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Arachidonate 15-lipoxygenase is required for chronic myeloid leukemia stem cell survival

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Cancer stem cells (CSCs) are responsible for the initiation and maintenance of some types of cancer, suggesting that inhibition of these cells may limit disease progression and relapse. Unfortunately, few CSC-specific genes have been identified. Here, we determined that the gene encoding arachidonate 15-lipoxygenase (Alox15/15-LO) is essential for the survival of leukemia stem cells (LSCs) in a murine model of BCR-ABL–induced chronic myeloid leukemia (CML). In the absence of Alox15, BCR-ABL was unable to induce CML in mice. Furthermore, Alox15 deletion impaired LSC function by affecting cell division and apoptosis, leading to an eventual depletion of LSCs. Moreover, chemical inhibition of 15-LO function impaired LSC function and attenuated CML in mice. The defective CML phenotype in Alox15-deficient animals was rescued by depleting the gene encoding P-selectin, which is upregulated in Alox15-deficient animals. Both deletion and overexpression of P-selectin affected the survival of LSCs. In human CML cell lines and CD34+ cells, knockdown of Alox15 or inhibition of 15-LO dramatically reduced survival. Loss of Alox15 altered expression of PTEN, PI3K/AKT, and the transcription factor ICSBP, which are known mediators of cancer pathogenesis. These results suggest that ALOX15 has potential as a therapeutic target for eradicating LSCs in CML.

Introduction
Cancer stem cells (CSCs) in a variety of hematologic malignancies and some solid tumors are required for cancer initiation and are responsible for disease relapse (1–7). Accumulating evidence suggests that CSCs must be targeted to achieve effective and curative therapies for these malignant diseases. A number of genes have been shown to regulate CSC proliferation, including Wnt/β-catenin, Hedgehog, Notch (3, 4, 8–11), Bim1 (12, 13), p53 (8), p16INK4a (14), p19ARF (15), Pten (16), PML (17), PP2A (18), Alox5 (19), TGFB/FOXO (20), and Musashi (21). A major challenge is to identify effective target genes for developing anti-CSC strategies in cancer treatment. Because CSCs often express similar markers and are identified in a manner similar to that of their normal stem cell counterparts (22, 23), it is difficult to develop a therapeutic strategy aimed at selectively targeting CSCs, although Alox5 is specifically required for the survival of leukemia stem cells (LSCs) in chronic myeloid leukemia (CML) (19). There are some examples showing that although certain genes play roles in both cancer and normal stem cells, they are functionally more critical for cancer than for normal stem cells (24, 25). In this situation, the difference in the degree of dependence on the same genes for survival between cancer and normal stem cells provides a therapeutic window for more selective killing of CSCs.

It is reasonable to believe that although the list of aberrantly expressed genes in CSCs may be extensive, there exists a selective number of genes that play critical roles in regulating the survival of CSCs and that could be used as targets for eradicating these cells. In this study, taking advantage of our previous identification of CML LSCs in mice (26), we used BCR-ABL–induced CML as a stem cell disease model to identify effective target genes in LSCs, which have been shown to be insensitive to BCR-ABL kinase inhibitors in CML mice (27) and in human CML (28, 29). Here, we identify Alox15 as a critical regulatory gene for LSC survival. We show that Alox15 deficiency or inhibition of the function of this gene causes the depletion of LSCs and prevents the initiation of BCR-ABL–induced CML in mice. Alox15 encodes arachidonate 15-lipoxygenase (15-LO). Compared with Alox5, which we identified previously (19), Alox15 has similar but also distinct functions that are involved in numerous physiological and pathological processes, including bone development (30), regulation of inflammation and immune response (31), and inhibition of proliferation/survival of malignant cells (32, 33). Thus, it is unlikely that there is a complete functional redundancy between Alox5 and Alox15 in the maintenance of LSCs.

Results
Alox15 is required for CML induction by BCR-ABL. Because LSCs in CML are insensitive to BCR-ABL kinase inhibitors (28) and BCR-ABL kinase activity is not involved in all signaling pathways activated by BCR-ABL (26), we hypothesized that there is a group of genes whose expression is regulated by BCR-ABL but not restored by inhibition of BCR-ABL kinase activity with imatinib. To identify these genes in LSCs, we previously conducted a DNA microarray study (GEO GSE10912), in which we isolated total RNA from bone...
Figure 1. *Alox15* is essential for CML induction by *BCR-ABL*. (A) DNA microarray analysis showed upregulation of *Alox15* expression by *BCR-ABL* in LSCs compared with GFP-Lin’Sca-1c-Kit+ cells, which only expressed GFP, and this upregulation was not inhibited by imatinib. Results represent the mean ± SD (*P* < 0.05). (B) RT-PCR confirmed the upregulation of *Alox15* expression by *BCR-ABL* in LSCs in the presence and absence of imatinib treatment (*P* < 0.05). Results represent the mean ± SD. (C) Kaplan-Meier survival curves for recipients of *BCR-ABL*-transduced BM cells from WT or *Alox15*−/− mice (8 mice per group). (D) Left: Gross appearance of CML mice, lung, and spleen of recipients of *BCR-ABL*-transduced BM cells from WT and *Alox15*−/− donor mice. Right: Photomicrographs of H&E-stained lung and spleen sections from recipients of *BCR-ABL*-transduced BM cells from WT or *Alox15*−/− donor mice. Original magnification, ×10 (top), ×40 (bottom). (E) In the absence of *Alox15*, the percentage of GFP+Gr-1+ cells in PB started to decrease from day 20 and gradually disappeared after 50 days, whereas the GFP−Gr-1+ cells that did not express *BCR-ABL* increased. Results represent the mean ± SD for each group (n ≥5). (F) *Alox15* transgene rescues the defective CML phenotype. *BCR-ABL* and *Alox15* were coexpressed in *Alox15*−/− BM cells by retroviral transduction, followed by transplantation of the transduced cells into recipient mice. Left: *BCR-ABL* and 15-LO were detected by Western blotting using antibodies against ABL and 15-LO in cells transfected with *BCR-ABL-IRES-Alox15-pMSCV*. Right: Kaplan-Meier survival curves for recipients of *BCR-ABL-IRES-Alox15-pMSCV*-transduced BM cells from *Alox15*−/− donor mice.
marrow (BM) LSCs (GFP+Lin Sca-1–c-Kit+) in CML mice treated or untreated with imatinib and compared gene expression profiles between LSCs and normal hematopoietic stem cells (HSCs). The study led to our identification of the Alox5 gene (19). In this study, we attempted to identify other critical genes in LSCs by starting with in-depth analysis of the DNA microarray data. Besides Alox5, another gene upregulated by BCR-ABL in LSCs was Alox15, and its upregulation was not inhibited by imatinib treatment (Figure 1A). The upregulation of Alox15 by BCR-ABL in LSCs with and without imatinib treatment was confirmed by real-time PCR (RT-PCR) (Figure 1B). These results imply that Alox15 is involved in the regulation of LSC function by BCR-ABL.

