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CD150⁻ side population defines leukemia stem cells in a BALB/C mouse model of CML and is depleted by genetic loss of SIRT1

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Abstract

Leukemia stem cells (LSCs) of chronic myeloid leukemia (CML) are refractory to tyrosine kinase inhibitor treatment, persist in the residual disease and are important source for disease recurrence. Better understanding CML LSCs will help devise new strategies to eradicate these cells. The BALB/c mouse model of CML using retroviral bone marrow transduction and transplantation is a widely used mouse model system for CML, but LSCs in this model are poorly characterized. Here we show that lineage negative CD150⁻ side population (CD150⁻ SP), but not CD150⁺ SP, are CML LSCs in this model, although both CD150⁻ SP and CD150⁺ SP cells are enriched for long-term hematopoietic stem cells in normal BALB/c mice. We previously showed that BCR-ABL transformation activates protein lysine deacetylase SIRT1 and inhibition of SIRT1 sensitizes CML stem/progenitor cells to tyrosine kinase inhibitors by acetylating and activating p53. In this study we demonstrate that SIRT1 homozygous knockout substantially reduces CD150⁻ SP CML LSCs, and compromises the maintenance of CML LSCs in the BALB/c model. We identified several molecular alterations in CD150⁻ SP LSCs that included the elevated expression of cyclin-dependent kinase Cdk6 facilitating LSC activation and significantly reduced p53 expression. SIRT1 knockout suppressed Cdk6 expression and likely increases p53 protein functions through acetylation without increasing its expression. Our results shed novel insight into CML LSCs and support a crucial role of SIRT1 in CML LSCs. Our study also provides a novel means for assessing new agents to eradicate CML LSCs.

Keywords

leukemia stem cells (LSCs); hematopoietic stem cells (HSCs); side population; SIRT1; Chronic myeloid leukemia (CML)

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ZW, CCC and WYC designed the study.

ZW performed the study.

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Introduction

Leukemia stem cells (LSCs) are a biologically unique subset of cells distinct from the bulk of leukemia cells in that LSCs have the ability to propagate malignant clones indefinitely and produce overt leukemia [1]. LSCs are innately resistant to treatment that would kill the bulk of leukemia cells, and form a reservoir of malignant cells for disease recurrence. LSCs have been given different names when different experimental designs are deployed to study them [1], for example, leukemia-initiating cells that refer to a subset of neoplastic stem cells able to regenerate and sustain leukemia when engrafted in mice and measured with limiting dilution analysis. Leukemia-initiating cells also apply to this study, and for simplicity, LSCs will be used in the manuscript.

Chronic myeloid leukemia (CML) is a disease initiated by BCR-ABL oncogenic transformation of a normal hematopoietic stem cell (HSC) to a LSC [2]. At the chronic phase, CML LSCs share certain cellular features with normal HSCs; however, as the disease progresses to blast crisis, granulocyte-macrophage progenitors become CML LSCs in patients along with more complex molecular alterations [3, 4]. CML LSCs are refractory to BCR-ABL tyrosine kinase inhibitor treatment and represent an important source for disease relapse [5-7]. Better understanding mechanisms of CML LSC drug resistance will help devise new strategies to eradicate the disease.

Mouse bone marrow transduction by BCR-ABL retroviral vectors followed by transplantation is a well-established and widely used CML modeling system that recapitulates many hallmarks of human CML [8, 9]. This modeling system can be applied to several mouse strains with the BALB/c strain having the most efficient transduction and near 100% disease penetrance [9]. Mouse HSCs are best characterized in C57BL/6 mice for which a series of cell surface markers have been developed to enrich these rare cells including $\text{Lin}^{-/\text{low}}$ Sca1^+ c-Kit^+ $\text{Thy1.1}^{-/\text{low}}$ that also identify chronic CML LSCs [10-12]. However, the mouse strain-dependent expression of Sca1 and Thy1.1 prevents broad applicability of these cell markers to HSCs in some other strains such as BALB/c [13]. Additional approaches have been developed to isolate HSCs for broader application. Among them, Hoechst dye exclusion identifies HSCs as side population (SP) and can be used in various mouse strains [14]. The SLAM markers CD150^+ CD41/48^- provide another convenient cell labeling approach for HSC enrichment in different mouse strains including BALB/c [15]. In spite of these, CML LSCs in BALB/c mice remain poorly characterized.

SIRT1 is a mammalian protein lysine deacetylase that plays diverse roles in cellular stress response, metabolism, aging and cancer [16, 17]. We previously showed that SIRT1 is activated by BCR-ABL transformation of human and mouse hematopoietic stem/progenitor cells, and inhibition of SIRT1 by small molecule inhibitors sensitizes CML stem/progenitor cells to tyrosine kinase inhibitor imatinib [18, 19]. Mice with homozygous SIRT1 constitutive knockout are viable in the BALB/c, but not C57BL/6, strain. Taking advantage of this, we demonstrated that SIRT1 knockout significantly inhibits development of CML-like myeloproliferative disease in the retroviral transduction/transplantation model with BALB/c mice [18]. Since the identity of CML LSCs in these mice was not clear, it remained to be determined how genetic loss of SIRT1 would affect CML LSCs.

