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TOPIC HIGHLIGHT

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# Role of osteoclasts in regulating hematopoietic stem and progenitor cells

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## Abstract

Bone marrow (BM) cavities are utilized for hematopoiesis and to maintain hematopoietic stem cells (HSCs). HSCs have the ability to self-renew as well as to differentiate into multiple different hematopoietic lineage cells. HSCs produce their daughter cells throughout the lifespan of individuals and thus, maintaining HSCs is crucial for individual life. BM cavities provide a specialized microenvironment termed "niche" to support HSCs. Niches are composed of various types of cells such as osteoblasts, endothelial cells and reticular cells. Osteoclasts are unique cells which resorb bones and are required for BM cavity formation. Loss of osteoclast function or differentiation results in inhibition of BM cavity formation, an osteopetrotic phenotype. Osteoclasts are also reportedly required for hematopoietic stem and progenitor cell (HSPC) mobilization to the periphery from BM cavities. Thus, lack of osteoclasts likely results in inhibition of HSC maintenance and HSPC mobilization. However, we found that osteoclasts are dispensable for hematopoietic stem cell maintenance and mobilization by using three independent osteoclast-less animal models. In this review, I will discuss the roles of osteoclasts in hematopoietic stem cell maintenance and mobilization.



**Key words:** Osteoclasts; Hematopoietic stem and progenitor cell; Mobilization; Receptor activator of nuclear factor kappa B ligand; Osteomac; Osteopetrosis; *op/op*; C-Fos; Osteoprotegerin

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## INTRODUCTION

Bones reportedly play crucial roles in regulating bone marrow (BM) hematopoiesis by preparing BM cavities and bone marrow "niches" to support hematopoietic stem cell (HSC) maintenance[1-8]. Niches consist of osteoblasts, endothelial cells, reticular cells and osteomacs and are regulated by their products, such as Angiopoietin 1 and Cxcl12 (Figure 1)[9-19]. Functional BM cavities are required for HSPC mobilization from BM cavities to the periphery[20-24]. Thus, BM cavities play crucial roles in regulating HSC maintenance and HSPC mobilization to the periphery and loss of BM cavities is predicted to promote impaired HSPC maintenance and mobilization. However, the impact of lack of BM cavities on the hematopoietic system remains unclear.

Osteoclasts are unique in their capacity to resorb bones: perturbation of osteoclast differentiation or function results in loss of BM cavities, a condition termed osteopetrosis[25-30]. Macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL) are cytokines[31-35] that play a crucial

role in inducing osteoclastogenesis[36-41]. Loss of M-CSF and RANKL, and c-Fos, a transcription factor required for osteoclastogenesis, via mutation or gene-targeting in animal models, impairs osteoclastogenesis and BM cavity formation, an osteopetrotic phenotype[25,26,28]. In contrast, loss of osteoprotegerin (OPG), a decoy RANKL receptor that inhibits osteoclastogenesis[42-45], accelerates osteoclastogenesis in mice and promotes an osteoporotic phenotype[46,47]. Osteoclast activity is reportedly upregulated following serial granulocyte colony-stimulating factor (G-CSF) injection[48], which stimulates HSPC mobilization to the periphery[49-61]. Furthermore, osteoclasts reportedly induce HSPC mobilization[62-64]. Osteoclasts were also reportedly involved in regulating the HSC niche in the bone marrow[65-70]. Thus far, however, hematopoiesis and HSPC mobilization in animals lacking M-CSF, RANKL, c-Fos or OPG remained uncharacterized before our study.

## HEMATOPOIETIC STEM CELLS ARE MAINTAINED IN OSTEOPETROTIC MICE

The chemotherapeutic agent 5-fluorouracil (5-FU) kills cycling cells. Since hematopoietic stem cells (HSCs) are maintained in a quiescent state, HSCs are resistant to 5-FU induced cell death. Thus, serial 5-FU injection has been utilized to evaluate HSC cell function and maintenance *in vivo*[71-74]. We hypothesized that osteopetrotic mice show a reduced HSC pool and function due to lack of BM cavities and niches. Indeed, we found that *op/op* mice were lethally susceptible to serial 5-FU injection[75]. However, RANKL- and c-Fos-deficient mice were not as susceptible to serial 5-FU injection as *op/op* mice were[75]. These results suggest that osteoclasts and BM cavities are not required for HSC maintenance, while M-CSF likely functions in resistance to 5-FU-induced myelosuppression. Indeed, we found that serum M-CSF concentrations increase during 5-FU-induced myelosuppression and that various tissues from M-CSF-deficient *op/op* mice exhibit serious bacterial infections, suggesting that M-CSF expression antagonizes infection during myelosuppression[75].

