

# SIRT1 is dispensable for maturation of hematopoietic stem cell in the bone marrow niche

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**Abstract.** Sirtuin 1 (SIRT1) is a histone deacetylase implicated in stem cell homeostasis. Conditional *Sirt1* deletion in the hematopoietic stem and progenitor system promotes hematopoietic stem and progenitor cell (HSPC) expansion under stress conditions. In addition, SIRT1 activators modulate the capacity and HSPC numbers in the bone marrow (BM). To investigate the role of SIRT1 in the BM niche, a conditional *Sirt1* deletion in the BM niche was generated in a mouse model for the present study. Multicolor flow cytometric analyses were performed to determine HSC cell populations. Using 5-fluorouracil-induced proliferative stress, a survival curve was produced. In the present study, *Sirt1* deletion in the BM niche demonstrated that the production of mature blood cells, lineage distribution within hematopoietic organs and frequencies of HSPC populations were comparable to those of controls. Additionally, *Sirt1* deletion in the BM niche did not perturb HSC maturation under stress induced by transplantation. Therefore, these observations suggest that SIRT1 serves a dispensable role in HSC maturation in the BM niche.

## Introduction

The bone marrow (BM) is a complex tissue comprised of multiple subsets of stromal cells. Studies using a number of different experimental approaches are beginning to unravel the stromal cell profile in the BM (1). The BM niche was the first proposed and experimentally defined niche for stem

cells in mammals (2). Bone marrow stromal cells serve to regulate hematopoiesis in the hematopoietic BM niche (1-3). Hematopoietic stem cells (HSC) mainly reside in the BM niche and participate in tissue regeneration under hematopoietic stress such as transplantation (4). However, under stress condition including transplantation, these HSCs can lose their capacity for self-renewal, resulting in stem cell exhaustion. Under these circumstances, allogenic HSCs have to be engrafted into the BM niche and subsequently expanded in a process known as transplantation (5).

The histone deacetylase sirtuin 1 (SIRT1) regulates a number of cellular responses, including cell proliferation, apoptosis and inflammatory responses by protein deacetylation (6-8). In particular, SIRT1 has also been implicated in stem cell homeostasis (9-12). Previous studies suggest that SIRT1 serves a role in HSC maturation and lineage specification as demonstrated by a conditional deletion approach, where *Sirt1* expression was knocked out only in the hematopoietic system (13,14). However, little is known about the role of SIRT1 in the BM niche, since SIRT1 was found to be dispensable for HSC activity due to developmental HSC adaptation in surviving conventional *Sirt1* KO mice in another study (15). In addition, the SIRT1 activator Resveratrol modulates HSC capacity in the BM *in vivo* (16), whereas SRT3025, another SIRT1 activator, also altered the number of HSCs (17); these observations suggest that SIRT1 is important for HSC maturation. In the present study aim was to elucidate the role of SIRT1 in the BM niche to provide novel insight for HSC transplantation.

## Materials and methods

**Ethics statement and animals.** All animals were housed in an air-conditioned room (22-25°C; 12-h light/dark cycle; 50% humidity) with free access to food pellets and tap water. *Ocn-Cre* transgenic mice used in BM niches (18) and *Sirt1* mice (19) have been described (Fig. S1). *Ocn-Cre*, floxed *Sirt1* and wild-type C57BL/6 mice were purchased from Jackson Laboratory and employed for experiments. The *Ocn-Cre* mice are widely used to target osteoblasts in the BM niche (18). To investigate the role for HSC maturation of *Sirt1* in the BM

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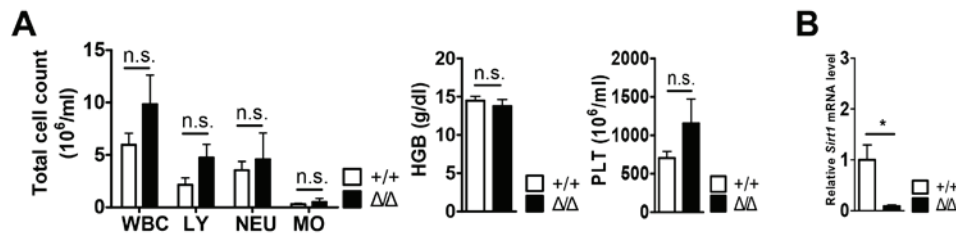