In the present study, we characterized LSCs in the BALB/c mouse model of CML and surprisingly found that CML LSCs reside exclusively in CD150⁻ SP cells, even though both CD150⁺SP and CD150⁻SP cells are enriched for HSCs and have long term reconstitution capability. We found that SIRT1 knockout significantly depleted CML LSCs and compromised the maintenance of LSCs in BALB/c mice, further supporting a crucial role of SIRT1 in CML LSCs.

Materials and Methods

Mouse colony maintenance

The use of animals was approved by the City of Hope Institutional Animal Care and Use Committee. For bone marrow transplantation (BMT) experiments, BALB/c mice (Taconic) at the age of 7-8 weeks were used as recipients. The SIRT1 knockout mice used in the study were backcrossed to BALB/c strain (Taconic) for at least nine generations. SIRT1^{+/-} mice were bred each other to produce SIRT1^{-/-} mice using a consecutive fostering protocol. In a litter with a new born SIRT1^{-/-} pup that appears visually smaller and thinner, the litter was reduced to 3 to 4 pups by discarding extra pups in normal body size. After one week, the SIRT1^{-/-} pup was transferred to a foster dam in the same breeding colony with pups in smaller body size than the SIRT1^{-/-} pup and kept the litter no more than 4 pups. This fostering procedure was repeated for additional two times to allow SIRT1^{-/-} pups to mature enough to wean, which would increase the survival of the knockout mice. SIRT1^{-/-} mice at the age of 3 to 6 months were used for the study. All mice were housed at the pathogen-free Animal Resources Center of City of Hope, and fed sterile mouse chow ad libitum.

Limiting dilution analysis of stem cells

To analyze normal HSC frequencies, 50, 20, and 5 male BALB/c donor cells per mouse per cell type of bone marrow SP, CD150⁺SP or CD150⁻SP cells were transplanted, with 4 female recipients per group. Sorted Lin⁺ cells, 20,000/mouse, were added as helper cells. Mice were followed for more than 16 weeks for long term reconstitution. To analyze LSC frequencies, unfractionated CML bone marrow nucleated cells with similar levels of GFP expression were used for BMT, 4×10⁶, 1×10⁶, 2.5×10⁵, 1.25×10⁵ and 5×10⁴ SIRT1^{+/+} CML cells and 4×10⁶, 1×10⁶, and 2.5×10⁵ SIRT1^{-/-} CML cells for each recipient, respectively, 4 mice per group. Stem cell frequency was calculated using Extreme Limiting Dilution Analysis as described [20].

Additional methods on the CML model, stem cell isolation and analysis, DNA, RNA and protein analysis, and statistical analysis are provided in supplemental information.

Results

LSCs reside exclusively in the CD150⁻ cells in the BALB/c mouse model of CML with bone marrow transduction and transplantation

In the presence of 5-fluorouracil priming of donor mice, retroviral transduction/transplantation of BALB/c mouse bone marrow cells by MIG210 BCR-ABL vector carrying green fluorescent protein (GFP) reporter cassette typically results in myeloproliferative

disease resembling human chronic CML [9, 18]. We initially set out to characterize CML LSCs in this model using SLAM markers, assuming that chronic CML LSCs would bear similar cell surface markers of normal HSCs as GFP⁺ Lin⁻CD150⁺CD41/48⁻ [15]. We first analyzed the frequency of GFP⁺Lin⁻CD150⁺CD41/48⁻ cells in CML mice. Similar to what we described previously [18], CML was typically established at 3-4 weeks after initial BMT with GFP⁺ cells ranging from 20% to 70% in peripheral blood and bone marrow, and total white blood cell (WBC) counts over 30,000/ μ l. Bone marrow and spleen were harvested from mice with the established CML. Lineage-positive cells were depleted by EasySep, and the enriched cells were then labeled with SLAM and lineage antibodies for flow cytometry analysis (Fig.1A). The average frequency of GFP⁺Lin⁻CD150⁺CD41/48⁻ cells was about 0.018% in CML mouse bone marrow and 0.066% in spleen. To determine functions of CML LSCs, we flow-sorted different fractions of SLAM labeled cells from bone marrow and spleen of CML mice that had total WBC counts over 50,000/ μ l for BMT study. With 40,000 purified cells from donor CML mice transplanted for each recipient, we found that all recipients developed CML-like myeloproliferative disease from GFP⁺Lin⁻ cells regardless the status of CD150 and CD41/48, whereas the recipients with GFP⁺Lin⁺ cells did not (data not shown). However, when the cell dosage was reduced to 1,500 cells per mouse, surprisingly only GFP⁺Lin⁻CD150⁻ cells regardless CD41/48 expression were able to generate the disease and died (Fig.1B-D), and the disease was passed onto tertiary recipients (data not shown). No disease was observed for mice receiving GFP⁺Lin⁻CD150⁺ cells over six-month observation (not shown). Similar results were observed for donor CML mice at the early phase of the disease (total WBC counts less than 50,000/ μ l) and at later stages (total WBC counts more than 50,000/ μ l) of the disease (not shown). These results suggest that CML LSCs reside in the Lin⁻CD150⁻ cell fraction, even though the reported Lin⁻CD150⁺ population may be enriched for normal HSCs.