To begin to examine whether Alox15 regulates the function of LSCs, we first tested the requirement of Alox15 for CML induction by BCR-ABL using Alox15 homozygous knockout (Alox15–/–) mice. Mice receiving BCR-ABL–transduced WT BM cells developed and died of CML within 4 weeks, whereas mice receiving BCR-ABL–transduced Alox15–/– BM cells were severely defective in their ability to induce CML (Figure 1C), which was not related to viral transduction efficiency (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI66129DS1). The failure of CML development in the absence of Alox15 correlated with a healthier physical appearance of the mice and much less infiltration of leukemia cells in the lung and spleen (Figure 1D).

In addition, FACS analysis of BCR-ABL–expressing (GFP+) myeloid (Gr-1+) cells showed that in the absence of Alox15, there was a transient growth of leukemia cells in peripheral blood (PB), but the number of these cells gradually declined to an undetectable level (Figure 1E). In the same animal, we observed a much smaller number of leukemia cells (GFP+) than nonleukemia cells (GFP–) (Figure 1E), suggesting that Alox15 signaling in maintaining LSC survival is much more critical for LSCs than for normal HSCs in vivo. To confirm the role of Alox15 in CML development, we conducted a rescue experiment by retrovirally coexpressing BCR-ABL and Alox15 in Alox15–/– BM cells to examine whether restoration of Alox15 expression reverts the defective CML phenotype caused by Alox15 deficiency. We transfected 293T cells and showed that the BCR-ABL–RES-Alox15–pMSCV construct expressed BCR-ABL and Alox15 (Figure 1F; left panel). We next showed that compared with no CML induction by BCR-ABL in the absence of Alox15, ectopically expressed Alox15 in Alox15–/– BM cells rescued the defective CML phenotype (Figure 1F; right panel); the development of CML caused by the ectopic expression of Alox15 with BCR-ABL was consistent with a high percentage of Gr-1+ myeloid cells in PB (Supplemental Figure 2A) and infiltration of leukemia cells in the lung and spleen (Supplemental Figure 2B). In contrast, Alox5 did not rescue the defective CML phenotype caused by Alox15 deficiency (Supplemental Figure 2C), indicating that Alox5 and Alox15 do not mutually compensate in function.

We analyzed GFP+ or GFP– cells to reflect the effects of Alox15 deficiency on growth of leukemia (GFP+) or normal (GFP–) donor cells in a recipient mouse (Figures 1 and 2). We used alternative approaches to exclude two possibilities after BM transplantation, namely: (a) could recipient cells contribute significantly to the GFP+ cell population? and (b) could the GFP cell population contain leukemia cells that had lost their GFP expression for unknown reasons? To determine the degree of host contribution to cellular compositions in recipient mice after BM transplantation, we transplanted CD45.1 donor BM cells into lethally irradiated CD45.2 recipient mice. Two weeks later, we analyzed BM cells of recipient mice by FACS and found that almost 95% of total ungrafted BM cells (Supplemental Figure 3A) and nearly 100% of HSC-containing LSK (Lin Sca-1–c-Kit–CD45.1) cells (Supplemental Figure 3B) were donor derived (CD45.1-positive). To examine whether BM reconstitution of recipient mice by donor BM cells was also true when we induced CML in mice, we transplanted BCR-ABL–transduced CD45.1 donor BM cells (GFP+) into lethally irradiated CD45.2 recipient mice. On day 14 after transplantation, we found that almost all BM cells in the leukemic mice were also donor derived (Supplemental Figure 2C). To confirm that all leukemia cells express GFP in these CML mice, we sorted GFP+ or GFP– leukemia cells from the PB of CML mice and assessed BCR-ABL transcripts by RT-PCR. We found that BCR-ABL transcripts were only detected in GFP+, but not GFP–, cells (Supplemental Figure 3D), indicating that leukemia cells did not lose GFP expression in CML mice.

Together, the results described above demonstrate that Alox15 is essential for induction of CML by BCR-ABL.

