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The Blk pathway functions as a tumor suppressor in chronic myeloid leukemia stem cells

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A therapeutic strategy for treating cancer is to target and eradicate cancer stem cells (CSCs) without harming their normal stem cell counterparts. The success of this approach relies on the identification of molecular pathways that selectively regulate CSC function. Using BCR-ABL–induced chronic myeloid leukemia (CML) as a disease model for CSCs, we show that BCR-ABL downregulates the Blk gene (encoding B-lymphoid kinase) through c-Myc in leukemic stem cells (LSCs) in CML mice and that Blk functions as a tumor suppressor in LSCs but does not affect normal hematopoietic stem cells (HSCs) or hematopoiesis. Blk suppresses LSC function through a pathway involving an upstream regulator, Pax5, and a downstream effector, p27. Inhibition of this Blk pathway accelerates CML development, whereas increased activity of the Blk pathway delays CML development. Blk also suppresses the proliferation of human CML stem cells. Our results show the feasibility of selectively targeting LSCs, an approach that should be applicable to other cancers.

RESULTS
Blk has tumor suppressor function in CML induction by BCR-ABL
LSCs in CML are insensitive to BCR-ABL inhibitors. Some genes are activated or inactivated by BCR-ABL in LSCs, but their expression is not affected by these inhibitors. Thus, expression of these genes is dependent on BCR-ABL protein but not on its kinase activity. To identify this type of gene in LSCs, we compared gene expression between normal Lin−Sca-1+c-Kit+ (LSK) cells and BCR-ABL–expressing LSK cells (LSCs) by DNA microarray, as described previously. We found that the Blk gene was lower in LSCs, and this downregulation was not significantly reversed by imatinib treatment. Real-time RT-PCR confirmed the downregulation of Blk by BCR-ABL and the inability of imatinib to restore Blk expression in LSCs. Short hairpin RNA (shRNA)-mediated knockdown of BCR-ABL restored Blk expression in leukemia cells (Supplementary Fig. 1a,b). Thus, BCR-ABL downregulates Blk in a kinase activity–independent manner.

These expression results raised the possibility that Blk suppresses CML development. We first studied the role of Blk in CML development using Blk homozygous knockout (Blk−/−) mice (Supplementary Fig. 2a). Wild-type or Blk−/− donor bone marrow cells in the C57BL/6 (B6) background were used to induce CML. Recipients of bone

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marrow cells transduced with a retrovirus encoding BCR-ABL from 5-fluouracil–treated Blk−/− donor mice developed CML significantly faster than did recipients of wild-type bone marrow cells transduced with BCR-ABL–encoding virus (Fig. 1c). Accelerated disease phenotype correlated with a higher percentage and number of myeloid leukemia (GFP+Gr-1−) cells in peripheral blood (Fig. 1d,e) and more severe infiltration of leukemia cells in the spleen (Fig. 1f). The lack of Blk did not affect the transduction efficiency of the retrovirus encoding BCR-ABL (Supplementary Fig. 2b) or the homing of normal (Supplementary Fig. 2c) and BCR-ABL–expressing (Supplementary Fig. 2d) cells to the bone marrow after transplantation. Conversely, we overexpressed Blk in donor bone marrow cells by transducing the cells with a retrovirus encoding both BCR-ABL and Blk (Supplementary Fig. 3), which resulted in greater survival of CML mice (Fig. 1g), correlating with a lower percentage of myeloid leukemia cells in peripheral blood (Fig. 1h) and lower infiltration of leukemia cells in the spleen and lung (Fig. 1i,j). To determine whether Blk inhibits CML progression, we induced CML and then transduced bone marrow cells, which included established leukemia cells, with vector expressing human CD4 marker (MSCV-ires-hCD4) or also expressing Blk (MSCV-Blk-ires-hCD4). After sorting hCD4+ cells by magnetic-activated cell sorting (MACS), we normalized and transplanted an equal number of GFP+hCD4+ cells into recipient mice (Fig. 1k). We observed that the percentage of GFP+hCD4+ leukemia cells in the two groups was initially similar (data not shown), but the percentage of Blk-expressing leukemia cells gradually decreased with time (Fig. 1k).

Suppression of LSCs in CML mice by Blk raised the possibility that restoration of Blk expression could synergize with BCR-ABL kinase inhibitor in CML treatment. We induced CML and then transduced bone marrow cells, which included established leukemia cells, with vector expressing human CD4 marker (MSCV-ires-hCD4) or also expressing Blk (MSCV-Blk-ires-hCD4). After sorting hCD4+ cells by magnetic-activated cell sorting (MACS), we normalized and transplanted an equal number of GFP+hCD4+ cells into recipient mice (Fig. 1k). We observed that the percentage of GFP+hCD4+ leukemia cells in the two groups was initially similar (data not shown), but the percentage of Blk-expressing leukemia cells gradually decreased with time (Fig. 1k).
combination with imatinib was much more effective, with approximately 40% of CML mice surviving longer than 130 d (Fig. 1). This therapeutic effect correlated with lower white blood cell counts (Fig. 1g), which was confirmed using real-time RT-PCR detection of BCR-ABL transcripts in cells from the peripheral blood of CML mice (Supplementary Fig. 4).

