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Review Article

Regulation of Osteoclast Differentiation and Bone Homeostasis

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Abstract

With numbers of osteoporosis patients increasing annually in developed countries, osteoporosis represents a major health issue to be resolved in the first world. As increases in the number and frequency of osteoporosis patients is closely correlated with age, osteoporosis is one of the most common targets in the "Anti-Aging" field. At present, a number of anti-osteoporosis drugs have been developed, such as bisphosphonates, selective estrogen receptor modulators (SERMs), parathyroid hormones, and active vitamin D_3; however, despite this range of treatment options, the number of osteoporosis patients continues to increase, suggesting the need to identify new targets for treatment. In this review, we describe new targets for inhibiting osteoclast differentiation or function, thereby increasing bone mass. Dendritic cell specific transmembrane protein (DC-STAMP) and osteoclast-stimulating transmembrane protein (OC-STAMP) are both seven-transmembrane proteins required for osteoclast cell-cell fusion. We generated DC-STAMP-deficient and OC-STAMP-deficient mice and found that osteoclast cell-cell fusion was completely abrogated in both strains without inhibiting osteoclast differentiation, indicating that both DC-STAMP and OC-STAMP are required for osteoclast cell-cell fusion but not for osteoclast differentiation. We also determined that B lymphocyte-induced maturation protein 1 (Blimp1) and B cell lymphoma 6 (Bcl6) play a critical role in regulating osteoclast differentiation and bone mass. Blimp1 inhibits Bcl6 expression in osteoclasts, thereby resulting in accelerated osteoclastogenesis and decreased bone mass; in turn, Blimp1-deficiency in osteoclasts promotes increased bone mass due to inhibited osteoclast differentiation.

KEY WORDS: osteoclast, DC-STAMP, OC-STAMP, Blimp1, Bcl6

Osteoclasts, as multi-nuclear cells

Cell-cell fusion and osteoclasts

Osteoclasts are multi-nuclear cells derived from hematopoietic stem cells or monocyte/macrophage lineage cells. Multi-nucleation of osteoclasts occurs by the cell-cell fusion of mono-nuclear osteoclasts, and this cell-cell fusion is the most characteristic feature of osteoclasts. Previously, multi-nucleation was considered necessary for osteoclast bone resorption and generation of cytoskeletal organs such as actin rings and ruffled borders; however, the molecules required for osteoclast cell-cell fusion or the roles of osteoclast multi-nucleation have not yet been clarified.

Identification of DC-STAMP, as an essential molecule for osteoclast cell-cell fusion

In vitro osteoclast differentiation was induced in a previous study in a co-culture system of osteoclast progenitor cells with osteoblastic cells in the presence of osteotropic factors such as vitamin D_3 [1]. While this system contributed to various advances in the osteoclast biology field, it was not suitable for isolating osteoclast-specific molecules due to the presence of osteoblastic cells and osteotropic factors. Identification of receptor activator of nuclear factor kappa B ligand (RANKL) enabled generation of osteoclasts free of osteoblastic cells [2-4]. Multi-nuclear osteoclasts were generated from osteoclast progenitor cells in the presence of macrophage colony-stimulating factor (M-CSF) and RANKL without osteoblastic cells, and we established a pure osteoclast culture system by isolating osteoclast and macrophage common progenitor cells via flow-cytometry and culturing in the presence of M-CSF plus RANKL [5,6]. Multi-nuclear osteoclasts were formed in the presence of M-CSF plus RANKL, while mono-nuclear macrophages were generated in the presence of M-CSF alone [5,6]. This culture system enabled us to isolate osteoclast-specific molecules via a subtractive screen between multi-nuclear osteoclasts generated in the presence of M-CSF plus RANKL and macrophages generated in the presence of M-CSF alone, and we isolated dendritic-specific transmembrane protein (DC-STAMP) as a candidate osteoclast cell-cell fusion regulator [7].

DC-STAMP has been found to be highly expressed in osteoclasts but not macrophages [7]. Here, we generated DC-STAMP knockout mice and found that osteoclast cell-cell fusion was completely abrogated in this strain both *in vivo* and *in vitro* [7]. DC-STAMP was reportedly involved in osteoclast differentiation [8]; however, osteoclast differentiation markers were equally expressed in DC-STAMP-deficient mono-nuclear osteoclasts and wild-type multi-nuclear osteoclasts [7], indicating that DC-STAMP was specifically required for osteoclast cell-cell fusion rather than differentiation (***Fig. 1***).