Loss of Alox15 causes a functional defect in LSCs. The impaired CML development in the absence of Alox15 (Figure 1) could be caused by a decrease in the number of LSCs. Therefore, we tested whether Alox15 regulates the function of LSCs. We compared the numbers of LSCs and normal HSCs (GFP Lin Sca-1–c-Kit–) in BM of the same CML mice by conducting FACs analyses on days 14 and 20 after CML induction. At both time points, Alox15 deficiency caused a marked reduction of LSCs as compared with LSCs in the BM of WT CML mice (Figure 2A). Loss of Alox15 also caused a reduction of normal BM HSCs (GFP+) but did so to a much lesser degree compared with GFP–LSCs (Figure 2A), suggesting that Alox15 deficiency has a more profound inhibitory effect on LSCs than on normal HSCs. This result was correlated with a more profound effect of Alox15 deficiency on BCR-ABL–expressing common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs), and megakaryocyte-erythroid progenitors (MEPs) than on the corresponding normal progenitor cells (Figure 2B). Alox15 deficiency did not cause a homing defect in HSCs (Supplemental Figure 4), which could cause a delay in CML development when the cells were transduced by BCR-ABL.

To test whether loss of Alox15 causes a decrease in the repopulating ability of LSCs, which could lead to a decrease in the number of BM LSCs (Figure 2A), we transduced WT (CD45.1) or Alox15–/– (CD45.2) BM cells with BCR-ABL to induce CML and transplanted equal numbers of WT and Alox15–/– BM cells (1:1 mixed) from the CML mice into each lethally irradiated secondary recipient mouse. On day 14 or 20 after transplantation, more than 80% of GFP+Gr-1+ cells in PB of the mice were WT (CD45.1+) leukemia cells (Figure 2C), and all these mice eventually died of CML (data not shown). These results showed that loss of Alox15 caused a severe reduction of the repopulating ability of LSCs. Cell cycle analysis of LSCs in the BM of CML mice showed that there was a higher percentage of LSCs in the S + G2/M phases in mice receiving BCR-ABL–transduced Alox15–/– BM cells than in mice receiving BCR-ABL–transduced WT BM cells (Figure 2D), presumably due to the compensatory response of Alox15–/– LSCs to
from CML mice into secondary recipient mice. We found that in the absence of Alox15, BCR-ABL–expressing cells grew at a significantly slower rate (Figure 2F) and had severely defective induction of CML (Figure 2G). Together, these results further indicate their reduced stem cell function (Figure 2C). Loss of Alox15 also caused increased apoptosis of LSCs but not of normal HSCs (Figure 2E). To further demonstrate the effect of Alox15 deficiency on LSC function, we induced CML and then transplanted BM cells from CML mice into secondary recipient mice. We found that in the absence of Alox15, BCR-ABL–expressing cells grew at a significantly slower rate (Figure 2F) and had severely defective induction of CML (Figure 2G). Together, these results further indicate
that Alox15 deficiency has a much more profound inhibitory effect on LSCs than on normal HSCs.

Inhibition of 15-LO impairs LSC function and attenuates CML in mice. Deletion of Alox15 caused an impairment of LSC function in CML mice (Figure 2), suggesting that Alox15 is a target gene in LSCs. Therefore, we examined whether inhibition of Alox15 function by the 15-LO inhibitor PD146176 also impairs LSC function. We isolated BM cells from CML mice and cultured the cells in the presence of different concentrations of PD146176 under stem cell culture conditions (34). We observed that PD146176 significantly suppressed LSCs (GFP+LSK) and CMPs (GFP+Lin−Sca-1−c-Kit+) in vitro (Figure 3A). To examine whether PD146176 suppresses LSCs in vivo, the CML mice were treated with a placebo or PD146176, and the percentages of BM LSCs in the treated CML mice were compared. A no-treatment control was used for FACS analysis. (C) PD146176 induced apoptosis of LSCs in CML mice. On day 14 after CML induction, BM cells from placebo- or PD146176-treated CML mice were stained with 7AAD and annexin V, and the percentage of double-positive LSCs was determined by FACS. Results represent the mean ± SD. (D) Kaplan-Meier survival curves for CML mice treated with placebo or with PD146176 alone. Inhibition of Alox15 by PD146176 significantly prolonged survival of the CML mice (n = 4 for each group). (E) In PD146176-treated CML mice, the effectiveness of PD146176 in treating CML mice correlated with a decreased percentage of GFP+Gr-1+ leukemia cells in PB. FACS analysis showed the disappearance of GFP+Gr-1+ cells in the PB of CML mice treated with PD146176. Results represent the mean ± SD. (F) Photomicrographs of H&E-stained lung and spleen sections from CML mice treated with placebo or PD146176. Scale bars: 100 μM (top); 50 μM (bottom). *P < 0.05; **P < 0.01.
also induced apoptosis of LSCs (Figure 3C). Next, we examined whether 15-LO serves as a potential LSC target for the treatment of CML. We found that PD146176 treatment reduced the plasma levels of 15S-hydroxyeicosatetraenoic acid (15S-HETE), a metabolic product of 15-LO, in CML mice (Supplemental Figure 5A), indicating that the metabolic function of Alox15 was inhibited in CML mice by PD146176. All placebo-treated mice died of CML within 4 weeks after CML induction, whereas almost all PD146176-treated CML mice survived (Figure 3D). The effectiveness of PD146176 in treating CML mice correlated with a decreased percentage of GFP+Gr-1+ leukemia cells in PB (Figure 3E) and a much lower degree of leukemia cell infiltration into the lung and spleen (Figure 3F). These drug effects were mostly related to inhibition of Alox15 function, because PD146176 treatment of mice receiving BCR-ABL–transduced Alox15−/− BM cells did not significantly reduce the percentage of BCR-ABL–expressing cells in the BM of the treated mice (Supplemental Figure 5B). In some treated CML mice, GFP+Gr-1+ leukemia cells in both the PB and BM dropped to an undetectable level after long-term treatment (Supplemental Figure 5C). To evaluate the impact of PD146176 on normal HSCs in CML mice, we treated CML mice with PD146176 for 90 days. BM cells from PD146176-treated CML mice were analyzed by FACS on day 90 after CML induction. We found that the number of GFP+LSK cells was reduced to 0.016% upon PD146176 treatment, whereas GFP−LSK cell numbers were 10 times higher than those of GFP+LSK cells in the treated CML mice, suggesting that targeting of Alox15 has a much less inhibitory effect on HSCs than on LSCs (Supplemental Figure 5D).