**Blk suppresses the function of LSCs**

The downregulation of Blk by BCR-ABL in LSCs and the ability of Blk to suppress CML development prompted us to test whether Blk suppresses LSCs. The percentages of total LSCs and long-term (CD34<sup>+</sup>) or short-term (CD34<sup>−</sup>) LSCs (LT-LSCs or ST-LSCs, respectively) in the bone marrow of recipients of BCR-ABL–expressing wild-type (WT) donor bone marrow cells (n = 5) 11 d after transplantation. Results are given as mean ± s.e.m. (n = 5). (c) RT-PCR analysis of expression of Blk, BCR-ABL and GFP in FACS-sorted LSCs 2 weeks after transplantation. (d) Kaplan-Meier survival curves for secondary CML mice receiving bone marrow cells obtained 15 d after transplantation from primary CML mice that expressed either BCR-ABL or BCR-ABL and Blk (n = 10 for each group). BMT, bone marrow transplantation. (e) Left, sorted LSCs from the bone marrow of primary CML mice (1 × 10<sup>5</sup>) expressing BCR-ABL (CD45.2) or BCR-ABL and Blk (CD45.1) were mixed at a 1:1 ratio and were engrafted into recipient mice. Right, percentages of cells expressing BCR-ABL alone and BCR-ABL and Blk in peripheral blood and bone marrow were compared (n = 3 for each time point). Results are given as mean ± s.e.m. (f) Cell cycle analysis of LSCs from the bone marrow of CML mice expressing BCR-ABL or BCR-ABL and Blk (n = 5 for each group) (BCR-ABL versus BCR-ABL and Blk, P < 0.05). (g) Percentage of apoptotic GFP<sup>+</sup>Lin<sup>−</sup>Sca-1<sup>−</sup>c-Kit<sup>−</sup> cells in the bone marrow of CML mice 14 d after transplantation. Results are given as mean ± s.e.m.
To more rigorously evaluate the inhibitory effect of Blk on LSC function, we examined whether Blk reduces the ability of LSCs to repopulate CML development. LSCs were sorted by FACS from the bone marrow of mice with primary CML induced by transplantation with CD45.2 donor bone marrow cells expressing BCR-ABL alone or with CD45.1 cells expressing BCR-ABL and Blk. Sorted CD45.2 and CD45.1 LSCs were mixed at a 1:1 ratio and were transplanted into recipient mice. At days 14, 23 and 28 after transplantation, fewer than 5% of GFP+Gr-1+ cells in the peripheral blood of the mice were CD45.1 leukemia cells that overexpressed Blk, whereas greater than 75–80% of GFP+Gr-1+ cells were CD45.2 leukemia cells that did not overexpress Blk (Fig. 2c). Consistent with these results, at day 28 after transplantation, the percentage of CD45.1+ leukemia cells that overexpressed Blk in the bone marrow was also very low (Fig. 2e). The suppression of LSCs by Blk can be explained, at least in part, by inhibition of cell cycle progression, as there were significantly fewer LSCs

Figure 3 Blk does not suppress normal HSCs. (a) FACS analysis of HSCs, CMPs, GMPs and MEPs in the bone marrow of wild-type (WT; n = 3) or Blk−/− (n = 4) mice. Percentages are indicated in the quadrants. (b) Percentages of LSK, LT-HSCs and ST-HSCs in the bone marrow of WT or Blk−/− mice. (c) Percentages of CMPs, GMPs and MEPs in the bone marrow of WT (n = 3) or Blk−/− (n = 4) mice. (d) Percentages of myeloid (Gr-1+Mac-1+) and lymphoid (B220+IgM+) cells in the bone marrow of WT or Blk−/− mice. Results in b–d are given as mean ± s.e.m. (*P < 0.05). (e) Cell cycle analysis of LSK cells in the bone marrow of WT or Blk−/− mice. (f) Apoptosis of bone marrow and LSK cells from WT (n = 3) or Blk−/− (n = 4) mice. Percentages are indicated in the quadrants. (g) Schematic of competitive repopulation assay and FACS analysis for different donor-derived cell lineages in recipient mice 8, 12 and 16 weeks after transplantation. Results are given as mean ± s.e.m. (h) Colony-forming assay of WT or Blk−/− bone marrow cells. BFU-E, burst-forming unit–erythroid; CFU-GM, colony-forming unit–granulocyte/macrophage; CFU-G, colony-forming unit–granulocyte; CFU-M, colony-forming unit–macrophage; CFU-GEMM, colony forming unit–granulocyte/erythroid/macrophage/megakaryocyte. Results are given as mean ± s.e.m. (i) The indicated amounts of wild-type (top) or Blk−/− (bottom) bone marrow cells were injected into lethally irradiated recipients, and survival of the mice was followed after bone marrow transplantation. (j) FACS analysis of cell lineages in the peripheral blood of recipients of bone marrow cells transduced with vector or vector expressing Blk 8, 12 and 16 weeks after transplantation. (k) Percentages of GFP+ LSK cells in the bone marrow of recipients of bone marrow cells transduced with vector or vector expressing Blk 16 weeks after transplantation. Results are given as mean ± s.e.m. (l) Cell cycle analysis of LSK cells from the bone marrow of recipients of bone marrow cells expressing GFP or Blk and GFP (P = 0.86 for G0-G1; P = 0.2 for S + G2/M).
that overexpressed Blk in the S + G2/M phases of the cell cycle than LSCs that did not overexpress Blk (Fig. 2f). In addition, we observed more apoptosis in LSCs from recipients of bone marrow cells transduced with viruses encoding both BCR-ABL and Blk (Fig. 2g).