Although osteoclast multi-nucleation was considered necessary for osteoclast bone resorption, we previously found that DC-STAMP-deficient mono-nuclear osteoclasts resorbed bone [7]. However, the bone-resorbing efficiency of DC-STAMP-

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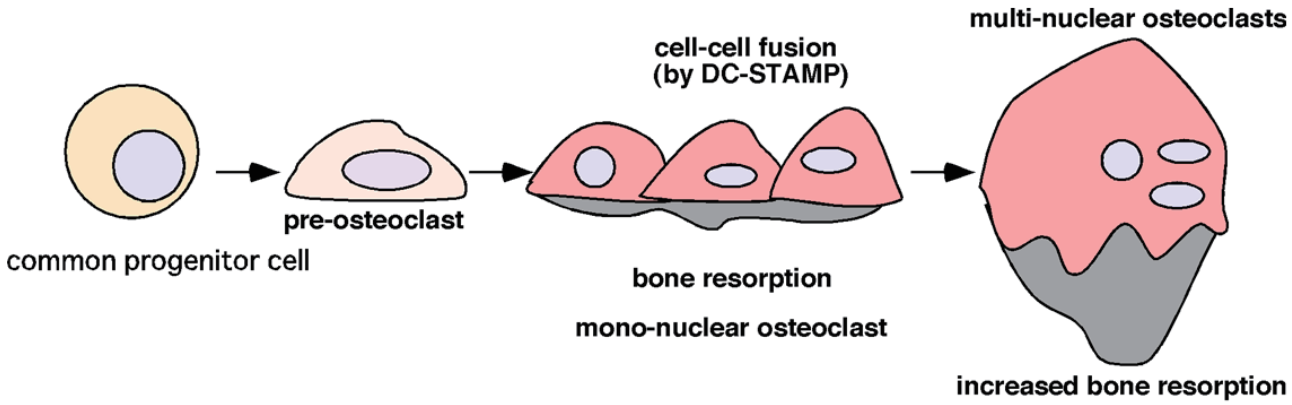

***Fig. 1*.** **Formation of multi-nuclear osteoclasts.**
Common progenitor cells for osteoclasts and macrophages differentiate into pre-osteoclasts and then mono-nuclear osteoclasts. Mono-nuclear osteoclasts fuse with each other to form multi-nuclear osteoclasts. Lack of DC-STAMP results in the complete abrogation of osteoclast cell-cell fusion.

deficient mono-nuclear osteoclasts was significantly lower than that of wild-type multi-nuclear osteoclasts [7], indicating that osteoclast cell-cell fusion upregulates osteoclast bone resorbing-efficacy. Indeed, DC-STAMP-deficient mice exhibited increased bone mass compared with wild-type mice [7].

Forced expression of DC-STAMP did not promote osteoclast cell-cell fusion

In a previous study [check], given that DC-STAMP was isolated using a subtractive screen between multi-nuclear osteoclasts induced by M-CSF plus RANKL and mono-nuclear macrophages induced by M-CSF alone [7]. Thus, DC-STAMP over-expression in M-CSF-induced mono-nuclear macrophages was predicted to be sufficient to promote cell-cell fusion in macrophages without RANKL. We then generated DC-STAMP-over-expressing transgenic mice (DC-STAMP Tg) under the control of an actin promoter [9]. We examined cell-cell fusion by isolating osteoclast progenitor cells from these mice and culturing in the presence of M-CSF alone; however, no fusion was found to be induced under these conditions, suggesting that DC-STAMP was not sufficient to promote cell-cell fusion in macrophages [9]. As such, we then attempted to determine the another cell-cell fusion regulator.

OC-STAMP is required for osteoclast cell-cell fusion

To isolate the another osteoclast cell-cell fusion regulator, we employed a microarray screen between multi-nuclear macrophage lineage cells [10]. Foreign body giant cells (FBGCs) are multi-nuclear cells formed from osteoclast and macrophage common progenitor cells in the presence of GM-CSF plus IL-4 [7]. On isolating those molecules highly expressed in both osteoclasts and FBGCs but not in macrophages, we picked up OC-STAMP as a candidate osteoclast cell-cell fusion regulator [10].

We next generated OC-STAMP knockout mice, and found that osteoclast cell-cell fusion was completely abrogated both *in vitro* and *in vivo*, as seen in DC-STAMP knockout mice [10]. Osteoclast differentiation markers were normally expressed in OC-STAMP-deficient mono-nuclear osteoclasts, as in wild-type osteoclasts, suggesting that OC-STAMP specifically regulates osteoclast cell-cell fusion rather than differentiation [10]. Bone resorption was significantly inhibited in OC-STAMP-deficient mono-nuclear osteoclasts compared with wild-type multi-nuclear osteoclasts, further supporting the idea that osteoclast cell-cell fusion regulates bone resorbing efficiency [10]. Fusion-related molecules, including DC-STAMP, were also normally expressed in OC-STAMP-deficient mono-nuclear osteoclasts, and OC-STAMP was normally expressed in DC-STAMP-deficient mono-nuclear osteoclasts, suggesting that DC-STAMP and OC-STAMP are both required for osteoclast cell-cell fusion and do not regulate each other's expression [10].