Figure 1. No changes in white blood cells, hemoglobin, and platelets counts of *Sirt1* $\Delta\Delta$  mice. (A) White blood cell counts, lymphocytes, neutrophil, monocytes, hemoglobin and platelet measurements from mice of the indicated genotype ( $n=4-5$ ). (B) Reverse transcription-quantitative PCR analysis of *Sirt1* expression in CD31 $^{+}$ CD45 $^{+}$ Ter119 $^{+}$  *Sirt1* $\Delta\Delta$  bone marrow cells ( $n=3$ ). Data are presented as mean + SEM. \* $P<0.05$ . n.s., not significant; Sirt1, sirtuin 1; *Sirt1* $\Delta\Delta$ , sirtuin 1 conditional knockout; WBC; white blood cells; LY, lymphocytes; NEU, neutrophils; MO, monocytes; HGB, hemoglobin; PLT, platelet; CD, cluster of differentiation.

niche, such as in osteoblast, *Sirt1*<sup>flxed/flxed</sup> mice were crossed with *Ocn-Cre*. A total of 70 mice (female; weight, 15-18 g; age, 8-10 weeks) were used for experiments.

Transplantations of BM cells were performed as described previously (20). Briefly, mice were euthanized then tibias and femurs were recovered. Scalpel was used to cut the ends of bone off and then a syringe with 27G needle (filled with PBS supplemented with 2% FBS and 1% P/S) was used to flush into a 50 ml tube covered with 40  $\mu$ M nylon filter. Recovered BM cells were lysed in RBC lysis buffer (Lonza Group, Ltd.) for 5-10 min on ice. Samples were then washed with 10 ml PBS supplemented with 2% FBS and 1% P/S and centrifuged 5 min at 1,500 rpm. Supernatant was aspirated and the cell pellets were collected for the experiments.

Control and *Sirt1* $\Delta\Delta$  mice received lethal doses of irradiation (9.5 Gy, Gammacell 3000) prior to the transplantation of 200  $\mu$ l  $1 \times 10^6$  BM cells by retro-orbital injection on the same day as previously described (21). For serial 5-FU treatments, 5-FU (150 mg/kg) was injected intraperitoneally every seven days until 100% animal mortality was achieved (22). For the duration of the present study, the following criteria were applied to define a mouse as moribund, which would require immediate euthanasia (23,24): i) Ruffled and/or matted fur; ii) weight loss of >15%; iii) hypothermia (detected by touching); iv) hunched posture; v) unable to eat or drink freely; vi) barrel rolling. No animals satisfied these criteria in the present study.

**Reverse transcription-quantitative PCR (RT-qPCR).** Reverse transcription and quantitative PCR were performed as previously described (14,25). Briefly, total RNAs were extracted from BM cells using QIAGEN RNeasy-Plus Mini-columns according to manufacturer's protocol (Qiagen, Inc.). cDNA synthesis were performed following the protocols of the manufacturer of the kit (Bio-Rad Laboratories, Inc.). qPCR was performed with SYBR Green Mix (Roche Diagnostics) and LightCycler 96 instrument (Roche Diagnostics) and data were normalized to the housekeeping gene *GAPDH*. The mRNA levels were measured using the  $2^{-\Delta\Delta Cq}$  method of quantification (26). RT-qPCR was performed using the following primers: *Sirt1* forward, 5'-CTGAAAGTGAGACCAGTACGA-3' and reverse, 5'-GATGAGGCAAAGGTTCCCTA-3' and *GAPDH* forward, 5'-GCACAGTCAAGGCCGAGAAT-3' and reverse, 5'-GCCTTCTCCATGGTGGTGAA-3'.

**Flow cytometric analysis.** Flow cytometry was performed as described previously (20). Briefly, BM cells were collected

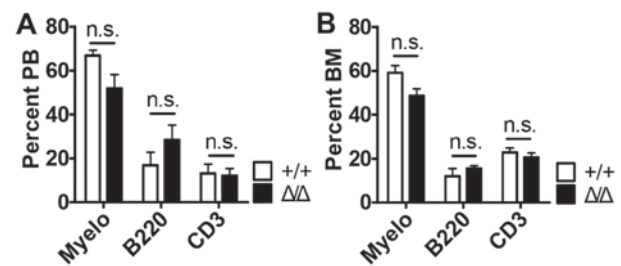


Figure 2. *Sirt1* deletion in the BM niche is dispensable for maintaining mature hematopoietic lineage cells. (A and B) The percentages of myeloid, B and T cells from (A) the peripheral blood, and (B) the bone marrow of *Sirt1* $\Delta\Delta$  and control mice ( $n=4-5$ ). Data are presented as mean + SEM. Mac1, macrophage-1 antigen; n.s., not significant; Sirt1, sirtuin 1; *Sirt1* $\Delta\Delta$ , sirtuin 1 conditional knockout; Myelo, Myeloid (Mac1 $^{+}$ ) cells; B220 $^{+}$  cells, B cells; CD3 $^{+}$  cells, T cells; PB, peripheral blood; BM, bone marrow.

