RANK Signaling Pathways and Key Molecules Inducing Osteoclast Differentiation

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Mononuclear osteoclast precursors derived from hematopoietic progenitors fuse together and then become multinucleated mature osteoclasts by macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B ligand (RANKL). Especially, the binding of RANKL to its receptor RANK provides key signals for osteoclast differentiation and bone-resorbing function. RANK transduces intracellular signals by recruiting adaptor molecules such as TNFR-associated factors (TRAFs), which then activate mitogen activated protein kinases (MAPKs), Src/PI3K/Akt pathway, nuclear factor- κ B (NF- κ B) and finally amplify NFATc1 activation for the transcription and activation of osteoclast marker genes. This review will briefly describe RANKL-RANK signaling pathways and key molecules critical for osteoclast differentiation.

Key Words: Osteoclast differentiation, RANK signaling, RANKL

INTRODUCTION

Bone undergoes continuous remodeling process throughout adulthood. Osteoblasts, the bone-forming cells, are derived from a mesenchymal progenitor cells, and osteoclasts, mineralized tissues-resorbing cells, are from hematopoietic progenitors of the monocyte/macrophage lineage (Suda et al., 1999; Chambers, 2000; Teitelbaum, 2000; Aubin, 2001). The functional balance between osteoblasts and osteoclasts is critical for bone homeostasis (Suda et al., 1999; Teitelbaum, 2000). The elevation of osteoclast numbers and/or activity results in bone diseases including osteoporosis, Paget's disease and rheumatoid arthritis.

To become multinucleated mature osteoclasts, mononu-

clear osteoclast precursors survive and fuse together by two main cytokines, macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B ligand (RANKL) (Chambers, 2000). M-CSF stimulates the proliferation and survival of osteoclast precursors via c-Fms, M-CSF receptor. The binding of RANKL to its receptor RANK provides key signals for osteoclast differentiation and bone-resorbing function as well as the survival of mature osteoclasts (Fuller et al., 1998). Co-stimulatory signaling induces Ca^{2+} oscillation for the robust production of NFATc1, which, cooperating with RANKL, finally induce complete osteoclast differentiation (Koga et al., 2004; Mócsai et al., 2004). RANKL- or RANK-deficient mice show similar osteopetrotic phenotypes due to a complete defect of osteoclasts, suggesting that RANKL-RANK signaling is essential

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for the osteoclast formation (Kong et al., 1999; Marx, 2004).

RANKL, also known as OPGL (osteoprotegerin ligand), ODF (osteoclast differentiation factor) and TRANCE (tumor necrosis factor-related activation-induced cytokine), is a type 2 membrane protein which belongs to the TNF superfamily and is synthesized by stromal cells/osteoblasts and activated T cells (Wong et al., 1997). RANKL has been shown to play important roles for lymph node formation, mammary gland development and the survival of immune cells as well as bone homeostasis in the 1990s (Anderson et al., 1997; Wong et al., 1997; Lacey et al., 1998; Naito et al., 1999; Fata et al., 2000). RANK, a type 1 transmembrane protein that belongs to the tumor necrosis factor receptor (TNFR) superfamily, is expressed primarily on monocytes/macrophages including osteoclastic precursors, activated T cells, dendritic cells, and mature osteoclasts (Anderson et al., 1997; Wong et al., 1997; Lacey et al., 1998). Osteoprotegerin (OPG) is a soluble decoy receptor for RANKL that is released from bone marrow stromal cells/osteoblasts as a soluble form (Simonet et al., 1997). OPG competes with RANK for RANKL, thereby inhibiting osteoclast differentiation and function. OPG-deficient knockout mice developed osteoporosis due to an excess of osteoclasts (Simonet et al., 1997; Bucay et al., 1998).

In this review, I will briefly describe RANK signaling pathways and key molecules inducing osteoclast differentiation. The elucidation on RANKL-RANK signaling pathways might give hints of potential drug targets for preventing bone-related diseases including osteoporosis.

TNF receptor-associated factors (TRAFs)

Stimulation of RANK by RANKL binding leads to trimerization of RANK and recruits TRAF adaptor proteins to the conserved TRAF domains within the C-terminal cytoplasmic tail of RANK (Darnay et al., 1998; Walsh and Choi, 2003). Whereas TRAF 2, 3, 5 and 6 can bind to RANK, TRAF4 was shown to be a nuclear protein so failed to interact with RANK (Darnay et al., 1998; Inoue et al., 2000; Walsh and Choi, 2003). Moreover, TRAF2, TRAF5 and TRAF6 activate transcription factors such as NF- κ B and AP-1 that are required for osteoclast differentiation, however, TRAF3 serves as an inhibitor of NF- κ B pathway (Kanazawa et al., 2003; Hauer et al., 2005; Kanazawa and Kudo, 2005).

Among the TRAFs, TRAF6 seems to be a major adaptor protein of RANKL-RANK signaling pathway for osteoclast formation and function since the phenotype of TRAF6-deficient mice is nearly similar with the bone phenotypes of RANKL- or RANK-deficient mice, severe osteopetrosis due to impaired osteoclast differentiation or bone resorbing function (Lomaga et al., 1999; Naito et al., 1999).