Furthermore, we performed a colony formation assay to test the effect of PD146176 on leukemic progenitor cells. PD146176 treatment significantly reduced colony formation both in vitro (Figure 4A) and in vivo (Figure 4B). Consistently, we observed that loss of Alox15 also significantly reduced the colony formation of leukemic progenitor cells (Figure 4C). We also conducted a secondary transplantation assay to further examine the effect of PD146176 on LSC function. We transplanted BM cells from PD146176-treated...
primary CML mice into secondary recipient mice and found that inhibition of Alox15 caused a significant reduction of leukemia cells in PB (Figure 4D) and delayed CML development in secondary recipient mice (Figure 4E). By contrast, PD146176 had a much weaker effect on normal HSCs (Figure 4F). Next, we tested whether PD146176 affects the homing of CML cells, which could affect treatment efficacy, and found no significant effect (Supplemental Figure 6). Because the BCR-ABL kinase inhibitor imatinib is the standard first-line therapy for CML patients, we tested the combinatorial effect of PD146176 and imatinib on LSCs in CML mice. Compared with imatinib alone, PD146176 alone or in combination with imatinib significantly reduced the percentage and total number of LSCs in both BM and PB (Figure 4G). We found that addition of imatinib did not further increase the inhibitory effect of PD146176, presumably because PD146186 is much more effective than imatinib at inhibiting LSCs. Together, these results indicate that inhibition of Alox15 function provides a novel therapeutic strategy for suppressing LSCs in CML treatment.

**Effect of Alox15 deficiency on normal HSCs.** Alox15 deficiency or inhibition of 15-LO by PD146176 severely impaired the function of LSCs in CML mice (Figures 2 and 3), with a much weaker inhibitory effect on normal HSCs in vitro and in CML mice (Figure 2, A and E). Here, we more directly examined the effect of Alox15 deficiency on normal HSCs by comparing the percentages and function of HSCs in WT and Alox15−/− mice. A recent study showed that HSC function requires Alox15 (35). We fully characterized hematopoietic cell lineages in the BM and PB of Alox15−/− mice in comparison with those of WT mice. The percentages of total HSCs (Lin Sca-1c-Kit+), long-term HSCs (Lin Sca-1c-Kit+CD34+), short-term HSCs (ST-HSCs) (Lin Sca-1c-Kit+CD34+Flt3+), and multipotent progenitors (MPPs) (Lin Sca-1c-Kit+CD34+Flt3+) in the BM of Alox15−/− and WT mice were similar (Figure 5A, left panel), but the total number of these cells was reduced in Alox15−/− mice (Figure 5A, right panel). This result suggests that Alox15 contributes to HSC self-renewal. However, the percentages of BM CMPs, GMPs, and MEPs in Alox15−/− mice were not reduced and were significantly higher than those in WT mice (Figure 5B), consistent with higher percentages of Gr-1+ and Mac-1+ myeloid cells in the PB of Alox15−/− mice (Figure 5C), although the percentage of B lymphoid cells (B220+) was significantly lower in the PB of Alox15−/− mice (Figure 5C). Furthermore, there were more HSCs in the S + G2/M phase of the cell cycle in Alox15−/− mice than in WT mice (Figure 5D), indicating that there were more proliferating HSCs in Alox15−/− mice, although the numbers of HSCs were significantly lower than those in Alox15−/− mice (Figure 5A, right panel). This result suggests that the higher cycling property of Alox15−/− HSCs is a compensatory response to the reduced number of HSCs in Alox15−/− mice, which likely explains the higher percentages of myeloid progenitor and mature myeloid cells in the BM and PB of Alox15−/− mice.
Alox15–/– plantations (Figure 6, B and C). These results indicate that Alox15–/– were significantly lower than in WT controls 4 months after transplanta-