## HSPCS ARE MOBILIZED FOLLOWING SERIAL G-CSF INJECTION INTO OSTEOPETROTIC MICE

Serial G-CSF injection is utilized clinically to mobilize HSPCs for transplantation[49-51]. Similarly, serial G-CSF injection in mice induces HSPC mobilization to the periphery and is often utilized to evaluate HSPC mobilization capacity in mouse models[20,52-61]. We analyzed HSPC mobilization capacity following serial G-CSF injection in three independent osteoclast-less and thereby BM cavity-less animals, *op/op* mice (M-CSF-deficient mice), c-Fos and RANKL-deficient mice. HSPC mobilization to the periphery was evaluated by flow cytometry to detect the phenotypically HSPC-rich fraction; Lineage-negative, Sca1-positive and c-Kit-positive (LSK), and functional assays such as colony formation and competitive repopulation assay. Since BM cavities or osteoclasts reportedly function in HSPC mobilization[20], we speculated that osteopetrotic animals do not show HSPC mobilization into the periphery due to their loss. Interestingly, however, we found that serial G-CSF injection mobilized HSPCs at levels in all three osteopetrotic mice, compared to control mice, indicating that osteoclasts and BM cavities are not required for HSPC mobilization to the periphery[75].

## HSPC MOBILIZATION IS IMPAIRED IN OSTEOPOROTIC OPG-DEFICIENT MICE

Since HSPC mobilization was detected in osteoclast-less osteopetrotic mice, we evaluated HSPC mobilization in OPG-deficient mice, which exhibit osteoporotic phenotypes due to accelerated osteoclastogenesis[75]. In contrast to osteopetrotic mice, osteoporotic OPG-deficient mice showed reduced HSPC mobilization capacity compared to wild-type mice, suggesting that osteoclasts negatively regulate HSPC mobilization following serial G-CSF injection.

## SPLEEN IS NOT THE PRIMARY TISSUE TO MAINTAIN HSPCS IN AN OSTEOPETROTIC CONDITION

In osteopetrotic patients and animal models, extramedullary hematopoiesis reportedly occurs in the spleen[76,77]. Indeed, we found an increased proportion of the LSK cell fraction in *op/op* mouse spleen compared to control mouse spleen[75]. Thus, we reasoned that there is a pool of mobilized HSPCs in osteopetrotic mice in the spleen prior to mobilization. We then removed spleens from *op/op* mice and analyzed HSPC mobilization capacity of HSPCs following serial G-CSF injection (Figure 2). HSPC mobilization was significantly elevated in splenectomized *op/op* mice compared to splenectomized controls or non-splenectomized *op/op* mice, suggesting that the spleen is not the primary tissue to maintain HSPCs and that it may even antagonize HSPC mobilization in osteopetrotic mice[75]. At present, the localization of HSPCs in osteopetrotic mice is not clear, but we found that small bone lacunae are distributed in osteopetrotic bones and that c-Kit-positive hematopoietic cells are located in such lacunae[75]. These lacunae in osteopetrotic bones likely contribute to the HSPC pool in these animals.

## F4/80-POSITIVE OSTEOMACS ARE REDUCED IN *OP/OP* MICE BUT ARE DETECTED NORMALLY IN C-FOS-, RANKL- AND OPG-DEFICIENT MICE

