

# Osteoclasts are dispensable for hematopoietic stem cell maintenance and mobilization

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**Hematopoietic stem cells (HSCs) are maintained in a specific bone marrow (BM) niche in cavities formed by osteoclasts. Osteoclast-deficient mice are osteopetrotic and exhibit closed BM cavities. Osteoclast activity is inversely correlated with hematopoietic activity; however, how osteoclasts and the BM cavity potentially regulate hematopoiesis is not well understood. To investigate this question, we evaluated hematopoietic activity in three osteopetrotic mouse models: *op/op*, *c-Fos*-deficient, and RANKL (receptor activator of nuclear factor kappa B ligand)-deficient mice. We show that, although osteoclasts and, by consequence, BM cavities are absent in these animals, hematopoietic stem and progenitor cell (HSPC) mobilization after granulocyte colony-stimulating factor injection was comparable or even higher in all three lines compared with wild-type mice. In contrast, osteoprotegerin-deficient mice, which have increased numbers of osteoclasts, showed reduced HSPC mobilization. BM-deficient patients and mice reportedly maintain hematopoiesis in extramedullary spaces, such as spleen; however, splenectomized *op/op* mice did not show reduced HSPC mobilization. Interestingly, we detected an HSC population in osteopetrotic bone of *op/op* mice, and pharmacological ablation of osteoclasts in wild-type mice did not inhibit, and even increased, HSPC mobilization. These results suggest that osteoclasts are dispensable for HSC mobilization and may function as negative regulators in the hematopoietic system.**

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Abbreviations used: 5-FU, 5-fluorouracil; BMD, bone mineral density; HSC, hematopoietic stem cell; HSPC, hematopoietic stem and progenitor cell; LSK, lineage negative, c-Kit positive, and Sca1 positive; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor kappa B ligand.

Hematopoietic stem cells (HSCs) have the ability to self-renew as well as to produce multiple lineages of daughter cells throughout an animal's life (Adams and Scadden, 2006; Kiel and Morrison, 2008). Hematopoietic stem and progenitor cells (HSPCs) are thought to reside in specific niches, which are specialized microenvironments within the BM cavity (Calvi et al., 2003; Zhang et al., 2003; Arai et al., 2004; Kiel et al., 2005; Stier et al., 2005; Adams and Scadden, 2006; Sugiyama et al., 2006; Kiel and Morrison, 2008; Lympieri et al., 2010). Niches consist of various cell types such as osteoblastic cells, vascular endothelial cells or reticular cells, and associated factors such as

angiopoietin1, N-cadherin, osteopontin, and Cxcl12 (Calvi et al., 2003; Zhang et al., 2003; Arai et al., 2004; Kiel et al., 2005; Stier et al., 2005; Adams and Scadden, 2006; Sugiyama et al., 2006; Kiel and Morrison, 2008; Lympieri et al., 2010). A functional BM is reportedly required for HSPC mobilization to the periphery from BM cavities (Katayama et al., 2006). Thus, these cavities are considered crucial for HSPC

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maintenance and mobilization; however, the impact of loss of BM cavities on the hematopoietic system remains unclear.

BM cavities are formed by osteoclasts, which are bone-resorbing multinuclear cells, and loss of osteoclasts results in loss of BM cavities (Yoshida et al., 1990; Grigoriadis et al., 1994; Kong et al., 1999). Various factors, such as M-CSF, *c-Fos*, and receptor activator of nuclear factor kappa B ligand (RANKL), are reportedly essential for osteoclastogenesis; mutational inactivation or genetic ablation of these molecules results in a lack of osteoclast differentiation and consequent defects in BM cavity formation, a condition termed osteopetrosis in which BM cavities are filled with bone (Yoshida et al., 1990; Grigoriadis et al., 1994; Kong et al., 1999). Osteoprotegerin (OPG) is a decoy receptor of RANKL that inhibits osteoclastogenesis (Bucay et al., 1998; Mizuno et al., 1998). Although bone phenotypes of *op/op*, *c-Fos*-deficient, RANKL-deficient, and OPG-deficient mice have been described, hematopoietic phenotypes in these mice have not yet been fully characterized. Clinically, osteoclastic activity increases after serial G-CSF injection administered before peripheral HSC implantation (Takamatsu et al., 1998; Watanabe et al., 2003), and such activity is reportedly required to drive HSPCs from the marrow to the periphery (Kollet et al., 2006). Therefore osteoclast-deficient mice, which lack a BM niche, are predicted to be defective in hematopoiesis as a result of impaired osteoclast activity required to mobilize HSPCs to the periphery. Indeed, osteopetrotic patients, as well as animal models, reportedly show extramedullary hematopoiesis in the spleen (Freedman and Saunders, 1981; Lowell et al., 1996).

Osteoclastogenesis is accelerated with age, resulting in decreased bone mass (Manolagas and Jilka, 1995; Teitelbaum, 2007). Hematopoietic activity also decreases with age (Geiger and Rudolph, 2009; Waterstrat and Van Zant, 2009), suggesting that osteoclast activity is inversely correlated with hematopoietic activity; however, precise roles of osteoclasts in regulating hematopoiesis are largely unknown.