To understand the underlying mechanism caused by Alox15 deficiency (Figure 2A), we compared gene expression profiles between WT and Alox15–/– HSCs by performing DNA microarray analyses. We intentionally did not compare BCR-ABL–expressing WT and Alox15–/– HSCs, because BCR-ABL–expressing Alox15–/– HSCs have a profound survival defect in vivo (Figure 2A), which makes it difficult to obtain a sufficient number of cells for the analysis. On the other hand, Alox15–/– HSCs had a functional defect in rescuing lethally irradiated mice (Figure 6B), which may be mediated through the molecular pathway related to the failure of BCR-ABL–expressing Alox15–/– HSCs to induce CML (Figure 1). The DNA microarray analysis showed that in the absence of Alox15, the Selp gene, among others, was upregulated (Figure 7A). We have previously shown that Selp, a cell surface adhesion molecule, plays a suppressive role in CML development (36, 37), providing a rationale for studying the functional relationship between Alox15 and Selp in CML. We treated K562 cells with PD146176, and FACs analysis showed upregulated Selp expression on the cell surface (Figure 7B), suggesting that Alox15 suppresses SELP expression. This idea was supported by our finding that treatment of the cells with 15s-HETE and lipoxin A4, the metabolic products of the Alox15 pathway, inhibited the expression of Selp on K562 cells (Figure 7C). These results suggest that Selp is a downstream target gene of Alox15 and mediates the inhibition of LSC function by Alox15 deficiency (Figure 2A). To definitively show the regulation of Selp by Alox15, we crossed Alox15–/– mice with Selp+/− mice to generate Alox15−/− Selp+/− homozygous double-knockout mice. If Selp played a critical role in suppressing the function of LSCs in the absence of Alox15, we would expect to see a rescue of the defective CML development caused by Alox15 deficiency. Indeed, Alox15−/− Selp+/− BM cells transduced with BCR-ABL efficiently induced CML in recipient mice (Figure 7D), whereas Alox15−/− BM cells transduced with BCR-ABL failed to do so (Figure 1C and Figure 7D). As compared with the nonrescue control group, the rescue of the defective CML phenotype by the deletion of Selp in Alox15−/− BM cells correlated with a higher number of LSCs in BM (Figure 7E) (P < 0.05), a higher number of leukemia cells (GFP+Gr-1+) in PB (Figure 7F) (P < 0.01), and severe infiltration of myeloid leukemia cells into the lung and spleen (Figure 7G). A colony-forming assay also showed that Selp deficiency largely rescued the defective CML phenotype by the deletion of Selp in Alox15−/− BM cells (Figure 8A). This result was consistent with the significant reduction of Alox15−/− BM cell apoptosis (Figure 8B). To confirm whether Selp has an effect on normal HSCs, we generated the MSCV-Selp-GFP construct and used MSCV-GFP as a control (Supplemental Figure 7). We transduced BM cells with MSCV-Selp-GFP or MSCV-GFP retrovirus and found that the percentages of GFP+ cells in PB (Figure 8C) and Lin Sca-1+ c-Kit+ cells in
BM (Figure 8D) were much lower in MSCV-Selp-GFP–transduced cells than in GFP-transduced cells at day 120 after transplantation. These results indicate that SELP inhibits HSC function. To understand the underlying mechanism by which SELP regulates the function of HSCs, we compared gene expression profiles in WT and Selp–/– HSCs by conducting DNA microarray analysis. We found that loss of Selp led to upregulation and downregulation of a group of genes associated with cell adhesion and apoptosis (Figure 8E), suggesting that Selp regulates HSC function through altering the movement and survival of HSCs.

Inhibition of Alox15 function suppresses human CML cells. To investigate whether Alox15 is a potential target gene in human CML cells, we treated K562 cells with PD146176 in culture for 48 or 96 hours, respectively, and found that the total cell numbers were greatly reduced (Figure 9A). We also tested the effect of PD146176 on the non–BCR-ABL–expressing leukemia cell line
CD34+ cells (greater than 95% of the cells were FISH-positive for BCR-ABL translocation) were treated in culture with PD146176 alone or in combination with imatinib or nilotinib, followed by analyses of survival, apoptosis, and colony formation of the cells. PD146176 markedly reduced survival (Figure 10A, top panel) and induced apoptosis (Figure 10A, bottom panel) of CML CD34+ cells. PD146176 in combination with imatinib or nilotinib showed a slightly augmented effect as compared with that of single agents in the induction of apoptosis (Figure 10B). The colony-forming assay also revealed that PD146176 significantly inhibited the ability of CML CD34+ cells to form progenitor colonies, with a lesser inhibitory effect on normal human CD34+ cells (Figure 10C). PD146176 in combination with imatinib or nilotinib did not further inhibit the colony-forming ability of the cells within 24 hours of PD146176 treatment, whereas at 72 hours, PD146176 in combination with nilotinib more significantly inhibited colony formation than did PD146176 alone (Figure 10C). To further test the effect of PD146176 on human CML stem cells, CD34+CD38–CD90+ CML cells were sorted and treated with PD146176. We found that PD146176 inhibited the proliferation of HL60 cells, its effect on HL60 (Supplemental Figure 8) was smaller than on K562 cells (Figure 9A). In addition, PD146176 induced apoptosis of K562 cells (Figure 9B). To determine the signaling pathways involved in the apoptosis of K562 cells, we analyzed protein lysates of K562 cells treated with PD146176 for expression of a group of signaling molecules that have been shown to be involved in the regulation of CSC functions (11, 27, 38–42). We found that PD146176 markedly induced expression of PTEN, ICSBP, and caspase 9 and reduced the expression of β-catenin, PI3K, and AKT (Figure 9C). PD146176 did not significantly alter the expression of BCL2 or BAX (Figure 9C). To more definitively demonstrate that inhibition of Alox15 function inhibits proliferation of human leukemia cells, we conducted an Alox15 shRNA knockdown experiment using lentivirus. We found that Alox15 knockdown led to growth inhibition (Figure 9D) and apoptosis induction (Figure 9E) of BV-173 cells.

