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Invited Review

Negative regulators in RANKL-induced osteoclastogenesis

Junwon Lee[1], Kabsun Kim[1], and Nacksung Kim[1,2]

[1]*Medical Research Center for Gene Regulation, Chonnam National University Medical School, Gwangju 501-746, Korea*
[2]*Medical Research Center for Gene Regulation, Chonnam National University Medical School, Hak-Dong 5, Dong-Ku, Gwangju 501-746, Korea*

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Receptor activator of nuclear factor κB ligand (RANKL) induces osteoclast formation from hematopoietic cells via up-regulation of positive regulators, including NF-κB, c-Fos, microphthalmia transcription factor (Mitf), PU.1, and nuclear factor of activated T cells (NFAT) c1. In addition to the positive regulation by these transcription factors, RANKL appears to regulate negative regulators such as MafB and inhibitors of differentiation (Ids). Ids and MafB are abundantly expressed in osteoclast precursors, bone marrowderived monocyte/macrophage lineage cells (BMMs). Expression levels of these genes are significantly reduced by RANKL during osteoclastogenesis. Overexpression of these genes in BMMs inhibits the formation of tartarate-resistant acid phosphatase (TRAP)-positive multinuclear osteoclasts by down-regulation of NFATc1 and osteoclast-associated receptor (OSCAR), which are important for osteoclast differentiation. Furthermore, reduced expression of these genes enhances osteoclastogenesis and increases expression of NFATc1 and OSCAR. Taken together, RANKL induces osteoclastogenesis via up-regulation of positive regulators as well as down-regulation of negative regulators.

Key words: osteoclasts, negative regulation, transcription factor, Id, MafB

Introduction

Bone is continuously remodeled by osteoclasts and osteoblasts, and their balanced activity is important for maintaining bone density. Osteoclasts are specialized cells possessing the unique ability to resorb bone matrix. Two key cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL; also called TRANCE [tumor necrosis factor (TNF) activation-induced cytokine], ODF [osteoclast differentiation factor], and OPGL [osteoprotegerin ligand]) induce and sustain osteoclast differentiation from monocyte/macrophage precursors of hematopoietic origin (Lacey *et al*., 1998; Suda *et al*., 1999; Wong *et al*., 1997; Yasuda *et al*., 1998).

RANKL, a TNF family member, supports osteoclast differentiation, survival, and activation. Binding of RANKL to its receptor, receptor activator of nuclear factor κB (RANK), initiates signals mediated by TNF receptor-associated factor (TRAF) adaptors and activates NF-κB, c-Jun N-terminal kinase (JNK), p38 MAP kinase, extracellular signal-related kinase (ERK), and AKT (Boyle *et al*., 2003; Lee and Kim, 2003).

RANKL induces and/or activates transcription factors including NF-κB, c-Fos, microphthalmia transcription factor (Mitf), PU.1, and nuclear factor of activated T cells (NFAT)c1, which are known to be important positive regulators for osteoclastogenesis in vitro and in vivo (Boyle et al., 2003; Teitelbaum, 2000; Teitelbaum and Ross, 2003). Recently, it has been shown that costimulatory signals mediated by immunoreceptor tyrosine-based activation motif (ITAM)-harboring adaptors, including DNAX-activating protein (DAP)12 and Fc receptor common γ chain (FcRγ), cooperate with RANKL during osteoclastogenesis, and their activation enhances the induction of NFATc1 via calcium signaling (Koga *et al*., 2004; Mocsai *et al*., 2004; Takayanagi, 2005). Osteoclast-associated receptor (OSCAR) is a member of the Ig-like surface receptor family and plays an important role as a costimulatory receptor for osteoclast differentiation by activating NFATc1 via association with

*Corresponding author: Nacksung Kim, Ph.D., Medical Research Center for Gene Regulation, Chonnam National University Medical School, Hak-Dong 5, Dong-Ku, Gwangju 501-746, Korea Tel: +82-62-220-4418 Fax.: +82-62-223-4018: E-mail: nacksung@jnu.ac.kr

the FcRγ chain (Ishikawa *et al.*, 2004; Kim *et al.*, 2002; Merck *et al.*, 2004). In addition, NFATc1 synergistically induces OSCAR gene expression with Mitf (Kim *et al.*, 2005; So *et al.*, 2003). Therefore, a positive feedback circuit involving RANKL, NFATc1, and OSCAR appears to be important for efficient differentiation of osteoclasts (Kim *et al.*, 2005; Kim *et al.*, 2005).