In this paper, we show that HSPCs are maintained even in osteopetrotic animals and that they are mobilized to the periphery after G-CSF stimulation, indicating that osteoclasts and BM cavities are both dispensable for HSPC maintenance and egress to the periphery. Pharmacological inhibition of osteoclastic activity did not inhibit but rather stimulated HSPC mobilization. Splenectomy of *op/op* mice increased HSPC mobilization, suggesting that spleen is not the primary tissue that maintains HSCs in osteopetrotic animals. HSCs were also detected in the osteopetrotic bone. OPG-deficient mice, which show increased osteoclastic activity, exhibited reduced HSPC mobilization. Thus, accelerated osteoclastic activity decreases both bone mass and hematopoietic activity, and both can be manipulated pharmacologically *in vivo*.

## RESULTS AND DISCUSSION

### HSPCs are maintained and mobilized in *op/op* mice

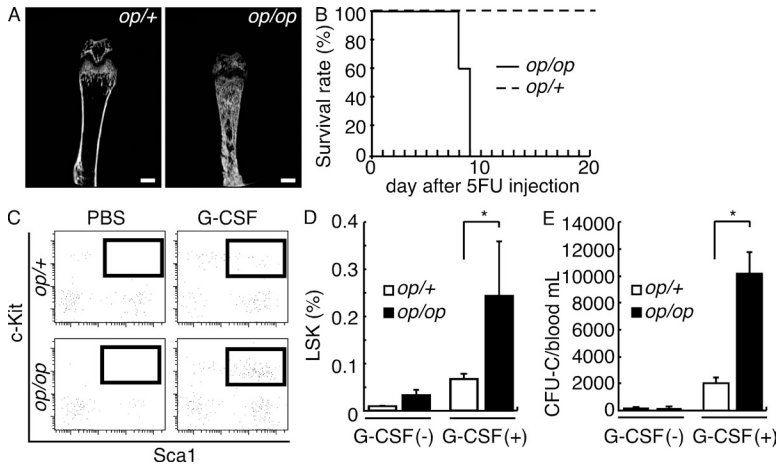
To determine whether loss of the BM niche and osteoclasts influences hematopoiesis, we analyzed osteopetrotic *op/op* mice, which carry a loss-of-function mutation in M-CSF, a cytokine essential for osteoclast differentiation (Yoshida et al., 1990).

These mice lack a BM cavity and exhibit impaired osteoclastogenesis (Fig. 1 A). Previously, the cell cycle-specific cytotoxic agent 5-fluorouracil (5-FU), which is used in chemotherapy, has been used to evaluate HSC function because HSCs are resistant to 5-FU-induced cell death as a result of their quiescence (Cheng et al., 2000; Miyamoto et al., 2007). *op/op* and wild-type mice were serially treated with 5-FU, and we found that *op/op* mice were lethally susceptible to 5-FU treatment compared with control *op/+* mice (Fig. 1 B), suggesting that osteoclasts and BM cavities are required to maintain HSCs.

To confirm roles of osteoclasts and BM cavities in the hematopoietic system, *op/op* mice were serially treated with G-CSF and HSPCs mobilization was analyzed (Fig. 1, C–E). Because a functional BM niche or osteoclasts are reportedly required to mobilize HSPCs to the periphery from the BM (Heissig et al., 2002; Katayama et al., 2006; Kollet et al., 2006), we predicted that *op/op* mice would show inhibited HSPC mobilization after G-CSF injection. However, we found that mobilization of the HSC-enriched fraction (lineage negative, *c-Kit* positive, and *Sca1* positive [LSK]) to peripheral blood, as analyzed by flow cytometry, was induced to levels even higher in *op/op* mice than in control littermate mice (*op/+*; Fig. 1, C and D). HSPCs mobilized in *op/op* mice were functional: colony-forming assays showed that greater numbers of colonies formed from the peripheral blood of G-CSF-treated *op/op* mice than from that of wild-type mice (Fig. 1 E). This observation suggests that *op/op* mice accumulate a greater number of HSCs than do wild-type mice, and that neither BM cavities nor osteoclasts are required for HSC maintenance and mobilization and may even be inhibitory to the process.

### HSPC mobilization is induced in *c-Fos*- and RANKL-deficient mice

To confirm these findings, *c-Fos*-deficient mice, which are also osteoclast deficient and lack BM cavities, were analyzed (Fig. S1 A). We observed that *c-Fos*-deficient mice were less susceptible to serial 5-FU treatment than were *op/op* mice (Fig. S1 B), suggesting that M-CSF, rather than BM cavities and osteoclasts, is required to resist 5-FU treatment. Indeed, injection of M-CSF into *op/op* mice rescued mice from a lethal response to 5-FU (Fig. S1 C). M-CSF regulates osteoclast formation as well as macrophage differentiation and innate immunity (Miyamoto et al., 2001; Chitu and Stanley, 2006). In wild-type mice, M-CSF concentration in sera was significantly up-regulated after 5-FU treatment, suggesting a protective role for M-CSF against myelosuppression induced by chemotherapy (Fig. S1 D). In fact, both Gram-positive and -negative bacteria were detected in multiple organs of 5-FU-treated *op/op* mice (Fig. S1 E and not depicted). These results indicate that by inducing macrophages, M-CSF likely plays a critical role in resistance to hematopoietic suppression after 5-FU treatment. They also suggest that serum M-CSF concentrations should be carefully monitored during myelosuppressive chemotherapies to prevent opportunistic infections.