Both CD150⁺SP and CD150⁻ SP cells are enriched for HSCs in BALB/c mice

With the surprise of CD150 marker in the CML LSC analysis, we sought to validate normal HSCs in BALB/c mice using Lin⁻SP cells, which were further separated into CD150⁺ and CD150⁻ SP subfractions (Fig.2A). The purity and viability of the sorted SP subfractions were confirmed by post sort (Fig. 2B). It has been reported that SP cells are heterogeneous for CD150 marker but quite homogeneous for many other markers [21, 22]. Indeed SP cells in BALB/c mice were mostly negative for CD16/32, a marker for granulocyte-macrophage progenitors, and CD41, a marker for megakaryocyte progenitors (Fig. 2C). For functional studies of CD150⁺ and CD150⁻ SP subfractions, we used 7-8 week old BALB/c mice for both donors and recipients. Syngenic BMT was carried out using 500 male donor cells to female recipients, and donor-derived cells were confirmed by male SRY analysis of peripheral blood cells from recipients (Supplemental Fig. 1A). We found that total SP, CD150⁺SP and CD150⁻SP had similar capability to give long term recipient mouse survival and reconstitution of myeloid (Gr-1/Mac1⁺) and lymphoid (B220⁺ or CD3e⁺) cells in peripheral blood and spleen, although total SP cells exhibited higher white blood cell outputs than the CD150 subfractions and CD150⁺SP cells had moderate change in the percentage of B and myeloid cells in bone marrow compared to total SP and CD150⁺SP cells (Fig. 2D, E and Supplemental Fig. 1B,C). In contrast, all control mice receiving non-SP cells died in 18 days after BMT. In secondary BMT, we also found that CD150⁺SP and

CD150⁻SP cells had similar capability to give long term blood lineage reconstitution and mouse survival (Supplemental Fig. 2). These results suggest that HSCs exist in both CD150⁺SP and CD150⁻SP cells.

We analyzed bone marrow SP fractions in the long-term reconstituted primary recipient mice with 500 cells of total SP, CD150⁺SP or CD150⁻SP. Bone marrow was harvested and analyzed at 5 months after BMT. Interestingly, we found that mice receiving CD150⁻SP donor cells exhibited less bone marrow SP than those receiving CD150⁺SP or total SP cells (Fig. 2F,G), suggesting that CD150⁺SP donor cells may have better reconstructive capability for HSC pool than CD150⁻SP cells. Donor CD150⁺SP cells produced both CD150⁺SP and CD150⁻SP populations, and vice versa for donor CD150⁻SP cells; however, donor CD150⁻SP cells tended to yield higher percentage of bone marrow CD150⁻SP in recipients (Fig. 2H). Considering that the efficiency of Hoechst dye efflux correlates with the degree of long term (LT)-HSC potential and more primitive LT-HSCs tend to be located in the lower SP [23], we examined the Hoechst efflux capacity of the SP cells. As shown in Fig. 2I, recipient mice receiving total SP cells had higher percentage of lower-SP than those receiving CD150⁺SP and CD150⁻SP cells, suggesting more efficient reconstitution capacity of total SP than separate subfractions. This was consistent with lineage cell outputs seen in recipient peripheral blood (Supplemental Fig.1B). Together, these data suggest that both CD150⁺SP and CD150⁻SP cells are enriched for LT-HSCs even though there is subtle difference between them, which is in line with a previous report showing that both CD150⁻SP and CD150⁺SP cells are LT-HSCs in C57BL/6 mice [22].

To further examine that CD150⁻ cells harbor LT-HSCs in BALB/c mice, we simply isolated Lin⁻ CD150⁺ and Lin⁻ CD150⁻ cells for BMT study with a stringent CD150 gate in the absence of Hoechst dye (Supplemental Fig.3A). Again, we observed long term reconstitution of lethally irradiated recipients by both Lin⁻ CD150⁺ and Lin⁻ CD150⁻ cells (Supplemental Fig.3B), although they were less efficient than more enriched Lin⁻CD150⁺SP and Lin⁻CD150⁻SP cells shown above. Under this setting, Lin⁻CD150⁺ cells had higher blood cell outputs than Lin⁻CD150⁻ cells (Supplemental Fig.3C,D). Similar to SP cell fractions, donor Lin⁻ CD150⁻ cells resulted in higher percentage of recipient bone marrow CD150⁻SP cells, and vice versa for Lin⁻CD150⁺ cells (Supplemental Fig.4). These data further supported that CD150⁻ cells carry LT-HSCs in normal BALB/c mice.

To better quantify frequencies of HSCs in SP subfractions, we performed limiting dilution assay. We transplanted 50, 20 and 5 male donor cells each of SP, CD150⁺SP or CD150⁻SP. By 12 weeks post BMT, mice receiving 5, but not 50, cells of SP and CD150⁻SP cells exhibited lower SRY DNA levels in peripheral blood (Fig. 3A). By 14 weeks, lower white blood cell counts were observed in CD150⁻SP recipients than in SP and CD150⁺SP recipients with both 50 and 20 donor cells, and some difference in the percentage of blood lineage cells was seen in mice receiving 20 donor cells (Fig. 3B,C). By 16 weeks, all mice receiving 50 or 20 donor stem cells survived, whereas in recipients with 5 cells, 4/4 mice from CD150⁺SP, 3/4 mice from SP and 2/4 mice from CD150⁻SP donors survived. The estimated HSC frequency was 1 in 1 (range 1.0 to 7.1) for CD150⁺SP, 1 in 3.5 (range 1.1 to 10.8) for SP, and 1 in 5.8 (range 2.1 to 15.9) for CD150⁻SP cells (Fig. 3D). There was no statistically significant difference among three groups, although CD150⁺SP trended to have

higher HSC frequency than CD150⁻SP ($p=0.076$). Together, our results suggest that both CD150⁺SP and CD150⁻SP fractions are enriched for LT-HSCs with subtle difference.

