expressing leukemic cells remained sensitive to inhibition by imatinib. Altogether, these results demonstrated that the LSC resistance to imatinib (Figure 1b) was not caused by the loss of BCR-ABL response to imatinib. We speculated that survival of LSCs in imatinib-treated mice might rely on a BCR-ABL kinase activity-independent mechanism.

β-Catenin is essential for survival of LSCs in imatinib-treated CML mice

To begin to identify the pathways that play a critical role in maintaining survival of LSCs, we used DNA microarray analysis to compare gene expression profiles between BCR-ABL-expressing

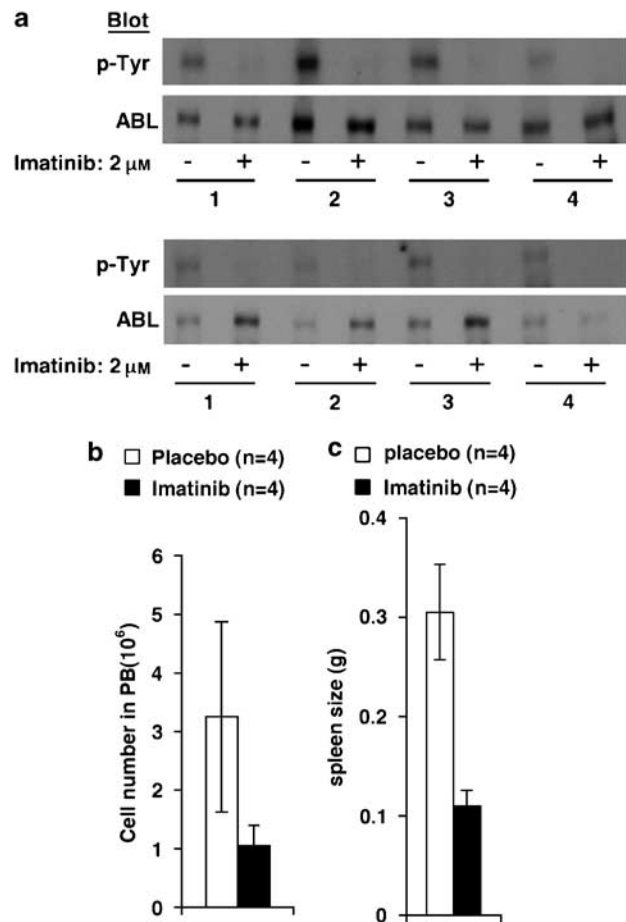

Figure 3 Resistance of LSCs to imatinib is not caused by the loss of response of BCR-ABL to imatinib. (**a**) Leukemic cell from the spleens of recipients of BM cells from primary CML mice treated with imatinib at 27 (top panel) or 55 (bottom panel) days before BMT were treated with imatinib (2 μM) for 12 h *in vitro*. Protein lysates were analyzed by western blotting with the antibodies indicated. (**b** and **c**) Recipients of BM cells from primary CML mice treated with imatinib at 55 days before BMT were treated with imatinib at a dose of 150 mg/kg, twice a day. Total leukemic cells in PB (**b**) and spleen weights were measured after 7 days of imatinib treatment. BMT, bone marrow transplantation; CML, chronic myeloid leukemia; LSC, leukemic stem cell; PB, peripheral blood.

LSCs (GFP$^+$Lin$^-$c-Kit$^+$Sca-1$^+$) in CML mice and normal HSCs (GFP$^+$Lin$^-$c-Kit$^+$Sca-1$^+$) in mice receiving empty vector-transduced normal bone marrow cells. Expression of the *β-catenin* gene was 2.5-fold upregulated in LSCs in CML mice (data not shown), suggesting the role of *β-catenin* in regulating survival of LSCs. The upregulation of *β-catenin* by BCR-ABL in LSCs was confirmed by real time-PCR (Supplementary Figure S2). Previous work supports the role of β-catenin in regulation of LSCs, as *β-catenin* was shown to play a role in acquisition of stem cell capabilities by granulocyte-macrophage progenitors in CML patients in blastic phase.[27] We next tested the effect of *β-catenin* on survival of LSCs in CML *in vivo*, using β-catenin conditional knockout mice by coexpressing *Cre* with *BCR-ABL* to remove the *β-catenin* gene in *BCR-ABL*-transformed cells (Figure 4a). We induced primary CML by transducing bone marrow cells from wild-type or β-catenin conditional knockout mice with retrovirus coexpressing the *BCR-ABL* and *Cre* genes, followed by the transplantation of transduced cells into wild-type recipient mice. At day 14, before bone marrow transplantation, equal numbers of bone marrow cells from both groups of