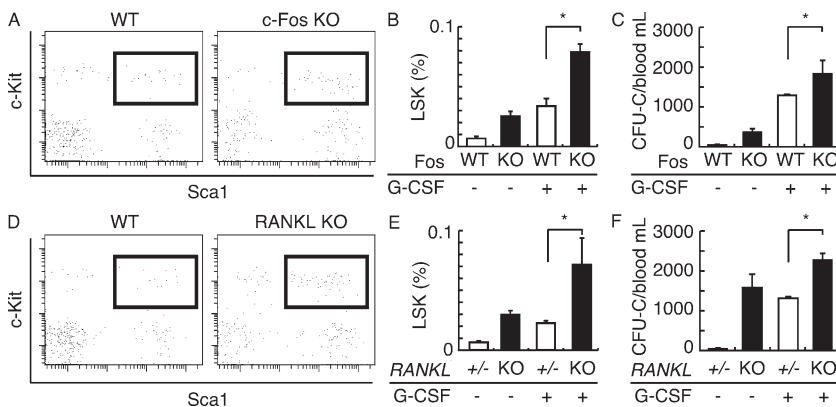


**Figure 1. *op/op* mice are osteopetrotic and exhibit increased numbers of HSPCs.** (A) Microradiographical analysis of femoral bones of 8-wk-old *op/op* and control (*op/+*) bone. Representative micro-CT data in three independent *op/op* or *op/+* mouse bones are shown. Bars, 1 mm. (B) 150 mg/kg 5-FU was injected into the peritoneal cavity of 8-wk-old *op/op* ( $n = 5$ ) and littermate control (*op/+*;  $n = 5$ ) mice on days 0 and 7, and the survival rate after 5-FU injection was analyzed. (C–E) 250  $\mu$ g/kg/d G-CSF or PBS was injected subcutaneously into 8–12-wk-old *op/op* and control (*op/+*) mice daily for 5 d ( $n = 3$  for each group). Peripheral blood was collected, and the frequency of the HSC fraction (LSK; C and D) and CFU-C (E) were analyzed. (C) Representative flow cytometric pattern of peripheral blood. Cells were gated on lineage (CD3, B220, TER119, Mac1, and Gr1)-negative cells. Data represent the mean LSK frequency (%)  $\pm$  SD in peripheral blood (\*,  $P < 0.051$ ; D) and CFU-C  $\pm$  SD in 1 ml peripheral blood (\*,  $P < 0.01$ ,  $n = 9$  for each group; E). Representative data of three independent experiments are shown (B–E).

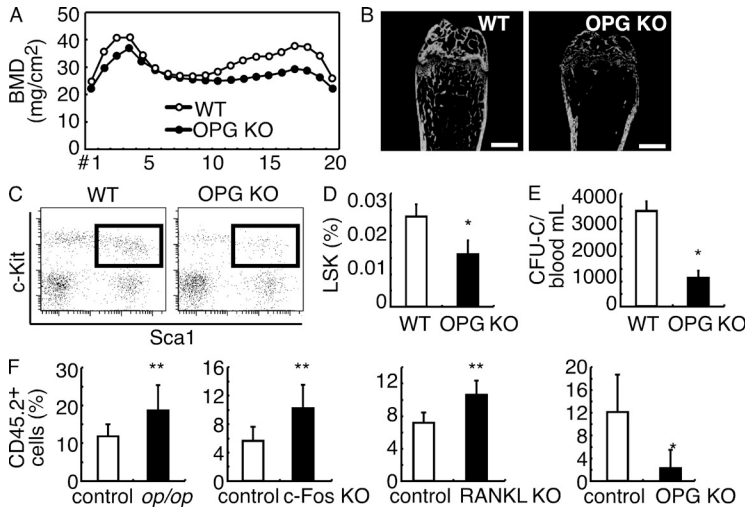
RANKL-deficient mice are also osteoclast deficient and lack BM cavities (Fig. S1 F). Interestingly, increased HSPC mobilization after serial G-CSF treatment was observed in both *c-Fos*-deficient and RANKL-deficient mice (Fig. 2), as it was in *op/op* mice, supporting the idea that osteoclasts and BM cavities are not required to maintain HSCs. Young female *PTP $\epsilon$* -knockout mice reportedly show a mild loss of osteoclast function but exhibit a BM cavity and impaired HSPC mobilization by G-CSF (Kollet et al., 2006). The three osteoclast- and BM cavity-deficient animal models evaluated in this study did not show defective HSPC mobilization after G-CSF treatment, suggesting that the function of osteoclasts in driving HSCs to the periphery is biphasic or BM cavity dependent. In wild-type mice, the number of osteoclasts reportedly does not increase during G-CSF treatment, but only after cessation of treatment (Takamatsu et al., 1998; Christopher and Link, 2008; Winkler et al., 2010), also suggesting that osteoclast loss likely does not affect HSPC mobilization in response to G-CSF treatment. All three osteopetrotic animal models show increased mobilization of HSPCs to the periphery without G-CSF administration (Figs. 1 and 2), suggesting that steady-state levels of circulating HSPCs are increased in these animals.