from femurs and tibias of the mice by flushing using fluorescence-activated cell sorting buffer, which consisted of phosphate buffered saline containing 2% fetal bovine serum (Hyclone; GE Healthcare Life Sciences) and 0.1% sodium azide. Peripheral blood cells were collected from the tail vein. Flow cytometry was performed using antibodies listed in Table S1. Data acquisition and analysis were performed with Cell Quest v.3.3 or Diva software v.6.1.3 (BD Biosciences) and with FlowJo software v.10.3 (FlowJo LLC) respectively.

**Statistical analysis.** The statistical significance of differences in the means between two populations were assessed using two-tailed unpaired Student's t-test.  $P<0.05$  was considered to indicate a statistically significant difference for pairwise comparisons (between indicated genotypes in the same population). The Kaplan-Meier log-rank test was used to analyze survival data.

## Results

**Effects of *Sirt1* deletion on the BM niche.** SIRT1 is a critical regulator of homeostatic adult HSCs in mice (13,14). Based on these observations, it is tempting to speculate that SIRT1 may also serve a role for HSC maturation in the BM niche. To investigate the role for HSC maturation of SIRT1 in the BM niche, *Sirt1*<sup>flxed/flxed</sup> mice were crossed with *Ocn-Cre* transgenic mice, resulting in *Sirt1* $\Delta\Delta$  mice. A previous study showed that the loss of *Sirt1* in the hematopoietic system resulted in anemia and specified lineage decision (myeloid

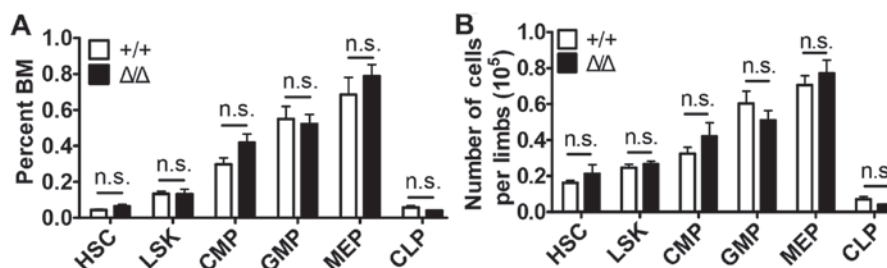


Figure 3. *Sirt1* deletion in the BM niche is dispensable for maintaining hematopoietic stem and progenitor cells. (A) The frequencies and (B) number of cells/limb of HSC, LSK, CMP, GMP, MEP and CLP; from the BM of *Sirt1*<sup>ΔΔ</sup> and control mice (n=4-5). Data are presented as mean + SEM. n.s., not significant; Sirt1, sirtuin 1; *Sirt1*<sup>ΔΔ</sup>, sirtuin 1 conditional knockout; Lin, lineage; Sca1, spinocerebellar ataxia type 1; cKit, mast/stem cell growth factor receptor kit; HSC, hematopoietic (Lin<sup>-</sup>Sca1<sup>+</sup>cKit<sup>+</sup>CD150<sup>+</sup>CD48<sup>-</sup>) stem cells; LSK, Lin<sup>-</sup>Sca1<sup>+</sup>cKit<sup>+</sup> cells; CMP, common myeloid progenitor (Lin<sup>-</sup>Sca1<sup>+</sup>cKit<sup>+</sup>CD34<sup>+</sup>CD16/32<sup>-</sup>) cells; GMP, granulocyte-macrophage progenitor (Lin<sup>-</sup>Sca1<sup>+</sup>cKit<sup>+</sup>CD34<sup>+</sup>CD16/32<sup>+</sup>) cells; MEP, megakaryocyte-erythroid progenitor (Lin<sup>-</sup>Sca1<sup>+</sup>cKit<sup>+</sup>CD34<sup>+</sup>CD16/32<sup>-</sup>) cells; CLP, common lymphoid progenitor (Lin<sup>-</sup>Sca1<sup>low</sup>cKit<sup>low</sup>CD127<sup>+</sup>) cells. BM, bone marrow.