Recently, F4/80-positive bone-lining cells termed "osteo-

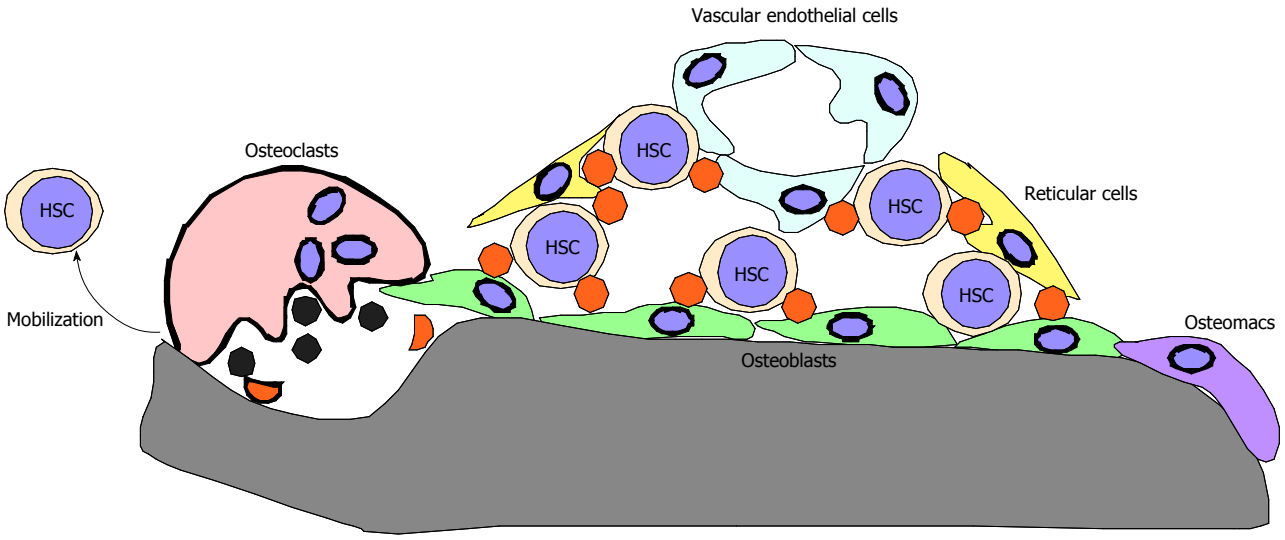


**Figure 1 Components of bone marrow niches.** Bone marrow niches are composed of cell types such as osteoblasts, reticular cells and vascular endothelial cells and factors expressed by these cells; HSC: Hematopoietic stem cell.

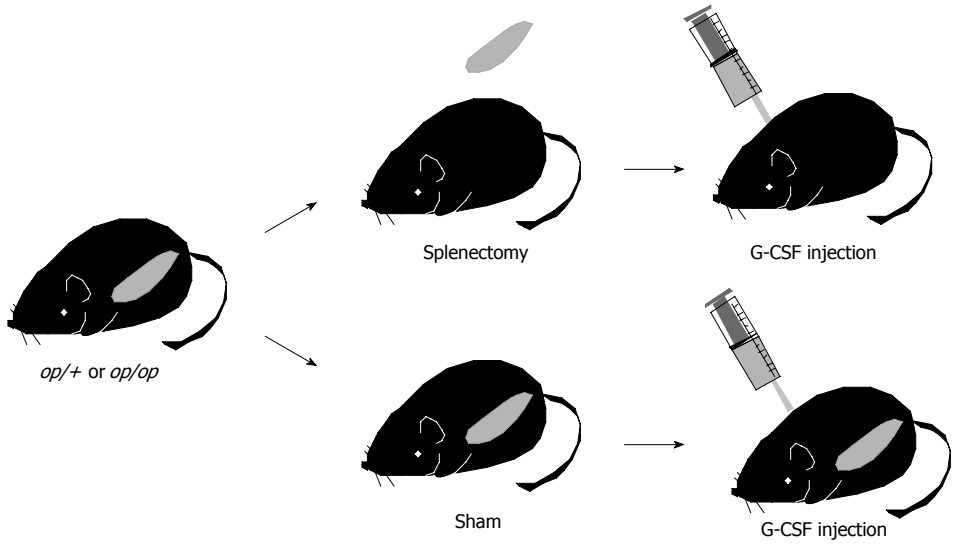


**Figure 2 Splenectomy strategy.** Splenectomy or sham surgery was performed on *op/op* and control mice. Seven days later, mice were injected with 250 μg/kg per day granulocyte colony-stimulating factor daily for 5 d and hematopoietic stem cell mobilization to peripheral blood was analyzed using flow cytometry and colony-forming assays. G-CSF: Granulocyte colony-stimulating factor.

macs" have been identified[78] and demonstrated to play a crucial role in regulating hematopoiesis *in vivo*[18]. Osteomacs reportedly regulate osteoblast function and help retain HSPCs in BM cavities and osteomac loss is predicted to mobilize HSPCs to the periphery[18]. Since osteomacs were once thought to be identical to osteoclasts, increased mobilization seen in osteoclast-less animals was considered due to the loss of osteomacs/osteoclasts. Indeed, we observed decreased numbers of osteomacs in *op/op* mouse bones[75]. However, F4/80-positive osteomacs were detected normally in RANKL-deficient and c-Fos-deficient mice, indicating that osteomacs are not osteoclasts[75]. F4/80 is reportedly not expressed in osteoclasts[79], further suggesting that osteomacs are a different cell type.