CD150⁻ SP cells are LSCs in the BABL/c mouse model of CML

We next used SP cells to define CML LSCs. We isolated GFP⁺Lin⁻SP cells from mice with established CML as described above, which were further separated into CD150⁺ and CD150⁻ subfractions (Fig. 4A-C). Notably, CD150⁺ cells were significantly expanded within the SP compartment in CML mice (Fig. 4B). Similar to normal SP, CML SP contained only a small percentage of CD16/32 or CD41 positive progenitors, although this percentage was higher in CML than in normal SP (Fig. 4D). The average frequency of GFP⁺Lin⁻SP cells in CML mouse bone marrow was around 0.059%. With 10,000 donor cells transplanted, CML developed more rapidly with total SP or CD150⁻SP donor cells than CD150⁺SP cells (Supplemental Fig. 5). With 1,000 donor cells, CML was exclusively developed with total SP or CD150⁻SP donor cells, but not CD150⁺SP cells (Fig. 4E, F). The recipient mice with total SP or CD150⁻SP cells developed typical CML-like myeloproliferative disease characterized by high white blood cell counts, high GFP⁺ cells in hematological organs, splenomegaly and extramedullary hematopoiesis with abundant neutrophils (Fig. 4F, G and Supplemental Fig. 5B,C). No disease was found in mice receiving 1,000 CD150⁺SP cells even after prolonged six-month observation (not shown). The disease from recipient mice with total SP or CD150⁻SP cells could be passed onto tertiary recipients (Fig. 4H). Together, these results suggest that CD150⁻SP cells are highly enriched for LSCs in this mouse model of CML.

We found that total SP and CD150⁻SP, but not CD150⁺SP, CML cells reconstituted CD150⁻SP and CD150⁺SP CML cells in recipient bone marrow (Fig. 5A). The total number (Fig. 5B) and percentage (Fig. 5C) of GFP⁺ cells in the SP compartment from recipient bone marrow or spleen were high and comparable for mice receiving total SP and CD150⁻SP, but not CD150⁺SP, CML cells. Consequently, mice receiving total SP and CD150⁻SP CML cells generated predominantly Gr1/Mac1⁺ myeloid cells with little differentiation potential towards B220⁺ cells in blood, whereas mice receiving CD150⁺SP CML cells maintained partial capability to differentiate into B220⁺ cells (Fig. 5D). These data further support CD150⁻SP cells as CML LSCs that maintain the LSC compartment and leukemic phenotypes in BALB/c mice.

We next examined if retroviral gene expression and transduction might affect the bias of CML LSCs in CD150⁻ but not CD150⁺ SP cells. As GFP⁺ cells in blood declined over time in mice receiving CD150⁺SP CML cells (Supplemental Fig. 6A), we treated the mice with histone deacetylase inhibitor SAHA in an attempt to overcome potential gene silencing of retroviral vectors [24], but we did not observe an increase of GFP expressing cells (not shown). Using SRY analysis of donor-derived cells in these mice, we found that the donor-derived genomic DNA content declined over time (Supplemental Fig. 6B), suggesting a loss of donor-derived cells but not gene silencing that may contribute to the declining of GFP⁺ cells.

CD150⁻SP HSCs have an increased rate of cell replication compared to CD150⁺SP HSCs [22]. To determine if retroviral vectors may have biased transduction or alter the functions

of CD150⁻ vs CD150⁺ in the SP compartment, we used MIGR1 control vector to transduce bone marrow cells. Three months after BMT, we sorted GFP⁺Lin⁻ SP, CD150⁻SP, and CD150⁺SP cells for secondary BMT (Fig. 5E). We found that both CD150⁻SP and CD150⁺SP cells gave similar long term reconstitution as confirmed by SRY analysis, and that GFP⁺ cells were similarly detected in secondary recipient mice transplanted with CD150⁻ and CD150⁺ SP fractions 2-4 months post BMT (Fig. 5E, F and Supplemental Fig. 7). Analysis of blood lineage cells showed that MIGR1 transduced CD150⁻SP cells had reduced myeloid differentiation potential and increased B lymphoid potential compared to SP or CD150⁺ SP cells (Fig. 5G), which is opposite to the above-described expansion of myeloid cells and block of B lymphoid differentiation in CD150⁻SP CML cells by MIR210. This suggests that retroviral transduction process per se unlikely causes the bias of CD150⁻SP cells for CML LSCs. In this regard, it is worth to note that in C57BL/6 mice CD150⁻SP cells have higher lymphoid potential whereas CD150⁺SP cells have higher myeloid potential [25].