versus lymphoid lineage specification) (14). However, *Sirt1* deletion in the BM niche demonstrated no differences in the numbers of white blood cells (WBCs), lymphocytes (LY), neutrophils (NEU), monocytes (MO), hemoglobin (HGB), and platelets (PLT) (Fig. 1A). It was also found that *Sirt1*<sup>ΔΔ</sup> mice did not result in significant differences compared with wild-type mice in their ability to produce mature myeloid cells (Mac1<sup>+</sup> cells), T cells (CD3<sup>+</sup> cells) and B cells (B220<sup>+</sup> cells) in the peripheral blood (PB) and bone marrow (BM) (Fig. 2 and Fig. S2). The expression of *Sirt1* was decreased in the CD31<sup>+</sup>CD45<sup>+</sup>Ter119<sup>-</sup> BM niche cells of *Sirt1*<sup>ΔΔ</sup> mice compared with *Sirt1*<sup>+/+</sup> mice, which confirmed *Sirt1* knock-down (Fig. 1B).

**Effects of *Sirt1* loss on HSPCs maturation were examined.** No differences in the numbers of HSCs (Lin<sup>-</sup>Sca1<sup>+</sup>cKit<sup>+</sup>CD150<sup>+</sup>CD48<sup>-</sup>, SLAM cells), Lin<sup>-</sup>Sca1<sup>+</sup>cKit<sup>+</sup> (LSK) progenitor cells, common myeloid progenitor (CMP) cells (Lin<sup>-</sup>Sca1<sup>+</sup>cKit<sup>+</sup>CD34<sup>+</sup>CD16/32<sup>-</sup>), granulocyte-macrophage progenitor (GMP) cells (Lin<sup>-</sup>Sca1<sup>+</sup>cKit<sup>+</sup>CD34<sup>+</sup>CD16/32<sup>+</sup>), megakaryocyte-erythroid progenitor (MEP) cells (Lin<sup>-</sup>Sca1<sup>+</sup>cKit<sup>+</sup>CD34<sup>+</sup>CD16/32<sup>-</sup>) and common lymphoid progenitor (CMP) cells (Lin<sup>-</sup>Sca1<sup>low</sup>cKit<sup>low</sup>CD127<sup>+</sup>) were observed in the *Sirt1*<sup>ΔΔ</sup> mice compared with control mice (Fig. 3 and Fig. S3).

***Sirt1* deletion in the BM niche does not alter HSC maturation.** To assess the role of HSC maturation following *Sirt1* deletion in the BM niche under stress condition, wild-type BM cells were transplanted into *Sirt1*<sup>ΔΔ</sup> mice, which had been lethally irradiated to remove endogenous blood cells (Fig. 4 and Fig. S4). Compared with control mice, *Sirt1*<sup>ΔΔ</sup> mice exhibited no detectable effects on peripheral blood (PB) donor chimerism (Fig. 4A), bone marrow (BM) donor chimerism and the numbers of mature hematopoietic cell types up to 16 weeks post-transplantation (Fig. 4B). In addition, *Sirt1*<sup>ΔΔ</sup> mice demonstrated no differences in the numbers of WBCs, Lys, NEUs, MOs and the levels of hemoglobin (HGB) four weeks post-transplantation compared with control mice (Fig. S5).

Next the *Sirt1*<sup>ΔΔ</sup> mice were repeatedly treated with 5-FU weekly to assess their sensitivity to stress under non-transplantation stress. Exposure of *Sirt1*<sup>ΔΔ</sup> mice to serial proliferative stress induced by 5-FU (22) exerted in no significant differences

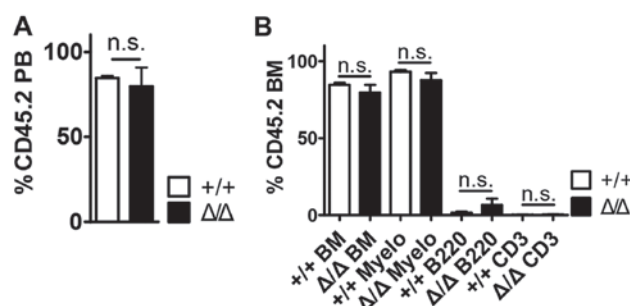


Figure 4. Hematopoietic stem cells were preserved in *Sirt1*<sup>ΔΔ</sup> mice under transplantation stress. (A) Peripheral blood was collected and analyzed for CD45.2<sup>+</sup> chimerism at 4 months post-transplantation (n=5). (B) The frequencies of myeloid, B and T cells (CD3<sup>+</sup>) in the BM from transplant recipients are shown 4 months post-transplantation (n=5). Data are presented as mean + SEM. n.s., not significant; Sirt1, sirtuin 1; *Sirt1*<sup>ΔΔ</sup>, sirtuin 1 conditional knockout; Mac1, macrophage-1 antigen; Myelo, Myeloid (Mac<sup>+</sup>) cells; B220, B cells; CD3, T cells; PB, peripheral blood; BM, bone marrow.