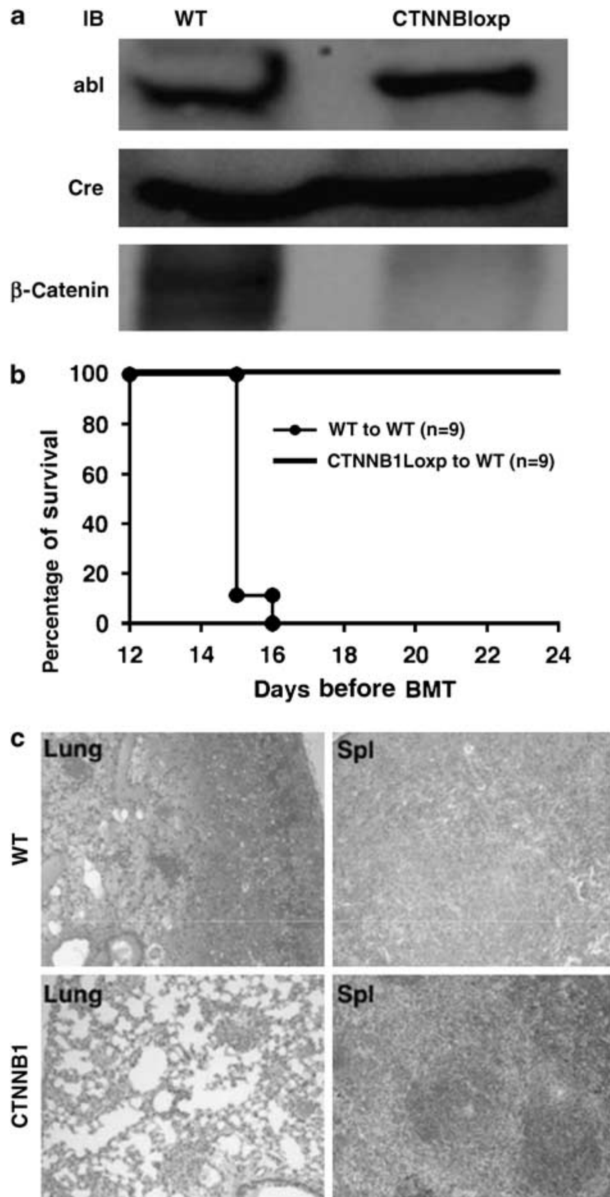

Figure 4 β-Catenin is essential for the survival of LSCs in imatinib-treated CML mice. (**a**) MSCV-*BCR-ABL*-IRES-*Cre*-transduced BM cells from C57LB/6J or *CTNNB1loxp* mice were cultured under the Whitlock–Witte conditions for 1 week. Protein lyates were analyzed by western blotting with the antibodies indicated. Cre-induced deletion of the *β-catenin* gene resulted in the removal of β-catenin protein. (**b**) Kaplan–Meier-style survival curves for recipients of BM cells from primary wild-type or CTNNB1loxp CML mice. All recipients of BM cells from primary wild-type CML mice developed and died of CML, and, in contrast, all recipients of BM cells from primary CTNNB1loxp CML mice survived. (**c**) Pathological analysis of the lungs and spleens of recipients of BM cells from primary wild-type or CTNNB1loxp CML mice. Recipients of BM cells from primary wild-type CML mice showed complete infiltration of the lungs with myeloid leukemic cells and complete disruption of follicular architecture of the spleen by infiltrating leukemic cells, whereas recipients of BM cells from primary CTNNB1loxp CML mice showed no lung or spleen pathology. BM, bone marrow; BMT, BM transplantation; CML, chronic myeloid leukemia; LSC, leukemic stem cell.

primary CML mice were transferred into wild-type secondary recipient mice to examine whether *β-catenin* affects the survival of LSCs in CML mice. In the presence of β-catenin, all mice developed and died of CML, whereas in the absence of β-catenin mice were disease-free (Figure 4b). This survival result was consistent with the pathology phenotypes of the lungs and spleen in the two groups of mice (Figure 4c).