Genetic loss of SIRT1 depleted CML LSCs

We previously reported that SIRT1 knockout in young BALB/c mice does not affect colony formation of bone marrow cells, blood lineage differentiation, and in the BMT setting, engraftment and hematological profiles in transplantation recipients [18]. In addition, both SIRT1^{+/+} and SIRT1^{-/-} bone marrow cells can be equally transduced by MIG retroviral vector. However, stem cell frequency and cell cycle were previously analyzed with SLAM markers [18]. In this study, we re-examined the impact of SIRT1 homozygous knockout on HSCs and CML LSCs using the stem cell markers described above. We found that SIRT1 knockout did not significantly change the percentage of bone marrow SP cells in young mice, although SIRT1^{-/-} SP cells exhibited moderately elevated cell cycle entry (Fig. 6A, B). We previously showed that SIRT1 protein expression was increased in Lin⁻CD150⁺ but was not detected in Lin⁻CD150⁻ CML cells [18]. Re-examination of SIRT1 expression revealed that SIRT1 protein was also increased in Lin⁻CD150⁻ CML cells compared to their normal counterparts (Fig. 6C). However, the basal levels of SIRT1 protein in normal Lin⁻CD150⁻ cells were extremely low compared to Lin⁻CD150⁺ cells, and the SIRT1 protein levels in CML Lin⁻CD150⁻ cells were much lower than that in CML Lin⁻CD150⁺ cells, which may account for the missed detection previously [18]. Lower levels of SIRT1 activation were similarly observed in human chronic CML stem/progenitor cells [18, 19]. Since the number of Lin⁻CD150⁻SP cells was very low, to further determine SIRT1 expression change in CML LSCs, we analyzed mRNA expression in SP fractions. In line with SIRT1 protein change, SIRT1 mRNA expression was increased in both GFP⁺Lin⁻CD150⁻SP CML LSCs and GFP⁺Lin⁻CD150⁺SP CML cells compared to their normal counterparts (Fig. 6D).

We next examined the impact of SIRT1 knockout on CML LSC frequency. Bone marrow from both wild type and SIRT1 knockout littermate mice at the age of 3 to 6 months were subjected to MIG210 transformation and transplantation. When CML was established as described above in the wild type control group, all mice were sacrificed for LSC analysis. We found that SIRT1 knockout substantially reduced GFP⁺Lin⁻ SP, CD150⁻SP and CD150⁺SP cells (Fig. 6E). This result suggests that the knockout not only depletes leukemic

stem cells but also those CD150⁺SP CML cells. This is in line with that SIRT1 knockout significantly inhibits BCR-ABL transformation and CML development we reported previously [18].

To definitively determine the effect of SIRT1 knockout on CML LSCs, we performed limiting dilution analysis using unfractionated bone marrow nucleated cells derived from CML mice with or without SIRT1 knockout. We found that the estimated LSC frequency was 1/31,883 for SIRT1^{+/+} CML and 1/558,746 for SIRT1^{-/-} CML ($p=1.0\times 10^{-5}$), nearly 20-fold reduction with the knockout, and mice receiving SIRT1^{-/-} CML cells had substantially better survival than mice receiving the same doses of SIRT1^{+/+} CML cells (Fig. 6 F and Supplemental Fig. 8).

Finally, we assessed the role of SIRT1 in CML LSC maintenance by comparing the same numbers of GFP⁺Lin⁻ CD150⁻SP and CD150⁺SP cells from CML mice with wild type or SIRT1 knockout. Because the number of CML LSCs with SIRT1 knockout was low, in order to obtain sufficient amount of these cells, we doubled the cell dosage of MIG210-transduced SIRT1^{-/-} bone marrow cells in the primary recipient mice to accelerate CML development, and pooled bone marrow from multiple CML mice with SIRT1 knockout for cell enrichment. We then sorted GFP⁺Lin⁻ CD150⁻SP and CD150⁺SP cells from CML mice and transplanted 1,000 sorted cells each to secondary recipients. Again, CML was developed only in mice receiving CD150⁻SP cells and SIRT1 knockout significantly increased recipient mouse survival (Fig. 6G, H). The results indicate that SIRT1 is crucial for maintenance of CML LSCs, which is in line with our previous finding that SIRT1 inhibition helps eradicate CML LSCs [19].

Molecular changes of side population fractions in HSCs and LSCs in BABL/c mice

To gain further mechanistic insight of normal and leukemic stem cells, we examined expression of a panel of genes involved in regulating HSCs and LSCs. Cell cycle analysis showed that normal CD150⁻SP cells were less quiescent than CD150⁺SP, consistent with a previous report [22]. Further significant reduction of quiescence occurred in both CML SP subfractions compared to their normal counterparts, with more CML CD150⁻SP cells entering cell cycle (Fig. 7A). Cyclin D1 (Ccdn1) was similarly expressed in normal CD150⁻SP and CD150⁺SP, but diminished in CML counterparts (Fig. 7B), consistent with increased cell cycling after BCR-ABL transformation and higher pyronin Y staining (indicating more G1/S transition). Cyclin-dependent kinase inhibitor 1C (Cdkn1c, p57 or Kip2) maintains quiescence of normal HSCs [26, 27]. Cdkn1c expression was lower in normal CD150⁻SP cells than in normal CD150⁺SP cells, in line with less quiescence of normal CD150⁻SP than CD150⁺SP cells; not surprisingly, Cdkn1c was depleted in all CML SP cells (Fig. 7B). Cyclin-dependent kinase 6 (Cdk6) promotes quiescence exit and is required for activation of both normal HSCs and CML LSCs [28, 29]. Interestingly, Cdk6 expression was lower in normal CD150⁻SP than in normal CD150⁺SP cells; but upon transformation, Cdk6 was reduced more drastically in the CD150⁺ compartment, leading to significantly lower Cdk6 in CML CD150⁺SP than in CML CD150⁻SP (Fig. 7B), which is consistent with CD150⁻SP as CML LSCs for active output of CML cells. SIRT1 knockout

reduced Cdk6 in CML CD150⁻SP cells (Fig. 7B), in line with the reduced leukemia cell production.