DC-STAMP and OC-STAMP are both targets of NFATc1

Osteoclastogenesis is induced by RANKL, and DC-STAMP and OC-STAMP were both induced by RANKL stimulation, suggesting that these molecules are targets of RANKL [10,11]. Nuclear factor of activated T cells 1 (NFATc1) is an essential transcription factor for osteoclastogenesis and is induced and activated under stimulation by RANKL [12]. Thus, DC-STAMP and OC-STAMP were considered targets of NFATc1 in osteoclasts. Indeed, both DC-STAMP and OC-STAMP expression were significantly inhibited by treatment with NFAT inhibitor FK506, and NFATc1 was recruited to both DC-STAMP and OC-STAMP promoter regions in the presence of RANKL. Thus, DC-STAMP and OC-STAMP are both targets of NFATc1 in osteoclasts [10,11].

Osteoclast cell-cell fusion is a target of osteoporosis treatment that does not excessively inhibit bone turnover

Bone volume and homeostasis are regulated by a delicate balance between bone-resorbing osteoclasts and bone-forming osteoblasts. Bisphosphonate, a therapeutic agent of osteoporosis, strongly inhibits osteoclast bone resorption, thereby increasing bone mass and inhibiting osteoporotic fracture. However, osteoclast bone resorption is required following osteoblast bone formation, and inhibition of osteoclasts results in the inhibition of osteoblastic bone formation. Excess inhibition of osteoclasts is considered a cause of disease states such as bisphosphonate-related osteo-necrosis of the jaw (BRONJ) and atypical femoral fracture [13,14]. Inhibition of osteoclast cell-cell fusion results in significant but not complete inhibition of bone resorption, suggesting that cell-cell fusion is a favorable target for treating osteoporosis while avoiding excess inhibition of osteoclasts.

New pathway for regulating osteoclast differentiation and bone mass

Bcl6, a transcriptional repressor, is downregulated by RANKL in osteoclasts

Osteoclastogenesis is promoted under stimulation by RANKL. Various factors or pathways such as c-Fos, TRAF6, and calcium signaling are activated by RANKL, subsequently activating NFATc1 [12], which directly promotes the expression of osteoclastic molecules such as Cathepsin K and DC-STAMP. Interestingly, NFATc1 reportedly activates NFATc1 itself in an auto-amplification manner under a stimulation of RANKL [15]. As such, inhibition of NFATc1 results in the significant inhibition of osteoclastogenesis.

At present, the NFAT inhibitor FK506 is used as an immunosuppressant to treat autoimmune diseases such as rheumatoid arthritis or organ transplantation, and patients treated with FK506 have exhibited decreased bone mass [16]. The discrepancy of inhibited osteoclastogenesis but decreased bone mass was explained by the fact that NFATs are also required for osteoblastogenesis, and the inhibition of NFATs results in significant inhibition of bone formation, thereby reducing bone mass [17].

We therefore attempted to identify another pathway regulating osteoclast differentiation, focusing on negative regulators of osteoclastogenesis, and identified B cell lymphoma 6 (Bcl6), a transcriptional repressor which was significantly downregulated under stimulation by RANKL in osteoclasts [18].

Bcl6 is a negative regulator of osteoclastogenesis

Since Bcl6 is downregulated by RANKL during osteoclast differentiation, this downregulation was deemed necessary for normal osteoclastogenesis. Indeed, forced expression of Bcl6 strongly inhibited osteoclast differentiation by RANKL [18]. We therefore used Bcl6 knockout mice to analyze the roles of Bcl6 in osteoclast differentiation [18].

Bcl6 knockout mice exhibited a significant reduction in bone mineral density accompanied by a significant elevation in osteoclast formation *in vivo* [18]. In *in vitro* culture, accelerated osteoclast differentiation and osteoclast bone resorption were evident in Bcl6 knockout osteoclasts compared with control osteoclasts [18]. These results indicate that Bcl6 is a negative regulator of osteoclastogenesis, and that lack of Bcl6 promotes significant acceleration of osteoclast differentiation and bone resorption, in turn resulting in a significant reduction in bone mass [18].

Blimp1 is upregulated by RANKL and required for osteoclastogenesis

Next, we attempted to isolate a transcription repressor which suppress Bcl6 expression in osteoclasts, ultimately isolating B lymphocyte maturation protein 1 (Blimp1) as a candidate Bcl6 repressor in osteoclasts [18].