**Blk does not suppress the function of normal HSCs**

We asked whether Blk has a similar inhibitory effect on normal HSCs. Using real-time RT-PCR, we first assessed Blk expression in different hematopoietic stem and progenitor populations, including long-term HSCs (LT-HSCs; CD34+Flt-3+LSK), short-term HSCs (ST-HSCs; CD34+Flt-3−LSK), multipotential progenitors (MPPs; CD34+Flt-3+ LSK), CMPs, MEPs and GMPS. We found that Blk was highly expressed in LT-HSCs but not in ST-HSCs, MPPs and progenitors, excluding MEPs with a higher level of Blk expression (Supplementary Fig. 5a). Next, we examined the effect of Blk on normal hematopoiesis and HSCs. The percentages of total LSK cells, LT-HSCs, ST-HSCs, CMPs and MEPs were similar in the bone marrow of wild-type and Blk−/− mice, although the percentage of GMPs was higher in Blk−/− (0.19%) than in wild-type (0.12%) mice (Fig. 3a-c). Notably, however, there was no significant difference in the percentage of more mature myeloid cells (Gr-1+Mac-1−) in the bone marrow of wild-type and Blk−/− mice (Fig. 3d). We also found that Blk deficiency did not affect cell cycle progression (Fig. 3e) or apoptosis (Fig. 3f) of LSK cells.

To examine whether Blk affects the function of normal HSCs, we performed a competitive repopulation assay. We transplanted 2 × 10^5 bone marrow cells from wild-type or Blk−/− mice (CD45.2) into each lethally irradiated wild-type recipient (CD45.1) along with an equal number of wild-type competitor cells (CD45.1). The lineage contribution of wild-type or Blk−/− cells in recipient mice was evaluated 8, 12 and 16 weeks after transplantation. We observed similar percentages of donor-derived myeloid (Gr-1+ and Mac-1+) and T-lymphoid (CD4+ and CD8+) cells (Fig. 3g), indicating that Blk did not affect the function of normal HSCs. Although Blk deficiency affected the percentage of B cells (B220+) (Fig. 3g), this effect is likely due to the known role of Blk in B-cell development. We also performed a colony-forming assay to examine the effect of Blk on progenitor cell function in vitro using sorted LSK cells from the bone marrow of wild-type and Blk−/− mice. Similar numbers and types of colonies were formed in the presence and absence of Blk (Fig. 3h). Further, there was no significant difference in the ability of wild-type and Blk−/− bone marrow cells to rescue lethally irradiated mice (Fig. 3i).

To provide additional evidence for the role of Blk in the regulation of HSC function, we tested whether overexpression of Blk suppresses HSCs. We transduced bone marrow cells from wild-type mice with retroviruses encoding GFP alone or Blk and GFP and then transplanted these cells into recipient mice (Fig. 3j). Overexpression

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**Figure 4** Pax5 is an upstream partner of Blk in LSCs. (a) Real-time RT-PCR analysis showing expression of Pax5 in LSCs compared to in normal HSCs. Results show mean ± s.e.m. (b) Protein blot analysis showing expression of Pax5, Blk and BCR-ABL in 293T cells transfected with vectors expressing BCR-ABL alone or BCR-ABL and Pax5. (c-g) Recipient mice were transplanted with donor bone marrow cells expressing BCR-ABL or BCR-ABL and Pax5. (c) FACS analysis of the number of total LSCs, LT-LSCs and ST-LSCs. Results are given as mean ± s.e.m. (d) Kaplan-Meier survival curves. (e) FACS analysis showing the percentage (indicated in the quadrant) of GFP+Gr-1+ cells in the peripheral blood and gradual disappearance of GFP+Gr-1+ cells in the peripheral blood of recipients of bone marrow cells expressing BCR-ABL and Pax5 but not in those only expressing BCR-ABL. (f) Gross appearance of the lungs and spleens 14 d after transplantation. (g) Real-time RT-PCR analysis monitoring Blk expression in LSCs from the bone marrow. Bone marrow cells from these mice with MCL were cultured under stem cell conditions for 6 d, and LSCs were sorted by FACS for the isolation of total RNA. Results are given as mean ± s.e.m. (h) Kaplan-Meier survival curves for recipients of wild-type (WT) or Blk−/− bone marrow cells expressing BCR-ABL or BCR-ABL and Pax5.
of Blk in GFP-LSK cells was confirmed by real-time RT-PCR (Supplementary Fig. 5b). The lineage contribution of cells expressing GFP alone or Blk and GFP in recipient mice was evaluated 8, 12 and 16 weeks after transplantation. The percentages of mature myeloid cells (GFP+Gr-1+Mac-1+) B cells (GFP+B220+) and T cells (GFP+CD3ε+) in the peripheral blood of recipients of bone marrow cells transduced with viruses expressing GFP alone or Blk and GFP were similar (Fig. 3), and the percentage of GFP-LSK cells in the bone marrow of recipients of cells expressing GFP or Blk and GFP at 16 weeks was also similar (Fig. 3k). In addition, there was no significant difference in cell cycle progression (Fig. 3l). Next, we conducted an in vivo limiting dilution analysis. At 16 weeks after transplantation, GFP-LSK cells were sorted from recipients of bone marrow cells transduced with retroviruses expressing GFP alone or Blk and GFP and were injected into secondary recipients. After 12 weeks, we analyzed GFP+ cells. Poisson statistics showed no significant difference in the frequency of long-term repopulation ability in control and Blk-expressing cells (Supplementary Table 1).