**Impaired HSPC mobilization in OPG-deficient mice**

To further explore the role of BM cavities and osteoclasts in HSC maintenance, HSPC mobilization in OPG-deficient mice was analyzed by serial G-CSF injection (Fig. 3). OPG is a decoy receptor for RANKL and therefore acts as a RANKL inhibitor (Bucay et al., 1998; Mizuno et al., 1998). OPG-deficient mice show severe osteoporosis as a result of accelerated osteoclastogenesis (Fig. 3, A and B). As expected, in contrast to osteoclast-deficient osteopetrotic mice, reduced HSPC mobilization was seen in OPG-deficient mice, as indicated by flow cytometry and colony-forming assays, compared with wild-type mice (Fig. 3, C–E). Furthermore, long-term competitive repopulation assays using mobilized cells from *CD45.2<sup>+</sup> op/op*, *c-Fos*-deficient, or RANKL-deficient mice versus *CD45.1<sup>+</sup>* BM competitors showed higher chimerism relative to that induced using mobilized cells from control mice (Fig. 3 F). In contrast, mobilized cells from OPG-deficient mice induced lower chimerism than those from wild-type mice (Fig. 3 F). *CD45.2* reconstitution was multilineage; *CD45.2* reconstitution from mobilized donor



**Figure 2. *c-Fos*-deficient and RANKL-deficient mice show elevated HSPC pools.** (A–F) 250  $\mu$ g/kg/d G-CSF or control PBS was injected subcutaneously into 8–12-wk-old *c-Fos*-deficient (*c-Fos* KO;  $n = 5$  for each group) or RANKL-deficient (RANKL KO;  $n = 5$  for each group) mice or into respective control littermates (wild-type [WT];  $n = 5$  for each group) daily for 5 d. Peripheral blood was collected, and the proportion of HSCs (A, B, D, and E) and CFU-C (C and F) were analyzed. (A and D) Representative flow cytometry pattern of peripheral blood. Cells were gated on lineage-negative cells. Data represent the mean LSK frequency (%)  $\pm$  SD in peripheral blood (\*,  $P < 0.01$ ,  $n = 5$  for each group; B and E) and mean CFU-C  $\pm$  SD in 1 ml peripheral blood (\*,  $P < 0.01$ ,  $n = 6$  for each group; C and F). Representative data from one of three independent experiments are shown (A–F).



**Figure 3. OPG-deficient mice are osteoporotic and have reduced HSPC pools.** (A and B) BMD assays and microradiography analysis were undertaken in femurs of 8–12-wk-old OPG-deficient (OPG KO;  $n = 5$ ) and wild-type (WT;  $n = 5$ ) mice using dual-energy x-ray absorptiometry (mg/cm<sup>2</sup>; A) and micro-CT (B), respectively. BMD was shown in 20 longitudinal divisions of femurs. Bars, 1 mm. (C–E) 250  $\mu$ g/kg/d G-CSF was injected subcutaneously into OPG-deficient (OPG KO;  $n = 5$ ) and wild-type control (WT;  $n = 5$ ) mice daily for 5 d. Peripheral blood was then collected, and the frequency of the LSK fraction (C and D) and CFU-C (E) were analyzed by flow cytometry and colony-forming assays, respectively. (C) Representative flow cytometry pattern of peripheral blood cells. Cells were gated with lineage-negative cells. Data represent the mean LSK frequency (%)  $\pm$  SD in peripheral blood (\*,  $P < 0.01$ ,  $n = 5$ ; D) and CFU-C  $\pm$  SD in 1 ml peripheral blood (\*,  $P < 0.01$ ,  $n = 12$ ; E). (F) 250  $\mu$ g/kg/d G-CSF was injected subcutaneously into OPG-deficient (OPG KO), c-Fos-deficient (c-Fos KO), RANKL-deficient (RANKL KO), and littermate control mice daily for 5 d. Peripheral blood was

collected from each mouse and transplanted into lethally irradiated Ly5.1 recipient mice with BM competitors isolated from Ly5.1 mice. Peripheral blood of recipient mice was collected after 3 mo of transplantation and stained with PE-conjugated anti-CD45.1 and FITC-conjugated anti-CD45.2, and leukocyte chimerism was determined by FACS. Data represent the mean CD45.2<sup>+</sup> frequency (%)  $\pm$  SD in peripheral blood (\*,  $P < 0.01$ ; \*\*,  $P < 0.05$ ,  $n = 8$ ). Representative data of two (A, B, and F) and three (C, D, and E) independent experiments are shown.