Positive regulators

RANKL rapidly activates NF-κB and subsequently induces gene induction of various target genes which contain the κB site on the promoter region (Anderson *et al.*, 1997). NF-κB refers to a group of five proteins, p65 (RelA), RelB, c-Rel, p50/p150 (NF-κB1), and p52/p100 (NF-κB2), which exist in unstimulated cells as inactive homo- or hetero-dimers bound to inhibitor of NF-κB (IκB) family proteins. Although p50- or p52-deficient mice have no obvious bone disorder, p50 and p52 double-deficient mice show severe osteopetrosis, suggesting a critical role of p50 and p52 in RANKL-mediated osteoclastogenesis (Iotsova *et al.*, 1997).

Mitf encodes a basic helix-loop-helix (bHLH) zipper protein that is important for differentiation of osteoclasts (Hodgkinson *et al.*, 1993). RANKL activates Mitf via the MKK6/p38 signaling cascade. Subsequently, activated Mitf induces the expression of target genes, including tartarate-resistant acid phosphatase (TRAP), cathepsin K, and OSCAR, which are important for osteoclast differentiation or function, by binding to the canonical E-box sequence in the promoter region of those genes (Kim *et al.*, 2002; Luchin *et al.*, 2000; Mansky *et al.*, 2002; Motyckova *et al.*, 2001; So *et al.*, 2003). Indeed, most osteoclasts derived from Mitf mutant precursor cells become TRAP-positive mononuclear cells, and show reduced expression of TRAP, cathepsin K, and OSCAR compared with osteoclasts from wild type cells (Luchin *et al.*, 2000; Motyckova *et al.*, 2001; So *et al.*, 2003).

The ETS family transcription factor PU.1 is preferentially expressed in the hematopoietic cell lineage and plays a pivotal role in the differentiation of a number of cell types, including B cells, macrophages, and osteoclasts (Simon, 1998; Singh *et al.*, 1999). Although PU.1 is expressed at all stages of osteoclast differentiation in wild type cells, osteoclasts from PU.1 knockout mice show a failure to differentiate at a very early stage, eventually leading to an osteopetrotic phenotype. It has also been reported that the direct interaction of Mitf and PU.1 permits efficient induction of their target genes in a synergistic manner (Luchin *et al.*, 2001; So *et al.*, 2003).

RANKL activates AP-1 transcription factors partly through induction of c-Fos (Wagner and Eferl, 2005). The AP-1 transcription factor is a dimeric complex composed of the Fos (c-Fos, FosB, Fra-1, Fra-2), Jun (c-Jun, JunB, JunD), and ATF (ATFa, ATF2, ATF3, ATF4, B-ATF) proteins (Wagner and Eferl, 2005). c-Fos-deficient mice develop osteopetrosis, a result of the absence of osteoclasts, demonstrating that c-Fos is required for the differentiation of monocyte precursors into osteoclasts (Wang *et al.*, 1992).

RANKL strongly induces NFATc1 expression during osteoclastogenesis (Takayanagi *et al.*, 2002). Interestingly, NFATc1 induction by RANKL is completely abrogated in c-Fos-deficient cells, indicating that c-Fos up-regulates NFATc1 (Takayanagi *et al.*, 2002). Later, NFATc1 and AP-1 complex containing c-Fos cooperate to enable the robust induction of NFATc1 (Asagiri *et al.*, 2005). Recently, it has been reported that ectopic expression of NFATc1 causes precursors to undergo efficient differentiation in the absence of RANKL, and that NFATc1-deficient embryonic stem cells fail to differentiate into osteoclasts in response to RANKL, suggesting that NFATc1 acts as a key modulator of osteoclastogenesis (Hirotani *et al.*, 2004; Takayanagi *et al.*, 2002).

Inhibitors of differentiation (Ids)

The helix-loop-helix (HLH) family of transcriptional regulatory proteins have important roles in developmental processes including neurogenesis, myogenesis, and hematopoiesis (Massari and Murre, 2000). Tissue-specific bHLH proteins form dimers with E proteins, ubiquitously expressed bHLH transcription factors, which bind to the E-box sequence CANNTG. HLH proteins can bind to bHLH transcription factors via their HLH domain; the resulting heterodimers are unable to bind to DNA because HLH proteins lack the necessary basic motif (Benezra *et al.*, 1990). Thus, HLH proteins act as dominant negative regulators of bHLH transcription factors. One of the HLH subfamilies is composed of Id genes 1 to 4. Id genes are thought to affect the balance between cell growth and differentiation in many cell types (Lasorella *et al.*, 2001).

Among four known members of the Id family, Id 1, Id 2, and Id 3 are abundantly expressed in osteoclast precursors (Lee *et al.*, 2006). The expression of these genes is significantly reduced by RANKL treatment. Overexpression of Id genes in BMMs inhibits RANKL-mediated osteoclastogenesis, but does not affect the phagocytic activity or differentiation of common precursors into dendritic cells (Lee *et al.*, 2006). Ids down-regulate the gene expression of OSCAR, through interaction with Mitf and subsequent inhibition of Mitf transactivation of the OSCAR gene (Lee *et al.*, 2006). Ids also down-regulate NFATc1 expression possibly by impairment of a positive feedback regulation between OSCAR and NFATc1 due to down-regulation of OSCAR by Ids. Furthermore, deficiency of Id 2 enhances RANKL-induced osteoclastogenesis (Lee *et al.*, 2006). Together, Ids may have a role in osteoclastogenesis as negative regulators.