**Pax5 is an upstream regulator of Blk in LSCs**

Pax5 binds to the Blk promoter and stimulates Blk expression. We therefore considered the possibility that the downregulation of Blk expression by BCR-ABL in LSCs is mediated by Pax5. We found that BCR-ABL markedly downregulates Pax5 expression in LSCs (Fig. 4a). To test whether Pax5 suppresses LSCs and CML development, we generated a retroviral construct that coexpressed BCR-ABL and Pax5 (Fig. 4b). We transduced bone marrow cells with viruses encoding BCR-ABL or BCR-ABL and Pax5 to induce CML. After 14 d, bone marrow cells from CML mice were analyzed for the percentages and numbers of LSCs. Pax5 overexpression caused marked lower numbers of total LSCs, LT-LSCs and ST-LSCs (Fig. 4c). We next compared survival between the two transplantation groups. All recipients of BCR-ABL-expressing bone marrow cells died of CML within 3 weeks of transplantation, whereas fewer than 20% of the recipients of bone marrow cells expressing both BCR-ABL and Pax5 developed CML and died (Fig. 4d), which correlated with the lower and gradually decreasing percentage of myeloid leukemia cells in peripheral blood during the course of the disease (Fig. 4e) and with less severe splenomegaly and leukemia cell infiltration in the spleen and lung (Fig. 4f).

We also found that ectopically expressed Pax5 caused higher Blk expression in LSCs (Fig. 4g), supporting the idea that Pax5 functions upstream of Blk to mediate the downregulation of Blk by BCR-ABL. To further test this idea, we transduced bone marrow cells from Blk−/− or wild-type mice with viruses expressing BCR-ABL alone or BCR-ABL and Pax5 to induce CML. Compared to recipients of Blk−/− bone marrow cells expressing BCR-ABL alone that had accelerated CML development (Fig. 4h), recipients of Blk−/− bone marrow cells expressing both BCR-ABL and Pax5 died of CML much more slowly, although these mice developed CML significantly faster than recipients of wild-type bone marrow cells expressing BCR-ABL and Pax5. These results suggest that Blk is one but not the only downstream functional target gene of Pax5 in LSCs.

We tested whether Pax5 suppresses normal HSCs. We first assessed Pax5 expression in different hematopoietic stem and progenitor populations using quantitative RT-PCR (qRT-PCR) and found that Pax5 was highly expressed in LT-HSCs but not in ST-HSCs, MPPs and progenitors, excluding MEPS (Supplementary Fig. 6a). Next, we transduced bone marrow cells from normal B6 mice with retroviruses encoding GFP alone or Pax5 and GFP, and the transduced cells were cultured under stem cell conditions for 4 d; FACS analyses of control or Pax5-expressing GFP+LSK cells were then performed. Pax5 overexpression was confirmed by RT-PCR (Supplementary Fig. 6b). Pax5 reduced the percentage of LSCs from 45.5% to 29.4% (Supplementary Fig. 6c), suggesting that, unlike Blk, Pax5 has some effect on normal HSCs. This result further suggests that Pax5 also regulates other downstream genes besides Blk. However, when we monitored the distribution of different lineages 8, 12 and 16 weeks after transplantation,
the initial decrease in mature myeloid cells (GFP×Gr-1+/Mac-1+) at 8 and 12 weeks was reversed at 16 weeks (Supplementary Fig. 6d), suggesting that the function of HSCs was not significantly affected. Development of lymphoid (GFP×B220+ or GFP×CD3ε+) cells was affected by Pax5 (Supplementary Fig. 6d), presumably due to the specific role of Pax5 in lymphoid development19 (for further discussion, see the Supplementary Note).