Furthermore, we found that Evi-1, a gene involved in maintaining normal HSC self-renewal and long-term multilineage repopulation [30, 31], were expressed in normal CD150⁺ and CD150⁻ SP cells, but depleted in CML counterparts (Fig. 7C). Nuclear factor κ B (NF- κ B) is important for HSC functions [32], and is activated in CML cells [33-35]. We found that NF- κ B was expressed in normal CD150⁻ and CD150⁺ SP cells; intriguingly, NF- κ B was activated by BCR-ABL in CD150⁺ SP but reduced in CD150⁻SP cells and SIRT1 knockout suppressed NF- κ B activation (Fig. 7C). Tumor suppressor p53 (Trp53) plays key roles in HSCs and CML LSCs [19, 36, 37]. We found that p53 was expressed in both normal CD150⁺ and CD150⁻ SP cells; upon transformation, significant reduction of its expression occurred in CD150⁻SP but not CD150⁺SP cells (Fig. 7C), consistent with the leukemogenic phenotype of CD150⁻SP cells. p53 mRNA expression remained low with SIRT1 knockout, but loss of SIRT1 can increase p53 protein acetylation and activation to inhibit CML LSCs as we described before [19]. Together, these results shed novel insight into an intricate molecular net work for regulating normal and leukemic stem cells in BABL/c mice.

Discussion

In this study we demonstrated that CD150⁻ SP cells are CML LSCs in the BALB/c mouse model of CML using bone marrow transduction/transplantation protocol. This mouse model has been widely used in CML study, but the identity of LSCs in this model has been poorly characterized except one previous study that also used SP to identify LSCs [38]. Interestingly, both CD150⁻SP and CD150⁺SP cells are enriched for LT-HSCs in BALB/c mice with moderately more enrichment in the latter, and both can be effectively transduced by retroviral vectors, but only the former are LSCs. In mouse models of chronic CML, LSCs typically have the same cell surface markers with normal HSCs [10, 11, 39, 40], but in mouse models of blast crisis CML created by combination of BCR-ABL with NUP98/HOXA9, or in the genetic E2A knockout background [41, 42], granulocyte-macrophage progenitors become LSCs. Notably, in the BCR-ABL and NUP98/HOXA9 blast crisis CML model, LSCs are also found in CD150⁻ cells [43]. Therefore, the results from our study suggest that in the BALB/c mouse CML model, although pathological phenotypes are displayed as chronic CML-like myeloproliferative disease, LSCs may actually share certain cellular phenotypes like blast crisis CML LSCs. Although CD150⁺SP CML cells were not LSCs, it is interesting to note that mice receiving 1,000 total SP CML cells trended to die faster than those receiving 1,000 CD150⁻SP CML cells (Fig.4E, $p=0.059$). This is reminiscent of a previous report that in the BMT setting mice receiving unpurified CML bone marrow cells develop more severe disease phenotypes than the purified CML stem cells [44]. It was suggested that more mature CML cells may facilitate the disease development from CML LSCs [44]. Although the mechanisms for such a phenomenon are not clear, it is possible that CD150⁺SP CML cells in total SP may aid CD150⁻SP LSCs to develop more severe disease phenotype and die faster.

Our molecular studies identified expression changes of several genes in normal HSCs and CML LSCs. In particular, cell cycle inhibitor Cdkn1c was reduced in normal CD150⁻SP

cells, which may contribute to their reduced quiescence and increased cell cycle entry. With depletion of Cdkn1c upon transformation, higher levels of Cdk6 in CML CD150⁻SP cells (than in CML CD150⁺SP) may activate these cells to become functional CML LSCs to produce leukemic progeny cells. On top of this, the LSC phenotype of CML CD150⁻SP cells was likely strengthened by the plunge of tumor suppressor p53 gene expression in these cells.

It is possible that strain-specific genetic factors may also play a role in affecting LSCs. BALB/c mice harbor two codon variants (A134C and G232A) of p16^{INK4a}, and these variants partially inactivate p16^{INK4a} functions to regulate cyclin D2/CDK4 [45]. In addition, BALB/c p16^{INK4a} promoter has an allelic variant that may also compromise p16^{INK4a} gene expression [46]. The INK4a/ARF locus encodes two key cell cycle regulators and tumor suppressors, p16^{INK4a} and p19^{ARF}, that have crucial roles in tumorigenesis [47]. INK4a/ARF mutations accelerate lymphomagenesis and promote chemoresistance by disabling p53 [48]. Homozygous deletion of the INK4a/ARF locus is a common mutation in blast crisis CML [49]. Loss of ARF gene enhances the oncogenicity in mouse models of BCR-ABL induced acute lymphoblastic leukemia [50]. Promoter hypermethylation of p16^{INK4a} has been reported by several studies in human CML, particularly those with lymphoid blast crisis [51]; however, the functional consequence of p16^{INK4a} inactivation in CML is unknown. It is possible that hypomorphic p16^{INK4a} in BALB/c may underlie the distinct features of CML LSCs in this model and perhaps cooperate with reduced p53 expression in CML CD150⁻SP cells to drive cellular change towards blast crisis LSCs. This raises an interesting question of potential role of p16^{INK4a} inactivation in promoting progression of CML to blast crisis, which remains to be tested.