## PHARMACOLOGICAL INHIBITION OF OSTEOCLASTS BY BISPHOSPHONATE AND RANKL NEUTRALIZING ANTIBODY DOES NOT INHIBIT HSPC MOBILIZATION

Although we analyzed osteoclast function in regulating hematopoiesis in osteopetrotic mice, the role of osteoclasts in hematopoiesis in adult animals remained unclear. Previously, osteoclast activity was reported to increase following G-CSF injection, but treatment with pamidronate, an osteoclast-inhibiting bisphosphonate, did not inhibit HSPC mobilization to the periphery, suggesting that increased osteoclast activity is not required for HSPC mobilization following G-CSF injection[48]. In contrast,

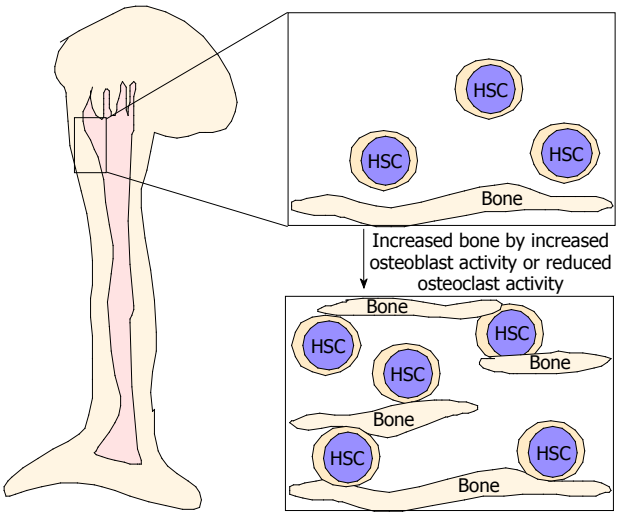


**Figure 3 Increased bone mass is associated with an increased hematopoietic stem cell pool.** Hematopoietic stem cells (HSC) are located in bone marrow cavities with surrounding niche cells. Increased bone mass due to either increased osteoblast activity or reduced osteoclast activity likely contributes to HSC expansion.

osteoclasts are reportedly required for HSPC mobilization since mobilization is induced by bleeding or LPS injection, which increases osteoclastogenesis[62]. Similarly, injection of HGF, SDF1 or RANKL also stimulated HSPC mobilization and increased osteoclast formation[62]. RANKL- or G-CSF-induced HSPC mobilization is abrogated in young female PTPe-deficient mice, which exhibit mild osteoclast dysfunction[62]. Thus, the role of osteoclasts in regulating HSPC mobilization in adults remained controversial and so we did the next experiments to resolve this controversy. We treated wild-type adult mice with an osteoclast-inhibiting agent: the bisphosphonate alendronate or a neutralizing antibody against RANKL (RANKL Ab). Both of these reagents strongly inhibit osteoclast activity and are used to treat osteoporosis patients[80-97]. Indeed, we observed increased bone mass following treatment with alendronate and RANKL Ab in wild-type adult mice[75]. Even in this osteoclast-inhibiting condition, HSPC mobilization to the periphery was normal or even highly induced compared with control mice, suggesting that osteoclast activity is neither required for nor antagonistic to HSPC mobilization[19].