c-Myc and Ebf1 mediate downregulation of Pax5 by BCR-ABL

We studied how BCR-ABL downregulates Pax5 expression. BCR-ABL induces c-Myc expression20–22, and analysis of the Pax5 promoter region identified a consensus c-Myc–binding motif 312 bp upstream of the transcription start site (Supplementary Fig. 7a). Chromatin immunoprecipitation (ChIP) analysis showed that c-Myc directly binds to this region but not to a region further upstream (Fig. 5a). Therefore, we investigated whether BCR-ABL downregulates Pax5 through c-Myc using a luciferase assay. We found that expression of c-Myc caused a reduction in Pax5 promoter activity in a dose-dependent manner in NIH3T3 cells (Fig. 5b). By mutating the c-Myc–binding site in the Pax5 promoter, we markedly rescued the suppression of luciferase activity by c-Myc (Fig. 5c). These results indicate that c-Myc directly binds to the Pax5 promoter to suppress Pax5 expression. To determine whether c-Myc downregulates Pax5 expression in HSCs, we transduced bone marrow cells with a retrovirus expressing c-Myc (Supplementary Fig. 7b) or, as a control, Pax5. qRT-PCR analysis showed that c-Myc significantly inhibited expression of Pax5 and Blk in LSK cells (Fig. 5d,e), whereas Pax5 markedly enhanced Blk expression in these cells (Fig. 5e).

The transcription factor Ebf1 binds to the Pax5 promoter and stimulates Pax5 expression23,24, and our microarray results indicated that Ebf1 expression was significantly downregulated in LSCs (Supplementary Fig. 8). ChIP analysis showed that Ebf1 directly binds to the Pax5 promoter within the region from 1,638 to 1,647 bp upstream of the transcription start site (Fig. 5f), consistent with previous results23,24. Also, expression of Ebf1 caused higher Pax5 luciferase activity (Fig. 5g). Although interferon regulatory factor 8 (Irf8) regulates the expression of Ebf1 and Pax5 (ref. 25,26), Irf8 had no effect on Pax5 promoter activity (Fig. 5g). We mutated the Ebf1-binding site in the Pax5 promoter and found that the increase in luciferase activity mediated by Ebf1 was markedly inhibited (Fig. 5h). It remained possible that c-Myc also downregulates Ebf1 expression, resulting in decreased Pax5 expression. Indeed, qRT-PCR analysis showed that BCR-ABL downregulated Ebf1 expression in LSCs (Fig. 5i), and c-Myc downregulated Ebf1 expression in LSK cells (Fig. 5j).

p27 functions downstream of Blk to suppress LSC proliferation

We attempted to identify genes required for Blk to suppress LSC proliferation and CML development. The mammalian cyclin-dependent kinase inhibitor 1b (Cdkn1b) p27 is a negative cell cycle regulator that blocks the G1-to-S phase transition27. BCR-ABL downregulates Cdkn1b expression through multiple mechanisms28–32. We compared the amount of p27 in 293T cells transfected with constructs expressing BCR-ABL alone or BCR-ABL and Blk and found that BCR-ABL downregulated p27 and that Blk restored p27 expression by inhibiting the expression of S-phase kinase–associated protein 2 (Skp2) (Fig. 6a). The inhibition of Skp2 by Blk was confirmed by qRT-PCR (Supplementary Fig. 9a). BCR-ABL and Blk did not alter the amounts of other cell cycle regulators, such as p21 and cyclin-dependent kinase 2 (Cdk2) (Fig. 6a). To confirm that BCR-ABL functions through Blk to reduce p27 expression, we transduced wild-type or Blk−/− bone marrow cells with retrovirus expressing BCR-ABL and found that p27 expression was significantly lower in the absence of Blk (Fig. 6b and Supplementary Fig. 9b). Conversely, we overexpressed Blk in LSCs, both to verify that Blk increases p27 expression and to identify other Blk target genes. Bone marrow cells were transduced with retroviruses expressing GFP alone, BCR-ABL and GFP or BCR-ABL, Blk and GFP, and, 14 d after transplantation, bone marrow cells were isolated, and LSCs were sorted by FACS for isolation of total RNA for DNA microarray analysis. BCR-ABL downregulated the expression of Cdkn1b (encoding p27), which was reversed by Blk overexpression (Fig. 6c). We also identified other genes that were significantly up- or downregulated by Blk in LSCs (Supplementary Table 2).

To test whether p27 suppresses LSC proliferation and CML development, we transduced wild-type or Cdkn1b−/− bone marrow cells with

![Figure 6](image-url)