Next, we tested whether inhibition of Alox15 function by PD146176 suppresses human CD34+ CML cells. Purified PB CD34+ cells (greater than 95% of the cells were FISH-positive for BCR-ABL translocation) were treated in culture with PD146176 alone or in combination with imatinib or nilotinib, followed by analyses of survival, apoptosis, and colony formation of the cells. PD146176 markedly reduced survival (Figure 10A, top panel) and induced apoptosis (Figure 10A, bottom panel) of CML CD34+ cells. PD146176 in combination with imatinib or nilotinib showed a slightly augmented effect as compared with that of single agents in the induction of apoptosis (Figure 10B). The colony-forming assay also revealed that PD146176 significantly inhibited the ability of CML CD34+ cells to form progenitor colonies, with a lesser inhibitory effect on normal human CD34+ cells (Figure 10C). PD146176 in combination with imatinib or nilotinib did not further inhibit the colony-forming ability of the cells within 24 hours of PD146176 treatment, whereas at 72 hours, PD146176 in combination with nilotinib more significantly inhibited colony formation than did PD146176 alone (Figure 10C). To further test the effect of PD146176 on human CML stem cells, CD34+CD38 CD90+ CML cells were sorted and treated with PD146176. We found that...
PD146176 had a significant effect on CML stem cell elimination after 24 hours of drug treatment compared with that observed in the untreated control ($P < 0.05$), whereas nilotinib had very little to no effect on this primitive cell population. The killing effect of PD14176 on CML stem cells was not enhanced by nilotinib (Figure 11A). In the highly stringent in vitro LT culture–initiating cell (LTC-IC) assay, purified CML CD34$^+$CD38$^-$CD90$^+$ cells were treated with the drug arms as indicated (Figure 11A) for 24 hours and further used to perform the LTC-IC assay. Treatment with PD146176 resulted in a significant ($P < 0.05$) decrease in CML stem cells in contrast to treatment with nilotinib (Figure 11B) and in increased apoptosis of these cells (Figure 11C). To further confirm the inhibitory effect of PD146176 on the proliferation of human CML stem cells, we labeled CD34$^+$ CML cells with CFSE to track cell divisions and treated them with PD146176 (2 μM), with or without imatinib (0.5 μM) or nilotinib (0.5 μM). PD146176 alone or in combination with imatinib or nilotinib dramatically inhibited proliferation, as shown by the fewer number of cell divisions completed in the presence of the drug. Overall recovery of CD34$^+$ CML cells across all cell divisions, including the CFSEmax undivided cell population, was clearly reduced by PD146176 (Figure 11D). These results demonstrate that Alox15 is a potential target gene for the treatment of human CML.

**Discussion**

The essential role of the Alox15 gene in regulating the function of LSCs in CML identifies Alox15 as a novel target gene in LSCs. It is striking that removal of a single gene or inhibition of its function alone causes a depletion of LSCs in CML mice and abolishes CML development. In agreement with our previously proposed hypothesis derived from the study of the specific role of Alox5 in functional regulation of LSCs (19), our results again support the
principle that targeting CSCs or cancer-initiating cells provides a curative therapeutic strategy for cancer treatment. In stem cell regulation, *Alox15* is different from *Alox5* in that it also regulates the function of normal HSCs. However, *Alox15* has a much more profound effect on LSCs than on HSCs, providing a therapeutic window for eradicating LSCs through targeting *Alox15*. The mechanism for the functional regulation of LSCs by *Alox15* remains largely unknown, and we show that inhibition of *Alox15* function by PD146176 alters β-catenin, PI3K, AKT, PTEN, and ICSBP levels in K562 cells. We and others have recently shown that β-catenin plays a critical role in CML LSCs (11, 27) and that PTEN is a tumor suppressor in LSCs in CML (42). Thus, the regulation of these LSC-related genes by *Alox15* indicates its role in the development of BCR-ABL–induced CML. Because *Alox5* also regulates the expression of β-catenin in LSCs (19), *Alox5* and *Alox15* could be functionally linked. However, we observed that *Alox5* did not rescue the defective CML phenotype caused by *Alox15* deficiency, suggesting that these 2 genes are independently involved in the regulation of LSCs in CML. As with *Alox5*, the expression of *Alox15* is upregulated by BCR-ABL and is not altered by imatinib, which may partially explain the insensitivity of LSCs to imatinib in principle that targeting CSCs or cancer-initiating cells provides a curative therapeutic strategy for cancer treatment. In stem cell regulation, *Alox15* is different from *Alox5* in that it also regulates the function of normal HSCs. However, *Alox15* has a much more profound effect on LSCs than on HSCs, providing a therapeutic window for eradicating LSCs through targeting *Alox15*. Although we observed a strong inhibitory effect of PD146176 on CD34+ human CML cells, future clinical trials will be required to validate whether *Alox15* is a useful target gene in CML therapy.