## ROLES OF OSTEOCLASTS AND BM CAVITIES IN HEMATOPOIESIS AND HSPC MOBILIZATION

In most mammalian and avian species, including humans and mice, hematopoiesis occurs in BM cavities and HSC daughter cells are mobilized to the periphery. To continuously supply hematopoietic cells throughout an animal's life, HSCs must self-renew and be capable of producing multiple lineages[98]. Protection of HSCs from various stresses is crucial to maintain lifelong hematopoiesis. To maintain function, HSCs locate in a specific microenvironment in BM cavities termed the "niche", where cells normally remain quiescent. Niches consist of various cell types, including osteoblasts, reticular cells, endothelial cells and osteoclasts, and corresponding products of these cells, such as Cxcl12 (SDF1), Angiopoietin 1 and N-Cadherin. Increased osteoblastogenesis reportedly increases the HSC pool, while degradation of Cxcl12 or surrounding extracellular matrix protein is required for HSPC mobilization to the periphery[9,10,20]. Since osteoclasts express high levels of matrix-degrading enzymes, such as matrix metalloprotenase 9 (MMP9) and Cathepsin K[99-105], and osteoclast activity increases following G-CSF injection[48], osteoclasts were predicted to be critical for HPSC mobilization through degradation of Cxcl12 and matrix protein[62]. In our study, we found that HSPC mobilization to the periphery was induced at comparable or even higher rates than that seen in controls following serial G-CSF injection of three independent osteoclast-less and therefore BM-less mice, phenotypes also seen in wild-type mice treated with two independent osteoclast inhibiting agents[75]. These findings suggest that osteoclasts and BM cavities are dispensable for HSPC maintenance and mobilization. However, it is important to note that we did not induce HSPC mobilization by bleeding or injection of LPS or cytokines, but rather treated wild-type adult mice with one bisphosphonate and one antibody. Nonetheless, our study, at least in part, demonstrates that osteoclast-less and BM cavity-less conditions or conditions in which osteoclasts are severely inhibited do not necessarily prohibit HSPC mobilization.

## HEMATOPOIESIS IN OSTEOCLAST-LESS, THEREBY BM-LESS, ANIMALS

As described above, even in osteopetrotic bones, HSCs were located in bones[75], suggesting that these bones play a role in providing an HSC pool. Parathyroid hormone (PTH) reportedly has dual effects in bone and single or intermittent PTH injection leads to increased osteoblastic activity, whereas continuous PTH stimulation results in increased osteoclast activity and decreased bone mass[106-111]. Interestingly, increased osteoblastic activity due to elevated PTH signaling in constitutive active PTH receptor transgenic mice reportedly increases bone mass and the size of the HSC pool *in vivo* and osteoblasts are thought to serve as critical niche components[9,10]. Similarly, we found that increased bone mass due to reduced osteoclastic activity may contribute to an increased HSC pool[75]. Thus, increased bone mass, due to either increased osteoblast activity or reduced osteoclast function, likely increases the HSC pool *in vivo* (Figure 3). Expanding HSCs *ex vivo* is considered difficult since senescence is crucial for maintaining HSCs and cell cycling disrupts HSC function. Increased niche size by either increased osteoblast activity or inhibited osteoclast function likely serves as a mechanism to increase the HSC pool *in vivo* (Figure 3). Further studies are needed to elucidate the

role of bones as a HSC niche.

## BM CAVITY FORMATION AND BONE STRENGTH

We have shown that osteoclasts and BM cavities are not required for HSPC maintenance and mobilization. So why do BM cavities develop with osteoclasts? We found that, indeed, bone mineral density was significantly higher in osteopetrotic bones than that seen in control bones since BM cavities of osteopetrotic bones were filled with bone[75]. However, osteopetrotic bone strength was low compared with control bones. Since cortical bone thickness is lower in osteopetrotic than in control bones, BM cavities likely developed along with development of cortical bones. At present, the roles of BM cavities are not well clarified and further studies are needed to elucidate their roles in bone and in hematopoiesis.

## PHARAMACOLOGICAL INHIBITION OF OSTEOCLASTS

Osteoclasts emerge in the presence of M-CSF and RANKL: mutational inactivation of M-CSF or targeted disruption of RANKL results in osteoclast differentiation failure and BM cavity-less osteopetrotic phenotypes[25,28]. Recently, a neutralizing antibody against RANKL, named Denosumab, was utilized to treat osteoporosis patients and found to promote significantly reduced osteoclast activity and elevated bone mineral density compared with non-treated placebo controls[90]. Similarly, neutralizing antibody against mouse RANKL increases bone mass[94]. That antibody could be a useful tool to analyze effects of osteoclast-inhibiting conditions in mouse models. The increasing number of osteoporosis patients is now a pressing problem in developed countries and many are treated with osteoclast-inhibiting agents such as bisphosphonates. Future analysis of the effects of these drugs on the systems other than the bone system, such as hematopoiesis, is required to evaluate potential adverse affects of these agents in the future.

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