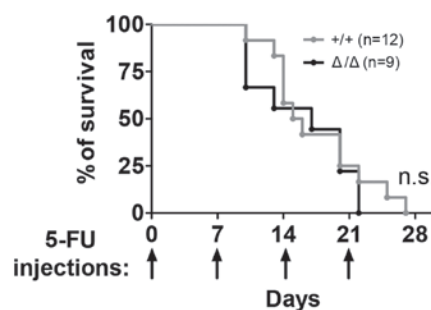


Figure 5. *Sirt1*<sup>ΔΔ</sup> mice did not respond differently to stress induced by 5-FU compared with control. Survival of *Sirt1*<sup>ΔΔ</sup> mice following weekly 5-FU (150 mg/kg) injections (n=9-12). Arrows indicate the day of 5-FU injection. n.s., not significant; Sirt1, sirtuin 1; *Sirt1*<sup>ΔΔ</sup>, sirtuin 1 conditional knockout; 5-FU, 5-fluorouracil.

in animal mortality compared with control mice, according to the Kaplan-Meier curve (Fig. 5).

## Discussion

In the present study, it was demonstrated that *Sirt1* deletion in the BM niche (*Sirt1*<sup>ΔΔ</sup>) was dispensable for maintaining mature hematopoietic lineage cells and HSC maturation.

Additionally, HSCs were preserved in *Sirt1*<sup>Δ/Δ</sup> under transplantation stress. Also, *Sirt1*<sup>Δ/Δ</sup> mice did not respond differently to 5-fluorouracil (5-FU)-induced stress compared with control mice. Collectively, these findings suggested that *Sirt1* deletion in the BM niche is dispensable for maturation of HSC.

First, a previous study showed that *Sirt1* deletion in the HSCs resulted in anemia, a significantly decreased number of lymphocytes and increased numbers of neutrophils, monocytes and eosinophils (16). SIRT1 is essential for the myeloid versus lymphoid lineage specification in the hematopoietic blood cells (14). However, in the present study it was found that *Sirt1* deletion in the BM niche lead to normal production of mature blood cells and lineage distribution within BM cells. In addition, no differences were observed between *Sirt1*<sup>Δ/Δ</sup> mice and wild-type mice in their ability to produce mature cells in the PB and BM.

A previous study showed that HSC expanded in response to the loss of *Sirt1* in the hematopoietic compartment (13), suggesting that SIRT1 was dispensable for HSC activity due to the developmental adaptation of HSCs (15). The question on the role of SIRT1 in HSC maturation has long been debated. However, the present study found that *Sirt1* deletion in the BM niche demonstrated pools of HSPC populations that were comparable to those of controls.

Finally, previous studies showed that SIRT1 is essential for HSC maturation under stress conditions, including resveratrol treatment and Fanconi anemia, SIRT1 activators enhanced HSPC capacity of the BM *in vivo* and increased the number of HSCs (Lin<sup>-</sup>Sca1<sup>+</sup>cKit<sup>+</sup> cells and Lin<sup>-</sup>Sca1<sup>+</sup>cKit<sup>+</sup>CD150<sup>+</sup>CD48<sup>-</sup> cells) (16,17). However, in the present study it was found that *Sirt1*<sup>Δ/Δ</sup> deletion in the BM niche did not perturb HSC maturation under transplantation stress or in the presence of 5-FU, unlike *Sirt1* deletion in the HSCs (13,14). Collectively, SIRT1 serves a dispensable role in HSC maturation in the BM niche, but not in the hematopoietic compartment. The findings of the present study contributed to better understanding of the molecular biology involved in the BM niche and hematopoiesis.

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#### Availability of data and materials

All data generated or analyzed during this study are included in this published article.

#### Authors' contributions

SMP, DHSh, JYK, DYP, DHSO, YHK, SMK, JHK, SSB, KK, CDKi, CDKa and DL contributed to the design of the study.

SMP, JYK and CMH acquired and analyzed the data. SMP and DL drafted the manuscript. DHSh, JYK, DYP, DHSO, YHK, SMK, JHK, SSB, KK, CDKi, CDKa and DL revised and edited the manuscript. DL acquired funding and resources. All authors read and approved the final version of the manuscript.

#### Ethical approval and consent to participate

The study was approved by the Ethics Committee of Pusan National University School of Medicine, Republic of Korea.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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