The requirement of *Alox15* for LSC survival and CML development in mice indicates that *Alox15* plays a stimulatory role in CML. This conclusion conflicts with a previous study showing that *Alox15* plays a suppressive role in a myeloproliferative disorder (MPD) (32). In that study, older *Alox15*–/– mice (>12 months of age) were shown to develop an MPD with an increase in Mac-1+ cells in PB and BM, and the mice also developed splenomegaly. In agreement with that study, we show an increase in the percentages of CMPs, GMPs, and MEPs in BM and of Gr-1+ and Mac-1+ cells in PB. However, we found that the total numbers of HSCs were reduced in *Alox15*–/– mice, suggesting that *Alox15* is required for the survival and self-renewal of HSCs. In addition, there were more HSCs in the S + G2/M phase of the cell cycle in *Alox15*–/– mice than in WT mice, showing that there were more proliferating HSCs in *Alox15*–/– mice, although the total number of HSCs was significantly lower in these mice. Furthermore, we found that the engraftment ability of *Alox15*–/– BM cells in lethally irradiated mice was lower than that of WT BM cells. Together, these results suggest that the MPD phenotype in *Alox15*–/– mice is likely a compensatory response to the reduced number of HSCs in the BM of *Alox15*–/– mice, which is consistent with our observation of more cycling myeloid cells in the spleen and upregulation of BCL2 in *Alox15*–/– mice (32). By contrast, we show that in K562 cells, inhibition of *Alox15* function by PD146176 markedly induced expression of PTEN, ICSBP, and caspase 9 and reduced the expression of β-catenin, PI3K, and AKT, with no significant effects on BCL2 or BAX. We believe that the different interpretations of similar results from the previous study (32) and our current study explain the conflicting role of *Alox15*.
inhibiting Alox15 function indicates the importance of the Alox15 pathway in regulating LSC function, and an in-depth study of this Alox15 network is critical to fully understanding the mechanisms for survival and self-renewal of LSCs. Our identification of SELP as a key downstream mediator of Alox15 provides the first clue to further dissecting the Alox15 pathway. Our previous work showed that loss of SELP accelerates BCR-ABL–induced CML in mice (36) and that SELP suppresses the functions of HSCs and LSCs (37), suggesting that Selp plays a suppressive role in hematopoiesis and CML development. Furthermore, we showed that SELP expression was upregulated in the absence of Alox15 and that loss of Selp rescued the defective CML phenotype in mice receiving BCR-ABL–transduced Alox15–/–BM cells, whereas overexpression of Alox15 diminished the function of HSCs. These results support the suppressive role of Selp in the functional regulation of LSCs. Mechanistically, lipid metabolites produced by Alox15 or Alox5 could mediate their regulation of LSC function. However, we have observed that expression of β-catenin, PTEN, and ICSBP is regulated by Alox15 or Alox5 (19), and no published work has indicated that the lipid metabolites produced by Alox15 or Alox5 affect the expression of these genes. It is possible that Alox15 and Alox5 regulate LSC function through novel but distinct mechanisms independently of production of their lipid metabolites.

The findings in this study are the first to our knowledge to demonstrate that Alox15 plays an essential role in the functional regulation of LSCs in CML mice and in human CD34+ CML cells, providing a novel strategy for targeting CML in patients. Human CML microarray studies have shown that Alox15 is differentially expressed in CD34+ CML cells (44), suggesting a role of Alox15 in human CML. In addition, eradication of LSCs in CML mice by inhibiting Alox15 function indicates the importance of the Alox15 pathway in regulating LSC function, and an in-depth study of this Alox15 network is critical to fully understanding the mechanisms for survival and self-renewal of LSCs. Our identification of SELP as a key downstream mediator of Alox15 provides the first clue to further dissecting the Alox15 pathway. Our previous work showed that loss of SELP accelerates BCR-ABL–induced CML in mice (36) and that SELP suppresses the functions of HSCs and LSCs (37), suggesting that Selp plays a suppressive role in hematopoiesis and CML development. Furthermore, we showed that SELP expression was upregulated in the absence of Alox15 and that loss of Selp rescued the defective CML phenotype in mice receiving BCR-ABL–transduced Alox15–/–BM cells, whereas overexpression of Alox15 diminished the function of HSCs. These results support the suppressive role of Selp in the functional regulation of LSCs. The regulatory role of Alox15 in LSCs appears to be cell autonomous, since the recipient mice in our leukemogenesis assay were WT mice. Because Alox15 plays a critical role in regulating LSC function and CML development, a complete understanding of the Alox15 pathway will help in the development of new therapeutic strategies for curing CML. We show that Alox15 function involves several important pathways such as PTEN, PI3K/AKT, and ICSBP, which play critical roles in cancer development. Thus, inhibition of Alox15 function may be beneficial in the treatment of other types of malignant diseases.

Figure 11. Inhibition of Alox15 function suppresses human CML stem cells. (A) CML stem cells (CD34+CD38−CD90+) sorted by FACS were treated with PD146176 or nilotinib or both for 24 hours. The number of treated and untreated CML stem cells was compared. Results represent the mean ± SEM. (B) CML stem cells were treated with PD146176 or nilotinib or both for 24 hours and plated on irradiated murine stromal cells for colony formation in the in vitro LTC-IC assay. Results represent the mean ± SEM. (C) CML stem cells were treated with PD146176 or nilotinib or both for 24 hours. Apoptotic cells (annexin V+DAPI+) were assessed by FACS. Results represent the mean ± SEM. (D) CML CD34+ cells from 3 patients were labeled with CFSE and then treated with PD146176 (2 μM) with or without imatinib (0.5 μM) or nilotinib (0.5 μM) for 6 days. The treated CD34+ CML cells were analyzed by FACS. *P < 0.05.
Methods. Mice, C57BL/6j-CD45.1, C57BL/6j-CD45.2, and homozygous Alox15-knockout (Alox15−/−) and homozygous Selp-knockout (Selp−/−) mice on a C57BL/6 background were obtained from The Jackson Laboratory. The Alox15−/− Selp−/− strain is viable and grows normally and was generated by crossing Alox15−/− mice with Selp−/− mice. Mice were maintained in a temperature- and humidity-controlled environment and given unrestricted access to a 6% chow diet and acidified water.

Cell lines. Human BCR-ABL-expressing K562, HL60, and BV-173 leukemia cell lines were grown in RPMI 1640 medium containing 10% FCS. 293T and NIH3T3 cell lines were grown in DMEM containing 10% FCS.

FACS and identification of leukemia and normal hematopoietic cell lineages. Hematopoietic cells were collected from the BM and PB of the normal and 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, Gr-1-APC for neutrophils, Mac-1-PE for macrophages, CD3E-APC for T cells, SELP-PE for Selp, and Sca-1-APC/c-Kit-PE/CD34-Pacific blue for hematopoietic stem cells. After staining, the cells were washed once with PBS and subjected to FACS analysis.