HSPCs was observed in CD11b<sup>+</sup> myeloid, B220<sup>+</sup> B cell, and CD3<sup>+</sup> T cell recipient lineages (Fig. S2). Thus, osteoclasts and BM cavities appear to antagonize HSC maintenance.

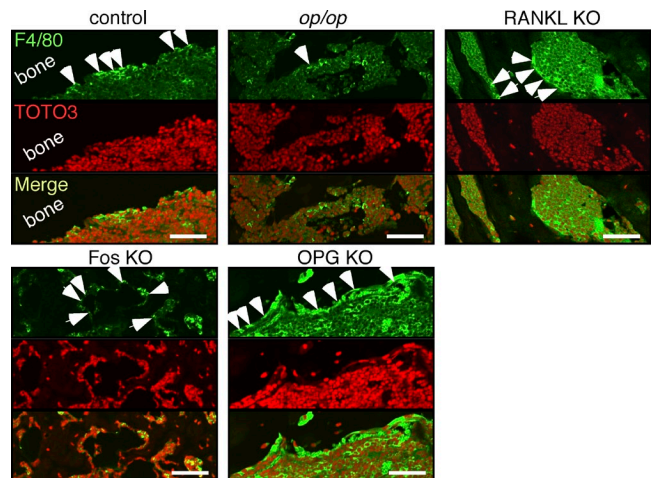
**F4/80<sup>+</sup> osteomacs are defective in *op/op* mice but appear normal in *c-Fos*<sup>-</sup> or OPG-deficient mice**

Recently, it was reported that F4/80<sup>+</sup> macrophages, termed osteomacs, reside in the endosteal (Chang et al., 2008) where they support osteoblast function, thereby contributing to retain HSCs in the BM niche (Winkler et al., 2010). Thus, increased HSPC mobilization seen in *op/op* mice after G-CSF injection could be a result of reduced levels of osteomacs. Indeed, we observed fewer F4/80<sup>+</sup> osteomacs in *op/op* mice, although their levels in *c-Fos*-deficient and RANKL-deficient mice were similar to those observed in wild-type mice (Fig. 4). These observations suggest that osteomac reduction is not a common property of osteopetrotic mice, all of which show increased HSPC mobilization after G-CSF administration. CD11b<sup>+</sup>F4/80<sup>+</sup>Ly6-G<sup>+</sup> macrophages, which also reportedly inhibit HSPC mobilization into the peripheral blood, are reduced after G-CSF treatment in wild-type mice (Winkler et al., 2010). However, like osteomacs, the CD11b<sup>+</sup>F4/80<sup>+</sup>Ly6-G<sup>+</sup> macrophage population was reduced in *op/op* mice compared with wild-type mice, but such a reduction was not seen in *c-Fos*-deficient mice (Fig. S3 A). In OPG-deficient mice, osteomac and CD11b<sup>+</sup>F4/80<sup>+</sup>Ly6-G<sup>+</sup> macrophage levels were similar to those seen in wild-type mice (Fig. 4 and Fig. S3 A). These results suggest that both of these cell types are M-CSF dependent and are deficient in the *op/op* model of osteopetrotic mice, but that this deficiency is not likely be the cause of increased mobilization of HSPCs seen in osteopetrotic animals. A more likely explanation is that HSPC mobilization seen in all osteopetrotic models is a result of loss of osteoclasts and BM cavities. Because F4/80<sup>+</sup>

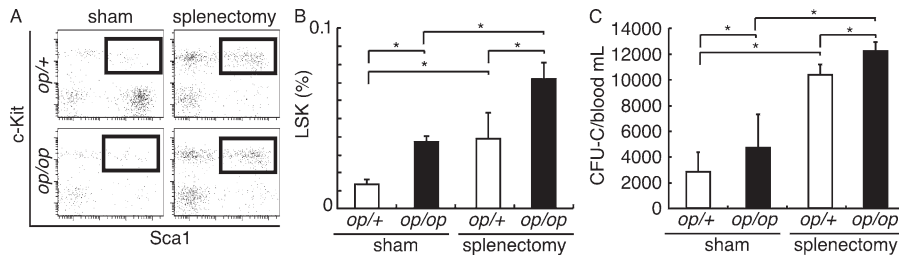
osteomacs and CD11b<sup>+</sup>F4/80<sup>+</sup>Ly6-G<sup>+</sup> macrophages were detected in osteoclast-deficient *c-Fos*-deficient mice, we do not consider F4/80<sup>+</sup> cells to be osteoclasts. Indeed, F4/80 is reportedly not expressed in osteoclasts (Quinn et al., 1999).