MafB

Maf proteins share a conserved basic region and leucine zipper (bZIP) motif which mediate DNA binding and dimer formation (Blank and Andrews, 1997; Cordes and Barsh, 1994). Members of the Maf family are divided into two subgroups: the large Maf proteins (MafA/L-Maf, MafB/Kreisler, c-Maf, and NRL), which contain an acidic transcription activating domain (TAD) located at their N terminus, and the small Maf proteins (MafK, MafF, and MafG), which contain only the bZIP region (Blank and Andrews, 1997; Motohashi *et al.*, 1997).

MafB is expressed selectively in monocytes and macrophages, but not in other hematopoietic cells (Eichmann et al., 1997; Sieweke *et al.*, 1996). During macrophage differentiation, MafB expression is undetectable in multipotent progenitors, but is expressed at moderate levels in myeloblasts, and strongly up-regulated in monocytes and macrophages (Sieweke *et al.*, 1996). Overexpression of MafB in transformed myeloblasts stimulates the rapid formation of macrophages, suggesting that MafB is important for macrophage differentiation (Kelly *et al.*, 2000).

MafB is abundantly expressed in osteoclast precursors. RANKL strongly down-regulates MafB expression via p38 and JNK pathway, which are important signaling cascades for osteoclastogenesis (Kim *et al.*, 2007). Overexpression of MafB in BMMs inhibits the formation of TRAP-positive multinuclear osteoclasts, but phagocytic activity of BMMs is retained (Kim *et al.*, 2007). Overexpression of MafB in BMMs attenuates the gene induction of NFATc1 and OSCAR during RANKL-mediated osteoclastogenesis. MafB proteins interfere with the DNA binding ability of c-Fos, Mitf, and NFATc1, inhibiting their transactivation of NFATc1 and OSCAR (Kim *et al.*, 2007). Furthermore, reduced expression of MafB by RNAi enhances osteoclastogenesis and increases expression of NFATc1 and OSCAR (Kim *et al.*, 2007). Thus, MafB can act as an important negative modulator of RANKL-mediated osteoclastogenesis.

Conclusion

After identifying osteoclast differentiation factor, which is widely called RANKL, many reports have been shown that RANKL activates various positive regulators, including NF-κB, c-Fos, Mitf, PU.1, and NFATc1 (Boyle *et al.*, 2003; Suda *et al.*, 1999; Teitelbaum and Ross, 2003). Recently, we showed that RANKL down-regulates negative regulators such as MafB and Ids, which are abundantly expressed in osteoclast precursors (Kim *et al.*, 2006; Lee *et al.*, 2006), suggesting that RANKL enhances osteoclast formation through activating positive regulators as well as overcoming the inhibitory effects of negative regulators on positive regulators such as NFATc1 and OSCAR (Fig. 1). Further study of the detailed mechanism of cross-interaction between positive and negative regulators during RANKL-induced osteoclastogenesis will provide molecular targets for therapeutics addressing bone diseases including osteoporosis and rheumatoid arthritis.

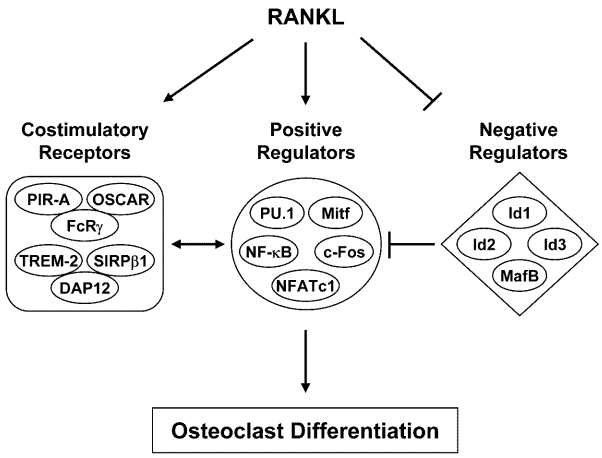

Fig. 1. Gene regulation by RANKL in osteoclastogenesis. RANKL activates positive regulators, including NF-κB, Mitf, PU.1, c-Fos, and NFATc1. Costimulatory signals through immuno-receptors such as OSCAR, PIR-A, TREM-2, and SIRPβ1, are also activated by RANKL, and subsequently cooperate with RANKL signals. In addition, RANKL down-regulates negative regulators such as Ids and MafB, subsequently enhances osteoclast formation via overcoming inhibitory effect of negative regulators on positive regulators.

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