BM transduction and transplantation. The retroviral vector MSCV-IRES-GFP carrying p210 BCR-ABL cDNA was used to make high-titer, helper-free, replication-defective ectropic virus stock by transient transfection of 293T cells using the kat system as previously described (45). The viral titers were evaluated by infecting 3T3 cells and analyzing protein lysates by Western blotting for detection of BCR-ABL protein expression levels. The viruses were normalized to a similar titer prior to induction of CML in mice. Six- to 8-week-old WT C57BL/6, Alox15−/−, or Selp−/− mice were used for leukemogenesis experiments. Induction of CML was performed as previously described (34, 46). Briefly, to model CML, BM from 5-FU-treated (200 mg/kg) donor mice was transduced twice with BCR-ABL retrovirus by co-transfection in the presence of IL-3, IL-6, and stem cell factor (SCF). WT recipient mice received 1,100 cGy gamma irradiation (given by 2 split doses), 0.5 × 106 (CML) cells were transplanted into the recipient mice twice with BM by clonal transplantation. The lungs from the placebo- or drug-treated mice were fixed in Bouin fixative (Fisher Scientific) for 24 hours at room temperature, followed by an overnight rinse in water. Sections (10-μm) were stained with H&E and observed using a model DMRE compound microscope (Leica). All sections were imaged with a 2.5 × PH1 objective (NPLan, NA 0.25) and 10 × PH1 objective (NPLan, NA 0.40). All images were imported into MetaMerath software (Molecular Devices) as a series of tagged image files and then constructed in Adobe Photoshop CS4 (Adobe).

Patient samples. The CD34+ cell population was enriched using ClinIMACS (Miltenyi Biotec) according to standard protocols, before storage in aliquots at −150°C. CML CD34+ samples (n = 4) were cultured in a humidified incubator at 37°C and 5% CO2 in serum-free medium (SFM) consisting of Iscove’s modified Dulbecco’s medium (Sigma-Aldrich) supplemented with serum substitute (BSA, insulin, and transferrin [BIT9500]; STEMCCELL Technologies), 2 mM L-glutamine, 10 units/ml penicillin, 100 mg/ml streptomycin, 0.1 mM 2-mercaptoethanol, and 0.8 μg/ml LDL (all from Sigma-Aldrich). SFM was supplemented with a growth factor cocktail of 0.20 ng/ml recombinant human (rh) SCF, 1 ng/ml rh IL-6, 0.20 ng/ml rh granulocyte-macrophage CSF (GM-CSF) (Chugai Pharma Europe), 0.05 ng/ml LIF, 0.2 ng/ml MIPα (all from STEMCCELL Technologies unless otherwise indicated). "Normal CD34+" or non-CML samples (n = 2) were CD34+ enriched leukapheresis products maintained and used as described for CML CD34+ samples.

Cell counting and apoptosis assays. After retrieval from −150°C aliquots and an overnight incubation, cells were seeded in 24-well plates at 1 × 106 cells/ml before drug exposure. After treatment, the aliquots were removed and counted in duplicate by trypan blue (Sigma-Aldrich) exclusion. Apoptosis was quantified by phosphatidylserine externalization. Briefly, samples were stained with annexin V-FITC and 7AAD (Viba-Probe solution; both from BD Biosciences) according to the manufacturer’s recommendations. FACSCalibur flow cytometry (BD Biosciences) enabled the distinction of viable cells (annexin V-FITC, 7AAD) from those in apoptosis (annexin V-FITC, 7AAD). Unless otherwise indicated, all results are expressed as the mean ± SEM.

Mouse colony forming cell assay. To evaluate the effect of PD146176 on CML progenitor cells, BCR-ABL-expressing cells from the BM of...
CML mice were treated with PD146176 in vitro in a colony-forming assay; also, CML mice were treated with PD146176, and then BM cells were isolated for analysis in the colony-forming assay, which was carried out as previously described (48).

**Human colony-forming cell assay.** Primary CML cells or non-CML CD34+ cells were treated for 24 or 72 hours with the indicated concentrations of PD146176, imatinib, or nilotinib, individually or in combination (Figure 10). Untreated and drug-treated cells and cells that had no prior culture or treatment (baseline) were seeded in methylcellulose medium (Methocult H4034; STEMCELL Technologies). The formation of colonies was assessed 14 days after plating 2,500 cells per plate.

**Human CML stem cell assay.** After purifying a primitive population of LSCs (CD34+CD38− and CD90+) using sorting techniques, the CML cells were treated with PD146176, nilotinib, or both. LSCs were identified and quantitated by FACS. In the highly stringent in vitro LTC-IC assay, purified CML cells that expressed CD34+CD38− and CD90− only were treated with the drug arms, as indicated (Figure 11), for 24 hours and were plated on irradiated murine stromal cells. After 6 weeks, cells were harvested, counted, and inoculated into methylcellulose media (Methocult H4435; STEMCELL Technologies). After 2 weeks, colonies were counted, and a functional readout was obtained for quantitation of the most primitive cells.

**Drug treatment.** To model CML, BM from 5-FU-treated (200 mg/kg) donor mice was transduced twice with BCR-ABL retrovirus by cocultivation in the presence of IL-3, IL-6, and SCF. WT recipient mice received 1,100 cGy gamma irradiation (administered by 2 split 550-cGy doses), and 0.5 × 10⁶ (CML) cells were transplanted into recipient mice via tail vein injection. After 8 days, CML mice with confirmed disease were randomly enrolled into the placebo or drug treatment groups. PD146176 (Cayman Chemical) was dissolved in DMSO, and access to Biobank samples was supported by the Glasgow National Health Service Greater Glasgow.

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