**The spleen is not the primary tissue that maintains HSPCs in *op/op* mice**

Hematopoiesis in osteopetrotic mice reportedly occurs in extramedullary spaces, such as spleen (Lowell et al., 1996). Indeed, a greater proportion of LSK cells was seen in the



**Figure 4. Osteomac levels are reduced in *op/op* but not *c-Fos*-deficient mice.** Paraffin specimens of *op/op*, *c-Fos*-deficient (*c-Fos* KO), RANKL-deficient (RANKL KO), OPG-deficient (OPG KO), and control mouse femoral bones were stained with Alexa Fluor 488-conjugated anti-F4/80 antibody and observed under a confocal microscope. TOTO3 served as a nuclear stain. Arrowheads indicate F4/80-positive osteomacs. Representative data of three independent experiments are shown. Bars, 100  $\mu$ m.



**Figure 5. HSCs do not accumulate in the spleen of *op/op* mice.** Splenectomy or sham surgery was performed on 8–12-wk-old *op/op* ( $n = 5$  for each group) and control (*op/+*;  $n = 5$  for each group) mice. 7 d later, mice were injected with 250  $\mu\text{g}/\text{kg}/\text{d}$  G-CSF daily for 5 d, and mobilization of HSCs to peripheral blood was analyzed using flow cytometry and colony-forming assays. (A) Representative flow cytometry pattern of peripheral blood

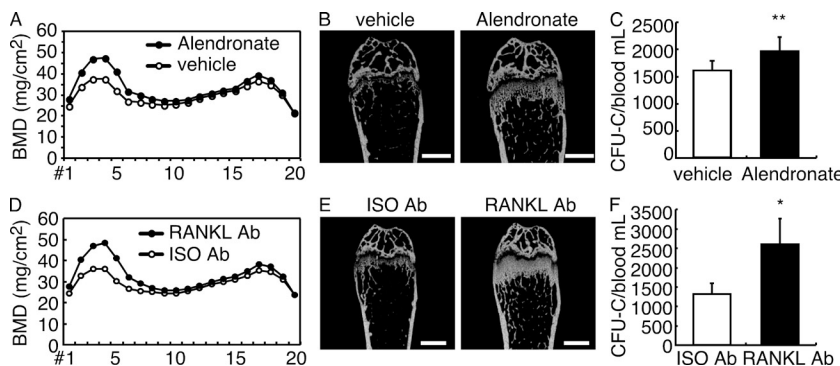
after G-CSF injection ( $n = 5$  for each group). Cells were gated on lineage-negative cells. (B and C) Data represent the mean LSK frequency (%)  $\pm$  SD in peripheral blood (\*,  $P < 0.01$ ,  $n = 5$  for each group; B) and mean CFU-C  $\pm$  SD in 1 ml peripheral blood (\*,  $P < 0.01$ ,  $n = 5$  for each group; C). Representative data of three independent experiments are shown.

spleen of *op/op* mice than in control (*op/+*) mice (Fig. S3, B and C). Thus, we removed the spleen from *op/op* mice and treated those mice with G-CSF serially to analyze HSPC mobilization (Fig. 5). Interestingly, HSPC mobilization was induced or even significantly higher in splenectomized *op/op* mice compared with sham-operated *op/op* mice (Fig. 5). These data suggest that HSCs are maintained in tissues other than spleen in osteoclast-deficient osteopetrotic mice. Interestingly, we found that bone tissues in osteoclast-deficient mice contained small spaces where c-Kit-positive hematopoietic cells are located (Fig. S4 A). Thus, hematopoiesis may be maintained in bone, even in osteopetrotic mice. In fact, flow cytometry analysis revealed an HSC-enriched LSK population in osteopetrotic bones of *op/op* mice (Fig. S4 B), and these cells were shown to be functional by an LTC-IC assay (not depicted). These results indicate that HSCs are maintained in bones of osteopetrotic mice, that increased bone mass as a result of impaired osteoclastogenesis may increase functionality of the HSC niche, and that osteoclasts could be a therapeutic target to expand HSCs as well as bone mass. Bones are formed by osteoblasts, and osteoblasts reportedly express various niche factors such as angiopoietin 1, osteopontin, Cxcl12, and KitL (Arai et al., 2004; Stier et al., 2005; Adams and Scadden, 2006; Katayama et al., 2006). We found that these niche factors were expressed in osteopetrotic bones of *op/op*, c-Fos-deficient, and RANKL-deficient mice (unpublished data).

**Osteoclast-inhibiting agents increase HSPC mobilization in wild-type mice**

Finally, we asked whether blocking osteoclast function or differentiation pharmacologically would inhibit or enhance hematopoietic activity in wild-type mice. Wild-type mice were pretreated with bisphosphonate (alendronate) or vehicle (PBS), and HSPC mobilization after serial G-CSF injection was analyzed (Fig. 6). Bisphosphonate treatment significantly increased bone mass (Fig. 6, A and B), and, interestingly, mobilization of HSPCs as determined by a colony-forming assay was highly induced in bisphosphonate- compared with vehicle-treated mice (Fig. 6 C). Thus, bisphosphonate therapy increased bone mass and up-regulated HSPC mobilization. Similar phenotypes were detected in mice treated with neutralizing RANKL antibody compared with isotype-matched control antibody-treated mice (Fig. 6, D–F). Thus our results demonstrate that HSCs can be expanded pharmacologically. Treatment with zoledronate, another bisphosphonate, also reportedly increased HSPC mobilization in response to G-CSF (Winkler et al., 2010), further suggesting that bisphosphonate, neutralizing RANKL antibody, or other osteoclast inhibitors might serve as adjuvants to increase HSPC mobilization.