Blimp1 expression was upregulated by RANKL during osteoclast differentiation. We generated osteoclast-specific Blimp1 conditional knockout mice (Blimp1 cKO) by crossing Blimp1 flox mice with Cathespin K Cre knockin mice, since Blimp1 null mice are early embryonic lethal. We found that Blimp1 cKO mice exhibited a significant elevation in bone mass accompanied by a significant reduction in osteoclast formation *in vivo* [18]. *In vitro* findings showed that osteoclast differentiation was significantly inhibited in Blimp1 cKO cells compared with control cells, and expression of various osteoclastic genes such as *Cathpesin K, DC-STAMP* and *NFATc1* was significantly inhibited in Blimp1 cKO cells [18]. Taken together, these findings indicate that Blimp1 is upregulated by RANKL and required for osteoclast differentiation.

Blimp1 is essential to suppress Bcl6 expression in osteoclasts

While Blimp1 expression in Bcl6 KO osteoclasts was unchanged compared with control osteoclasts, Bcl6 expression in Blimp1 cKO osteoclasts was significantly upregulated, indicating that Blimp1 lies upstream of Bcl6 and is required for Bcl6 suppression in osteoclasts by RANKL [18].

Without RANKL, Bcl6 was on the promoters of osteoclastic molecules such as *Cathpesin K, DC-STAMP*, and *NFATc1* and was dissociated from the promoters by RANKL stimulation. However, in Blimp1 cKO cells, Bcl6 was not dissociated from the promoters even in the presence of RANKL [17]. Further, the increased bone mass seen in Blimp1 cKO mice was partially reduced in Blimp1/Bcl6 double knockout mice (DKO). We therefore concluded that "RANKL-Blimp1-Bcl6-osteoclastic molecules" constitute a critical pathway regulating osteoclastogenesis and bone homeostasis (***Fig. 2***) [18].

Blimp1 is a therapeutic target to increase bone mass in adults

To test whether or not Blimp1 is a viable therapeutic target to increase bone mass in the adult stage, we generated inducible Blimp1-conditional knockout mice (iBlimp1 cKO) by crossing Blimp1 flox mice with Mx Cre mice [19]. Blimp was deleted by injecting polyI-polyC at an adult stage, with deletion resulting in significant inhibition of osteoclastogenesis and significant elevation in bone mass. Blimp1 null mice were reportedly early embryonic lethal, and Blimp1 was found to be required for the development of immune cells such as plasma cells and T cells; however, no adverse effects or inhibition of B- or T-cell development were evident in Blimp1-deleted iBlimp1 cKO mice [19]. Taken together, these results indicate that Blimp1 can indeed function as a therapeutic target for increasing bone mass at an adult stage, and some molecules that exhibit embryonic lethality in a null mutation can also function as therapeutic targets in adult models.

Conflict of interest statement

The authors declare no financial or other conflicts of interest in the writing of this paper.

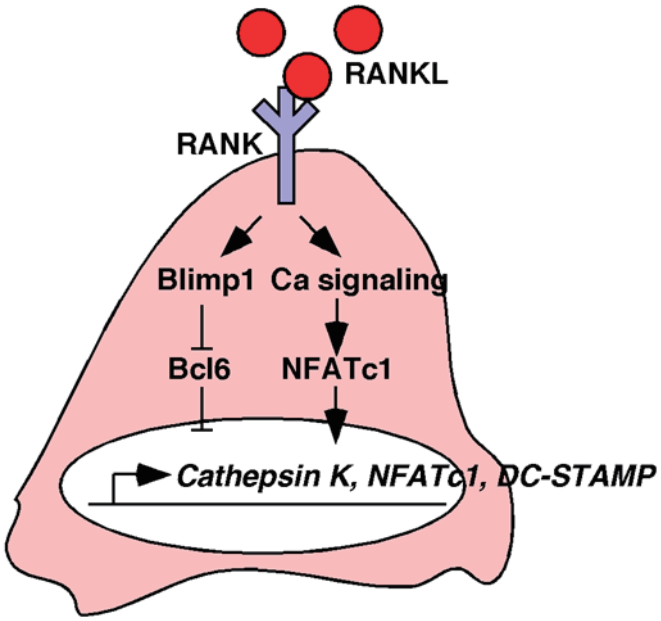

Fig. 2. **The signals for osteoclastogenesis.**
Osteoclast differentiation is induced via two different pathways under the stimulation of RANKL: a "positive axis" via Ca signaling-NFATc1-"osteoclastic genes", and a "negative axis" via Blimp1-Bcl6-"osteoclastic genes".

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