Figure 6 p27 is a downstream partner of Blk in LSCs. (a) Protein blot analysis of the expression of p27, p21, Cdk2 and Skp2 in 293T cells after transfection with plasmids expressing BCR-ABL or Blk, alone and in combination, or with empty vector (control). (b) Protein blot analysis of p27 expression. Bone marrow cells from wild-type or Blk−/− mice were grown in Whitlock-Witte culture for 7 d, and protein lysates were isolated in the presence or absence of BCR-ABL expression. (c) Microarray analysis showing Cdkn1b expression in LSCs transfected with vector or with plasmids expressing BCR-ABL or BCR-ABL and Blk. Results are shown as mean ± s.e.m. (d–g) Recipient mice were transplanted with donor bone marrow cells from wild-type (WT) or Cdkn1b−/− mice expressing BCR-ABL. (d) The number of total LSCs, LT-LSCs and ST-LSCs in the bone marrow 14 d after transplantation. Results are given as mean ± s.e.m. *P < 0.05. (e) Kaplan-Meier survival curves (n = 8 for each group). (f) FACs analysis showing the percentage (indicated in the quadrant) of GFP×Gr-1+ cells in the peripheral blood 12 d after transplantation (WT versus Cdkn1b−/−, P < 0.02). (g) The total number of GFP×Gr-1+ cells in the peripheral blood. Results are given as mean ± s.e.m.
Figure 7 The inhibitory effect of Blk on CML does not require Blk kinase activity. (a) Schematics of Blk mutants. SH, Src homology; TK, tyrosine kinase. (b) Protein blot analysis of the phosphorylation status of Blk at tyrosine (p-Tyr), showing lower phosphorylation of Blk-Y383F compared to wild-type Blk. (c) Protein blot analysis of the expression of Blk, BlkATk and BCR-ABL or empty vector (control) in 293T cells. (d) Kaplan-Meier survival curves for recipients of bone marrow cells expressing BCR-ABL alone (n = 19) or with Blk (n = 23), BlkDeltaK (n = 8), Blk-K263E (n = 14) or Blk-Y383F (n = 9). (e) Gross appearance of the lungs and spleens of recipients of the bone marrow cells described in d 14 d after transplantation. (f) Protein blot analysis indicated that Blk but not truncated Blk DeltaK regulated Skp2 and p27 expression.

Suppression of CML does not require Blk kinase activity

To determine whether Blk kinase activity is required for suppression of CML, we analyzed three Blk mutants that had deletion of the entire kinase domain (ΔTk), a p.Lys263Glu alteration (K263E) or a p.Tyr383Phe alteration (Y383F) (Fig. 7a). The K263E alteration causes a loss of Blk kinase activity, and the Y383F alteration reduces Blk autophosphorilation. We coexpressed these three mutants with BCR-ABL in 293T cells (Fig. 7b-c) and found that the kinase activity of Blk-K263E was almost completely absent and that autophosphorylation of Blk-Y383F was significantly lower than for wild-type Blk (Fig. 7b). We transduced bone marrow cells with viruses expressing BCR-ABL alone or expressing BCR-ABL with wild-type Blk, BlkATk, Blk-K263E or Blk-Y383F, which had similar viral titers (Supplementary Fig. 10). We found that recipients of bone marrow cells expressing BCR-ABL and BlkK263E or BCR-ABL and Blk-Y383F, CML development was also suppressed (Fig. 7d), correlating with decreased infiltration of leukemic cells into the spleen and lung (Fig. 7e). Thus, suppression of CML development by Blk requires its kinase domain but not its kinase activity, although we cannot rule out the possibility that the very low levels of kinase activity of the Blk mutants are sufficient for CML suppression.

It is possible that the ability of Blk to stimulate p27 expression involves Skp2 because p27 levels are inversely correlated with Skp2 expression. Also, BCR-ABL stimulates cell cycle progression by promoting Skp2-mediated degradation of p27 (ref. 28), and Skp2 is required for BCR-ABL–induced myeloproliferative disease. We also found that Blk prevented BCR-ABL–induced Skp2 expression, which was dependent on the Blk kinase domain (Fig. 7f).

BLK functions as a tumor suppressor in human CML cells

We first asked whether BLK expression was lost in human CML cells. We found that BLK expression was substantially lower in bone marrow cells from humans with CML compared to in normal human bone marrow cells (Fig. 8a). We also analyzed a publicly available gene expression profiling database derived from analysis of human bulk CD34+ cells in individuals with CML and found that BLK expression was markedly lower in the majority of persons with CML in the chronic, accelerated and blast crisis phases (Fig. 8b). BCR-ABL significantly lowered BLK expression in human cord blood CD34+ cells transduced with virus expressing BCR-ABL, and this effect was not reversed by imatinib (Fig. 8c), indicating that downregulation of BLK expression by BCR-ABL in human CML cells does not require BCR-ABL kinase activity. To examine whether the BCR-ABL kinase activity–independent regulation of BLK is at the level of CML stem cells, we first analyzed another publicly available DNA microarray study of human CML CD34+CD38− cells and found that BLK expression was not altered by imatinib treatment (Fig. 8d). We further analyzed BLK expression in quiescent and dividing CD34+ cells from individuals with CML on the basis of the results in a public database. We found that the levels of BLK expression in quiescent and dividing CD34+ human CML stem cells were significantly lower than those in quiescent and dividing normal CD34+ cells (Fig. 8e). In addition, the level of BLK expression in quiescent CML stem cells was significantly lower than that in dividing CML stem cells, but there was no difference in BLK expression between quiescent and dividing normal CD34+ cells (Fig. 8e). Further, we sorted CD34+CD38− human CML stem cells...
Supplementary Fig. 11