Collectively, our results challenge the dogma that BM cavity-deficient animals cannot maintain HSCs and raises the question of why mammalian and avian species developed BM cavities. Although the density of osteopetrotic bones in *op/op* mice was greater than that seen in controls (Fig. S5 A), strength



**Figure 6. Pharmacological inhibition of osteoclasts increases bone mass and expands the HSC pool in wild-type mice.** (A–C) Wild-type mice were treated with 5 mg per mouse of alendronate (bisphosphonate;  $n = 5$ ) or vehicle (PBS;  $n = 5$ ) daily for 14 d and then injected with 250  $\mu\text{g}/\text{kg}/\text{d}$  G-CSF daily for five more days. Mice were assessed for BMD (A), micro-radiography (B), and HSC mobilization in peripheral blood (C; \*\*,  $P < 0.05$ ,  $n = 5$ ). Bar, 1 mm. (D and E) Wild-type mice were treated with 5 or 2.5 mg/kg of neutralizing antibody against RANKL (RANKL Ab;  $n = 5$ ) or isotype control antibody (ISO;  $n = 5$ ) 14 and 1 d before G-CSF injection, respectively. Mice were then injected with 250  $\mu\text{g}/\text{kg}/\text{d}$  G-CSF daily for 5 d, and BMD (D), micro-

radiography (E), and mobilization of HSCs in peripheral blood (F; \*,  $P < 0.01$ ,  $n = 5$ ) were analyzed. BMD was shown in 20 longitudinal divisions of femurs (A and D). Data represent mean CFU-C  $\pm$  SD in 1 ml peripheral blood (C and F). Bars, 1 mm. Representative data of two independent experiments are shown.

tests indicated that bones were more fragile than control bones (Fig. S5 B). Fractures were induced in osteopetrotic bones earlier and by lower energy than in control bones, based on constant displacement tests (Fig. S5, B and C). Osteopetrotic bone also showed reduced elasticity (Fig. S5 D). H/E staining indicated that *op/op* mouse bones were filled with trabecular bone and that mutants exhibited thinner cortical bones than did control mice (Fig. S5, E and F). Indeed, osteopetrotic patients suffer from frequent fractures (Landa et al., 2007). Thus bones likely developed not only to maintain HSCs but to optimize bone strength and body support. To overcome the reduced ability to support HSCs in an open BM cavity created by osteoclasts, BM niches were likely developed by osteoblastic cells, vascular endothelial cells, reticular cells, or aggregates of these cells (Adams and Scadden, 2006; Kiel and Morrison, 2008), or by the products of these cells such as angiopoietin 1, osteopontin, Cxcl12, and thrombopoietin 1 (Calvi et al., 2003; Zhang et al., 2003; Arai et al., 2004; Kiel et al., 2005; Stier et al., 2005; Adams and Scadden, 2006; Sugiyama et al., 2006; Kiel and Morrison, 2008; Lymperi et al., 2010).

Manipulating HSCs *in vitro* to increase their number likely results in reduced stemness because quiescence is essential to maintain stem cell function (Cheng et al., 2000; Miyamoto et al., 2007). Thus, there is likely an antagonistic relationship between expansion and quiescence in HSC expansion. Bone mineral density (BMD) decreases with age, as osteoporosis is increased by osteoclastic activity (Manolagas and Jilka, 1995; Teitelbaum, 2007). Hematopoietic activity also decreases with age (Geiger and Rudolph, 2009; Waterstrat and Van Zant, 2009), suggesting that bone aging can cause both reduced bone mass and reduced hematopoiesis, and that antagonizing osteoclasts pharmacologically could promote increased bone mass and stimulate an induced niche *in vivo* to increase the HSC pool.

## MATERIALS AND METHODS

**Mice.** *op/op*, *c-Fos*-deficient, and RANKL-deficient mice were generated by crossing heterozygotes of respective genotypes. *c-Fos*-deficient mice were provided by E. Wagner (Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Vienna, Austria). Animals were maintained under pathogen-free conditions in animal facilities certified by the Keio University School of Medicine animal care committee. To analyze blood counts, peripheral blood from the postorbital vein was collected in heparinized microtubes (Drummond Scientific). 5-FU (Kyowa Hakko Kirin) was administered intravenously at 150 mg/kg to 8–12-wk-old *op/op*, *c-Fos*-deficient, or littermate control mice once a week, and mouse survival was monitored daily. Animal protocols were approved by the Keio University School of Medicine animal care committee.