Identification of LSCs in the BALB/c CML model will facilitate studies of CML LSC drug resistance. We have shown that SIRT1 activation by BCR-ABL transformation promotes CML LSC survival and resistance to tyrosine kinase inhibitors, and SIRT1 inhibition by small molecular inhibitor tenovin-6 sensitizes CML LSCs to imatinib [18, 19]. SIRT1 also promotes acquisition of BCR-ABL mutations in blast crisis CML cells for acquired resistance to tyrosine kinase inhibitors [52, 53]. Besides CML, we and others have recently shown that SIRT1 activation promotes LSC maintenance and drug resistance of FLT3-ITD acute myeloid leukemia [54, 55]. However, whether genetic loss of SIRT1 would affect LSCs has not been shown before because the identity of CML LSCs in BALB/c mice was not clear. Using CML markers established in this study, now we demonstrate that SIRT1 is activated in CML LSCs in the BALB/c CML model and SIRT1 homozygous knockout substantially depletes LSCs and compromises their maintenance, further supporting a crucial role of SIRT1 in leukemic stem cell functions.

Although we did not find dramatic hematological phenotypes in young SIRT1 knockout mice in BALB/c strain, two recent studies using conditional knockout in C57BL/6 strain reported that SIRT1 loss compromises hematopoiesis and HSC functions [56, 57]. The findings of these two studies are quite different from the other two previous SIRT1 knockout studies that were also in C57BL/6 strain and showed no significant impact on hematopoiesis and HSC functions [58, 59]. While the precise reasons for difference among these studies are not known, one possible explanation is that different ways to induce knockout either by

constitutive germline targeting, a ubiquitous promoter driving Cre expression to yield SIRT1 knockout or chemical induction of Cre expression for conditional knockout may impose different levels of stress on mouse bone marrow [60]. Since SIRT1 is a stress response gene, loss of SIRT1 may compromise hematopoiesis when significant stress is imposed by marrow-depleting agent tamoxifen or toxicity triggered by high expression of bacterial protein iCre (bacterial protein shock) during conditional knockout. In addition, phenotypic severity of SIRT1 knockout is strongly affected by mouse genetic background as discussed previously [61]. SIRT1 knockout has milder impact in BALB/c strain and the viable SIRT1^{-/-} mice can be generated though consecutive fostering as described in this study, but the fostering protocol does not work for the knockout mice in C57BL/6 strain (unpublished observation). Viable SIRT1 constitutive knockout mice in BALB/c strain allow functional study of SIRT1 loss without imposing additional stress on the mice.

Interestingly, we previously showed that combination of tenovin-6 with imatinib helps eradicate LSCs in a mouse model of chronic CML with inducible BCR-ABL expression [19], but such combination does not provide further survival benefit in the BALB/c transduction/transplantation model [18]. Our current study of LSCs in the BALB/c model may shed some new insight into this difference in that LSCs in the BALB/c model could lean towards certain features of blast crisis LSCs and could be more drug resistant. Therefore, more potent and specific SIRT1 inhibitors may be required to eradicate LSCs in the BALB/c CML model. In line with this notion, we showed that combination of SIRT1 homozygous knockout with imatinib provides additional survival benefit over individual treatment [18]. In conclusion, our study not only helps us better understand the biology of CML LSCs and their drug resistance, but may also provide a new tool for developing novel and potent SIRT1 inhibitors or other agents to eradicate CML LSCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. CML LSCs reside in CD150⁻ population in BALB/c mice

(A) Scheme for analysis and sorting of SLAM labeled cells. Lineage positive cells were depleted by EasySep and lineage-depleted cells were then labeled with lineage and SLAM marker antibodies for sorting. Lin⁻ cells were gated for GFP expression followed by SLAM marker separation. Fluorescence minus one (FMO) controls were performed with unfractionated bone marrow nucleated cells on the top row. FS, forward scatter. (B-D) Peripheral blood GFP positive cells (B), white blood cell counts (C) and survival curve (D) of mice receiving 1,500 sorted GFP⁺ leukemia cell populations. Six mice for each group. Two mice each died of CML in two CD150⁻ groups by the time of measurement and were excluded in B and C.

Figure 2. Both CD150⁺ and CD150⁻ side population are enriched for HSCs in normal BALB/c mice

(A) Gating strategy for sorting SP, CD150⁺SP and CD150⁻SP cells from lineage negative bone marrow progenitor cells. (B) Post sort analysis of sorted SP subfractions. (C) Analysis of CD16/32 and CD41 in SP cells. Lin⁺ cells were analyzed for comparison. (D) Four-month survival of lethally irradiated mice receiving 500 sorted SP, CD150⁺SP and CD150⁻SP cells, compared to 10,000 non-SP cells. Four mice each group. (E) Comparison of blood lineages of receipt mice by antibody staining and flow cytometry analysis at 5 months after transplantation with 500 sorted cells. (F) Representative flow cytometry profiles of bone marrow SP cells and their CD150 status in recipient mice reconstituted with 500 SP, CD150⁺SP or CD150⁻SP donor cells at 5 months after BMT. (G-I) Quantitation of SP frequency (G), CD150 status of SP cells (H), and distribution of lower vs upper fraction of SP cells from D (I). SP frequency was calculated by SP cell number per million total bone marrow cells that were harvested from four limbs (tibias and femurs). * for p<0.05; ** for p<0.01; n.s. for not significant.