Figure 8 BLK functions as a tumor suppressor in human CML cells. (a) Real-time RT-PCR analysis of BLK expression in bone marrow cells from humans with CML and normal donors. (b) Microarray analysis of BLK expression in bone marrow and peripheral blood CD34+ cells from 42 chronic-phase (light gray), 17 accelerated-phase (black) and 31 blast crisis-phase (dark gray) CMLs. (c) Real-time RT-PCR analysis of BLK expression in BCR-ABL–expressing human cord blood CD34+ cells. BCR-ABL–expressing CD34+ cells were also treated with imatinib (1 μM) for 24 h. (d) BLK expression in human CD34+CD38– CML stem cells was not affected by imatinib (n = 3 human samples). (e) Expression of BLK in normal and CML quiescent and dividing CD34+ cells. (f) Real-time RT-PCR analysis of BLK expression in bone marrow cells from humans with chronic-phase CML transduced with either an empty lentivirus (pLenti-puro) or with lentivirus expressing BLK (pLenti-BLK-puro). (g) FACs analysis showing inhibition of proliferation of CD34+CD38– CML stem cells. Equal numbers of human CML bone marrow cells transduced with empty lentivirus or with virus expressing BLK were plated in cytokine-supplemented methylcellulose in the presence of puromycin. (h) Growth curves of FACs-sorted human K562 cells expressing GFP alone or BLK and GFP. (k) Molecular model of the BLK pathway in LSCs. Results are given as mean ± s.e.m. in a,c,f,h,i.

stained with carboxyfluorescein diacetate succinimydyl ester (CFSE) by FACs into quiescent and dividing populations and isolated RNA for qRT-PCR analysis, confirming that BLK expression levels were lower in quiescent human CML stem cells than in dividing human CML stem cells (data not shown).

Next, we analyzed the functional effect of BLK on human CML stem cells. To infect quiescent cells, we used a lentiviral vector to express BLK in human CML cells. We purified lineage-negative cells from humans with primary CML, transduced the cells with virus encoding BLK (Fig. 8f) and subsequently labeled these transduced cells with CFSE to track quiescent and dividing CML stem cells39. We found that BLK overexpression inhibited the proliferation of CD34+CD38– CML stem cells, as shown by a lower percentage of CFSElo cells in BLK-expressing CML stem cells relative to CML stem cells that were transduced with vector alone (Fig. 8g). Also, BLK overexpression induced apoptosis of CD34+CD38– CML stem cells (Supplementary Fig. 11). Further, we performed a colony-forming assay to assess progenitor function40 and found that BLK overexpression inhibited the colony-forming ability of human CML cells but not normal bone marrow cells (Fig. 8h). BLK expression also inhibited the colony-forming ability of BCR-ABL–expressing human cord blood CD34+ cells (Fig. 8i). Finally, we transduced BCR-ABL+ human K562 cells with a retrovirus encoding GFP alone or BLK and GFP and showed that BLK-expressing K562 cells grew significantly slower than cells that did not express BLK (Fig. 8j).

**DISCUSSION**

We show that Blk functions as a tumor suppressor in CML (pathway summarized in Fig. 8k). Blk and BLK are downregulated by BCR-ABL in mouse and human CML hematopoietic cells, respectively. Of particular note, BLK expression is markedly downregulated in bulk CD34+ cells from the majority of humans with CML in the chronic, accelerated and blastic phases. Thus, suppression of BLK expression begins at an early stage of CML and is maintained throughout the course of disease.

Although BCR-ABL kinase inhibitors induce a complete cytopathic response in the majority of humans with CML in the chronic phase of CML, they are incapable of eradicating LSCs.13,41 We show that Blk suppresses LSCs without affecting normal HSCs or hematopoiesis. Thus, the BLK pathway provides a selective target for eradicating LSCs. CML could be treated by restoring BLK expression or upregulating the expression of other BLK pathway genes, such as Pax5 and CDKN1B. We note, however, that restoration of BLK expression in patients with CML would be technically challenging and might require, for example, gene therapy approaches.

The strategy of selectively targeting LSCs contrasts sharply with other therapeutic approaches that inhibit the function of gene products essential for both LSCs and normal HSCs.42–46 For example, the Wnt signaling pathway is critical in regulating hematopoietic stem and progenitor cell function43,44, and deletion of the Cimbal gene causes a profound defect in LSCs and subsequent induction of CML by BCR-ABL45,47. Inhibition of the Hedgehog pathway impairs both LSCs and normal HSCs.42,46.