**Analysis of skeletal morphology.** 8–12-wk-old mice were necropsied, and their hindlimbs were removed, fixed in 70% ethanol, and subjected to dual energy x-ray absorptiometry analysis to measure BMD and for micro-radiographic analysis. BMD was shown in 20 longitudinal divisions of femurs.

**Mobilization of hematopoietic progenitors.** To induce HSPC mobilization, mice were injected with recombinant human G-CSF (Kyowa Hakko Kirin Co.; 250 µg/kg/d for 5 d, subcutaneous injection). Peripheral blood was harvested 3 h after the last G-CSF injection and used for blood counts, determination of colony-forming units in culture (CFU-C), and flow cytometric (FACS) analysis. Blood counts were analyzed using CellTac (Nihon Kohden). For CFU-C assays, cells were cultured in methylcellulose medium containing recombinant mouse (rm) stem cell factor, rm IL-3, recombinant human (rh)

IL-6, and rh erythropoietin (MethoCult GF M3434; STEMCELL Technologies) in 35-mm culture dishes (Falcon) and incubated at 37°C in 5% CO<sub>2</sub>. The number of CFU-Cs was determined on day 7 using a microscope (Olympus). FACS analysis was undertaken as described. For some experiments, Alendronate or RANKL neutralizing antibody was administered. 5 µg alendronate/mouse/d was injected subcutaneously for 14 d before G-CSF injection. 5 mg/kg RANKL neutralizing antibody (Oriental Yeast Co.) or isotype control antibody (BD) at 14 d and 2.5 mg/kg at 1 d before G-CSF injection was injected into the peritoneal cavity. For competitive repopulation assays, peripheral blood from individual CD45.2<sup>+</sup> mice was pooled, and 20 µl of pooled blood was mixed with 500,000 competitive BM cells from untreated CD45.1<sup>+</sup> mice and injected intravenously into lethally irradiated CD45.1<sup>+</sup> recipient mice. After 3 mo of transplantation, peripheral blood reconstitution in multiple lineages was determined by FACS using CD45.1 (A20), CD45.2 (104), Mac1 (M1/70), CD3 (500A2), and B220 (RA3-6B2) antibodies (all BD).

**Flow cytometry.** mAbs (all BD) recognizing the following markers were used for flow cytometric analyses and cell sorting: c-Kit (2B8), Sca-1 (E13-161.7), CD3 (500A2) B220 (RA3-6B2), TER-119 (Ly-76), Gr-1 (RB6-8C5), and Mac-1 (M1/70). A mixture of mAbs recognizing CD3, B220, TER-119, Mac-1, or Gr-1 was used to identify lineage<sup>+</sup> cells. Mature myeloid cells were stained with PEcy7-conjugated anti-CD11b (M1/70; eBioscience), Alexa Fluor 488-conjugated anti-F4/80 (CI-A3-1; BioLegend), and PE-conjugated anti-Ly6-G antibody (1A8; BD). Stained cells were analyzed on a FACS Aria2 machine (BD).

**ELISA.** Serum M-CSF levels were assayed using the Mouse M-CSF ELISA kit (R&D Systems), according to the manufacturer's instructions.

**Immunohistochemical analysis.** Frozen sections of *op/op* mice femoral bone that had not been decalcified were stained with goat anti-c-Kit antibody (AF1356; R&D Systems), followed by Alexa Fluor 488-conjugated donkey anti-goat antibody (Invitrogen) to detect HSCs and with TOTO3 (Invitrogen) to detect nuclei. Samples were observed under a fluorescence microscope (IX70; Olympus). Paraffin sections of wild-type, *op/op*, *c-Fos*-deficient, and OPG-deficient mice femoral bone were stained with Alexa Fluor 488-conjugated anti-F4/80 antibody (BioLegend) and TOTO3 as a nuclear stain and then observed under a confocal microscope (FV1000; Olympus).

**Bone strength test.** The femur was removed from mice and placed on the lower supports of a three point bending fixture with the anterior side facing up using an apparatus called the Bone Strength Tester MZ-500S (Maruto) installed with a load of 50 N. The span between the two lower supports was set at 6 mm. For all femoral specimens, the upper loading device was aligned to the center of the femoral shaft and the load was applied at a constant displacement rate of 10 mm/min until breakage occurred.

**Online supplemental material.** Fig. S1 demonstrates the sensitivity of *c-Fos*-deficient and *op/op* mice to 5-FU, and the bone phenotype of RANKL-deficient mice. Fig. S2 shows multilineage reconstitution of donor-derived cells in recipient mice. Fig. S3 demonstrates the frequency of the CD11b<sup>+</sup>F4/80<sup>+</sup>Ly6-G<sup>+</sup> macrophage population in wild-type, *op/op*, *c-Fos*-deficient, and OPG-deficient mice, and the frequency of LSK cells in *op/op* mouse spleen. Fig. S4 demonstrates the presence of HSCs in osteopetrotic bone. Fig. S5 shows osteopetrotic bone strength. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20101890/DC1>.

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