Figure 3. Limiting dilution analysis of normal SP fractions

(A) SRY analysis of peripheral blood samples from recipients with 5 and 50 male donor SP cells at 12 weeks post BMT. One mouse died in the SPCD150⁻ group and was not included in analysis. (B, C) Blood cell counts (B) and lineage cell percentage (C) for recipients with 20 and 50 donor SP donor cells at 14 weeks post BMT. Two mice died in SPCD150⁻ group with 5 donor SP cells and data were not shown. (D) Stem cell frequency plots with extreme limiting dilution analysis. * for p<0.05; ** for p<0.01; n.s. for not significant.

Figure 4. CML LSCs are enriched in CD150⁻ SP cells in the BALB/c model

(A) Gating strategy for sorting GFP⁺CD150⁺ and GFP⁺CD150⁻ SP cells from lineage negative bone marrow progenitor cells from MIG210 BCR-ABL transformed CML mice. (B) Expansion of CD150⁺SP cells in CML mouse bone marrow compared to that in normal BALB/c mice. (C) Post sort analysis of sorted CML SP fractions. (D) Analysis of CD16/32 and CD41 for GFP⁺Lin⁻SP cells. GFP⁺Lin⁺ cells were analyzed for comparison. (E) Survival curves of recipient mice receiving 4 million total CML bone marrow cells, 1000 sorted non-SP or 1000 SP fractions of GFP⁺Lin⁻ cells as indicated. (F) The percentage of GFP⁺ nucleated cells in the blood of recipient mice as indicated. (G) H & E staining of spleen sections of the recipient mice from E. Disruption of splenic architecture was seen with SP, CD150⁻SP and total bone marrow (TBM), but not with CD150⁺SP or non-SP cells. Images were taken with a 40x objective. (H) Survival curves of tertiary BMT recipient mice with 4 million total BM cells from SP or CD150⁻ SP CML mice in E.

Figure 5. CD150⁻ SP cells reconstitute LSC compartments in the recipient BALB/c mice
Panels A-D for mice receiving MIG210 transduced cells, and panels E-G for mice receiving MIGR1 transduced cells. (A) Representative spleen side population analysis of recipient mice receiving 1000 SP, CD150⁻ SP or CD150⁺ SP cells of CML mice. (B) Total bone marrow GFP⁺SP cell number per mouse in different recipient groups from A. (C) The percentage of GFP⁺ cells in side population from bone marrow or spleen in the recipient mice. (D) Lineage analysis of peripheral blood of the recipient mice in A. (E) The scheme of MIGR1 transduction analysis: total male BM cells were transduced by MIGR1 and transplanted to female recipients. Three months after BMT, lineage negative SP fractions and non-SP cells were FACS sorted for secondary BMT with 100 sorted cells together with 0.3 million female helper bone marrow cells for each recipient. SRY analysis was performed for the recipient mice of MIGR1 transduced bone marrow at 4 months after secondary BMT. (F, G) The percentage of GFP⁺ blood nucleated cells in recipient mice receiving MIGR1 transduced SP fractions or non-SP cells at 4 months post secondary BMT (F), and lineage distribution of GFP⁺ blood nucleated cells at 2 months post secondary BMT (G). * for p < 0.05; ** for p < 0.01; n.s. for not significant.

Figure 6. CML stem cells are depleted by SIRT1 knockout

(A, B) SP cell number (A) and cell cycle status (B) from wild type (WT) and SIRT1 knockout (KO) mice. (C) SIRT1 protein expression in Lin⁻CD150⁺ and Lin⁻CD150⁻ cells from normal and CML mice. (D) Relative SIRT1 mRNA levels in CD150⁺ and CD150⁻ SP from normal and CML mice. (E) SIRT1 knockout significantly reduced CML CD150⁻SP LSCs and CD150⁺SP progenitors. CM stem or progenitor cell numbers were calculated from total bone marrow cells collected from four limbs. (F) Limiting dilution analysis of LSCs with unfractionated bone marrow nucleated cells from CML mice with or without SIRT1 knockout. (G,H) SIRT1 knockout inhibited maintenance of CML LSCs. CD150⁺ and CD150⁻ SP cells were sorted out from the GFP⁺Lin⁻ fraction of primary CML mice with or without SIRT1 knockout. One thousand cells each were transplanted into lethally irradiated recipient mice. The survival curve was shown in panel G and peripheral blood was analyzed for GFP-expressing nucleated cells at day 35 in panel H. * denotes $p < 0.05$; ** denotes $p < 0.01$.

Figure 7. Cell cycle and molecular analysis of normal and leukemic SP subfractions

(A) Cell cycle analysis of SPCD150⁺ vs SPCD150⁻ cells in normal and CML mice. (B) Nested RT-PCR analysis of genes regulating cell cycles for SPCD150⁺ vs SPCD150⁻ cells in normal mice and in CML mice with or without SIRT1 knockout. (C) Nested RT-PCR analysis of genes regulating stem cell self-renewal and tumorigenesis. * for P<0.05; ** for P<0.01; *** for P<0.001; **** for p<0.0001. n.s. for not significant.