The finding that Blk functions as a tumor suppressor is somewhat unexpected because some Src family kinases promote leukemogenesis.48–51. Paradoxically, Blk promotes normal B-cell development by cooperating with other Src family members. Pax5, which we show functions upstream of Blk, is also required for normal B-cell
Bmi-1 is required for maintenance of adult self-renewing Kip1

Effective targeting of quiescent chronic myelogenous leukemia -

Essential role of Src-family protein tyrosine kinases in NF-

of IRF8 regulates B-cell lineage specification, commitment, and Absence of SKP2 expression attenuates BCR-ABL–induced gene expression changes associated with progression and Kip1 Cdk-inhibitory activity and stability of p27

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For stem cell analysis, bone marrow cells were suspended in Iscove’s Modified Dulbecco medium (Sigma) supplemented with a serum substitute (BIT; StemCell), 40 μg/ml low-density lipoproteins (Sigma), 100 ng/ml recombinant human F3 ligand, 100 ng/ml steel factor, 20 ng/ml recombinant human interleukin-3 (IL-3), IL-6 and granulocyte colony-stimulating factor57 (all these cytokines were purchased from Prospec). For CML stem cell proliferation assay, sorted human CML lineage-negative (Lin⁻) cells were transduced with vector or with Blk-encoding lentivirus, labeled with 1 μM CFSE and cultured for 4 d in the presence of puromycin (2.5 μg/ml) to select the transduced cells. Informed consent was obtained from all subjects, and the study was approved by the institutional review boards at the University of Massachusetts Medical School.

Mice. Bk⁻/⁻ mice were kindly provided by A. Tarakhovsky. Cdkn1b⁻/⁻, C57BL/6j-Cd45.1 and C57BL/6j-Cd45.2 mice were obtained from Jackson Laboratory. All mice were in the C57BL/6j background.

Cell culture. K562 cells were obtained from ATCC and maintained in RPMI 1640 supplemented with 10% FBS. 293T cells were cultured in DMEM (Cellgro) supplemented with 10% FBS (HyClone).

Generation of retrovirus and lentivirus stocks. The retroviral constructs MSCV-IRES-GFP, MSCV-BAR-ABL-IRES-GFP, MSCV-BAR-IRES-GFP, MSCV-BR-ABL-IRES-Blk-luc-GFP and MSCV-BR-ABL-IRES-Pax5-IRES-GFP were used to generate high-titer helper virus–free, replication-defective ectropic viral stocks through transient transfection of 293T cells, as previously described58. With the retroviral constructs MSCV-IRES-hCD4 and MSCV-Blk-IRES-hCD4, human CD4 lacking the cytoplasmic domain can be expressed as a cell surface marker. Lentiviral vector (pLenti-Puro) was a kind gift from E. Campaus. Lentiviral particles were produced by cotransfection of 293T cells with plP1, plP2 and VSV-G and with empty vector or plP1-hBlk-Puro. Lentiviral shRNA vector plK0.1 was from Open Biosystems. The targeted BCR-ABL sequences are as follows: sense 5’-CTGACCAACTCGTGTGTGAAA-3’ and antisense 5’-TTTCACACAGGTGTGTGCA-3’. Bone marrow transduction and transplantation. Eight- to 12-week-old C57BL/6 mice were used for bone marrow transduction and transplantation. Retroviral transduction and transplantation of mouse bone marrow cells to induce CML by BCR-ABL has been described previously48,58,59.

Flow cytometry analysis. For stem cell analysis, bone marrow cells were suspended in staining medium (Hank’s Balanced Salt Solution (HBSS) with 2% heat-inactivated calf serum) and were incubated with biotin-labeled lineage antibody cocktail containing a mixture of antibodies against CD4, CD4, CD8, B220, Gr-1, Mac-1 and Ter119 (from Miltenyi Biotec, 30-092-613). After washing, fluorochrome-labeled secondary antibody (APC-Cy7–conjugated streptavidin) to recognize biotin and PE–conjugated antibody to c-Kit (clone ACK2) and APC–conjugated antibody to Sca-1 (clone D7) were added to the cells. FITC– and PE– or APC– conjugated antibodies were used to discriminate between LSK bone marrow cells from CML stem cells, and with and without imatinib treatment. Probe-level raw intensity data were normalized and renormalized into a single set–level using the probe logarithmic intensity error (PLIER) method. The significance of changes between relevant groups was assessed by t test.

Immunoprecipitation, protein blotting and antibodies. Protein lysates were prepared by lysing cells in RIPA buffer containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS. Blk was immunoprecipitated with antibody to Blk, and blotting was performed with antibody to phosphorylated tyrosine (Millipore). Antibodies against c-ABL (sc-131), Blk (sc-329), phosphorylated tyrosine (sc-508), Pax5 (sc-55515), p27 (sc-1641), p21 (sc-471), CDK2 (sc-163), Skp2 (sc-7164), C-Myc (sc-764) and β-actin (sc-1616) were purchased from Santa Cruz Biotechnology.

RT-PCR. Total RNA was isolated from GFP⁺LSK bone marrow cells from mice using the RNaseasy Mini kit. CDNA was synthesized using the Ovation Pico cDNA synthesis method. All RT-PCR reactions were performed using the Applied Biosystems 7500. Reaction system (2 μl) was composed of 12.5 μl of SYBR Green, 2.5 μl of 20 μM primer mixture, 10 ng of cDNA and nuclease-free water. All experiments were performed in triplicate. β-actin was the internal control. Specific primer sequences are given in Supplementary Table 3.
Statistical analysis. Statistical analysis was performed by Student’s $t$ test for all column statistics. For survival curves, $P$ values were obtained using a log-rank test.

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