To demonstrate whether the failure of CML induction in the secondary recipients is caused by the deletion of *β-catenin* or by a possible technical reason related to a failure of cell engraftment that is potentially caused by the *β-catenin* deficiency, we carried out an *in vivo* homing experiment comparing the ability of wild-type and β-catenin-deficient bone marrow cells to home to the bone marrow. In this experiment, we used a GFP strain as wild-type mouse, in which all blood cell lineages express GFP. We crossed *CTNNB1loxp* mice with *Mx-1-Cre* mice and used pIpC to induce the deletion of the *β-catenin* gene in *CTNNB1loxp-Mx-1-Cre* mice. Both GFP mouse and *CTNNB1loxp-Mx-1-Cre* mouse are positive for CD45.2. Bone marrow cells from GFP mice and those from pIpC-treated *CTNNB1loxp-Mx-1-Cre* mice were 1:1 mixed and then injected into CD45.1 mice. Percentages of CD45.2$^+$GFP$^+$ (wild type) and CD45.2$^+$GFP$^-$ (*β-catenin* deficient) cells in the PB of CD45.1 recipient mice were similar, indicating that β-catenin knockout cells are able to home the bone marrow of recipient mice (Supplementary Figure S3). This result indicates that the failure of *β-catenin*-deficient BM cells to induce CML in secondary recipients was not because of the failure of cell engraftment but because of the β-catenin deficiency. Altogether, these results indicated that β-catenin is essential for the survival of LSCs in CML mice.

Discussion

Our findings provide evidence that the inhibition of BCR-ABL kinase activity by imatinib effectively delays the development of CML in mice, but is not sufficient to achieve complete control of the disease. Significantly, our results also indicate that this is because of the inability of imatinib to completely eradicate LSCs. Although imatinib treatment reduced the LSC pool in CML mice, presumably through the inhibition of BCR-ABL kinase activity, LSCs were maintained at a level that could not be further reduced by imatinib. Importantly, this residual level of LSCs increased with time during imatinib treatment. This observation indicates that a stem cell pathway that is not inhibited by imatinib promotes survival of LSCs in CML mice. The LSCs in CML mice were Lin$^-$c-Kit$^+$Sca-1$^+$,[10] which are also expressed in normal HSCs. Thus, it is reasonable to believe that these LSCs utilize similar survival mechanisms as do normal HSCs to maintain their survival and self-renewal, and that these survival pathways would not be inhibited by BCR-ABL kinase inhibitors. These pathways may cooperate with BCR-ABL signaling pathways to maintain the survival of LSCs, and if this were the case, BCR-ABL would still be a target for inhibiting LSCs. This assumption is supported by our observation that imatinib treatment reduced the level of LSCs in CML mice. However, targeting BCR-ABL by kinase inhibitors is insufficient to completely eradicate LSCs, and the BCR-ABL kinase activity-independent or BCR-ABL-independent pathways in LSCs must be inhibited simultaneously or independently.

In this study, we used Lin$^-$c-Kit$^+$Sca-1$^+$ as cell surface markers for LSCs in CML mice. Thus, both long-term and short-term HSCs were included in this cell population. Comparing to normal HSCs, when HSCs express BCR-ABL to become LSCs, their biological functions may differ, and there have been no published studies that compare the similarities and differences in stem cell properties such as repopulation of recipients between normal and BCR-ABL-expressing long-term/

short-term-HSCs and BCR-ABL-expressing ST-HSCs and LT-HSCs, which expressed BCR-ABL, and both types of BCR-ABL-expressing HSCs could function as LSCs in CML mice.

A recently published study examined the role of β-catenin in self-renewal of LSCs in BCR-ABL-induced CML, and showed the requirement of β-catenin in LSC functions,[34] in agreement with our finding of this study. We further excluded a possibility that the loss of β-catenin may severely reduce the homing of LSCs, which could result in the lack of CML development. In addition, we coexpressed *BCR-ABL* with the *Cre* gene in bone marrow cells, allowing an acute deletion of *β-catenin* in BCR-ABL-expressing bone marrow cells to rule out a possibility that the early deletion of the *β-catenin* gene during embryogenesis may somehow affected HSC functions indirectly or nonspecifically through unknown mechanisms.

As described above, if LSCs in CML mice survived imatinib treatment through the BCR-ABL kinase activity-independent or BCR-ABL-independent pathways, the survival of these LSCs should depend on survival mechanisms utilized by normal HSCs, in which β-catenin plays an important role in regulation of self-renewal of normal HSCs.[17,18] In addition, overexpression of β-catenin in HSCs increases cell growth and promotes the survival of HSCs after withdrawal of steel factor, and on the other hand, degradation of β-catenin by axin reduces proliferation of HSCs in response to growth factor.[17] Our results showed that in the absence of β-catenin, LSC-containing bone marrow cells from primary CML mice were unable to induce CML in secondary recipient mice, indicating that β-catenin is essential for maintaining survival and self-renewal of LSCs. Targeting the Wnt/β-catenin pathway will provide a new strategy for curative therapy of CML.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)