However, it seems that RANKL could induce osteoclast differentiation through a TRAF6-independent signaling pathway since TRAF6-deficient hematopoietic precursors can differentiate into osteoclasts by RANKL if proper cofactors are provided, *in vitro*. Indeed, hematopoietic precursors from RANKL-, RANK-, or TRAF6-null mice can become osteoclasts *in vitro* when TNF α and cofactors such as TGF- β are supported (Kim et al., 2005). Nevertheless, RANKL-induced osteoclastogenesis is considerably reduced by TRAF6 deficiency, even cofactors are added, which implies that TRAF6 is a critical downstream mediator of RANKL-RANK signaling pathway to induce osteoclast differentiation (Kim et al., 2005).

RANK-TRAF6 interaction activates the I κ B kinase (IKK) complex to stimulate Nuclear factor kappa B (NF- κ B) via a signaling complex with TGF β -activated kinase 1 (TAK1), TAK-1-binding protein 1 (TAB1) and TAB2 or atypical protein kinase C (aPKC) (Mizukami et al., 2002; Duran et al., 2004). Dominant-interfering mutant forms of TAK1 inhibit RANKL-mediated activation of both I κ B kinase 1/2 (IKK1/2) and JNK1, leading to the inactivation of the NF- κ B and AP-1, respectively (Mizukami et al., 2002). The interaction of TRAF6 with aPKCs is through the mediation of scaffolding protein, p62 (Duran et al., 2004).

Mitogen activated protein (MAP) kinases

Recruitment of TRAF6 to RANK activates ERK, JNK, and p38 through activation of MEK1/2, MKK7, and MKK6 in osteoclast precursors, respectively (Kashiwada et al., 1998; Matsumoto et al., 2000; Yamamoto et al., 2002; He et al., 2011). The receptor for activated C kinase 1 (RACK1) acts as a scaffold protein to link the TRAF6-TAK1 complex with MKK6, which selectively facilitates the activation of p38 during the RANKL-initiated differentiation of osteoclast precursor cells into osteoclasts (Lin et al., 2015). Moreover,

it was reported that RANK-TRAF6-Rac1-NADPH oxidase1-dependent pathway-induced reactive oxygen species production is required for MAPK activation for osteoclastogenesis (Lee et al., 2005). TRAF6 deficiency abolishes RANKL-mediated JNK and p38 MAPKs activation (Kobayashi et al., 2001). Mice with genetic deletion of *erk1* exhibited reduced osteoclast formation *in vivo*, suggesting that ERK1 plays an important role in osteoclast differentiation (He et al., 2011). ERK1/2 induces activation of their downstream targets such as c-Fos. The lack of Fos (encoding c-Fos) results in increased numbers of bone marrow macrophages with the decrease of osteoclasts (Grigoriadis et al., 1994). Osteoclast precursor cells derived from *jnk1*-lacking mice but not from *jnk2*-lacking mice exhibited reduced ability to differentiate to osteoclasts (David et al., 2002). JNK1/2 induces activation of their downstream targets such as activator protein-1 (AP-1) transcription factors (David et al., 2002).

A specific inhibitor of p38, SB203580, suppressed RANKL-mediated osteoclast differentiation in RAW 264.7 cells (Li et al., 2002). Stimulation of p38 results in the downstream activation of the transcriptional regulator mi/Mitf, which controls the expression of the genes encoding tartrate-resistant acid phosphatase (TRAP, encoded by *Acp5*) and cathepsin K (CATK), a cysteine protease (Mansky et al., 2002). Mutant osteoclasts in *Mitf^{mi/mi}* mice are primarily mononuclear and express decreased levels of TRAP (Luchin et al., 2000; Luchin et al., 2001). Cathepsin K knockout mice develop osteopetrosis due to a deficit matrix degradation but not demineralization (Gowen et al., 1999). Similarly, mutations in the human cathepsin K gene have demonstrated an association with a rare skeletal dysplasia, pycnodysostosis (Gelb et al., 1996; Johnson et al., 1996). TRAP, an osteoclast differentiation marker, as well as cathepsin K also affect the functional activity of osteoclast by regulating bone matrix resorption and collagen turnover (Roberts et al., 2007).

Src/PI3K/Akt pathway

RANK associates with Src family kinase through interaction between TRAF6 and Cbl scaffolding proteins (Wong et al., 1999; Arron et al., 2001), activating the pro-survival factor PI3-kinase (PI3K)/Akt pathway (Wong et al., 1999). Activated PI3K generates phosphatidylinositol-(3,4,5)-phos-

phate (PIP3) at the plasma membrane, which leads to the recruitment of Akt/PKB via its pleckstrin homology (PH) domain and activation (Vanhaesebroeck et al., 2000). The activation of PI3K/Akt is Src-dependent because a genetic deletion of c-Src inhibits Akt activation by RANKL (Wong et al., 1999). The PI3K inhibitor LY294002 decreases osteoclast differentiation by reducing survival of osteoclast precursor cells during differentiation (Wong et al., 1999; Lee et al., 2002). Additionally, the association of the actin-binding protein gelsolin with PI3K showed that PI3K is important for the actin filament formation in osteoclasts (Chellaiah et al., 1998).

Akt induces osteoclast differentiation through regulating the GSK3 β /NFATc1 signaling cascade. Akt overexpression in osteoclast precursors induces the expression and nuclear localization of NFATc1 by GSK-3 β phosphorylation and inactivation (Moon et al., 2012).

Phosphatase and tensin homolog (PTEN) and SH2-containing inositol phosphatase 1 (SHIP1) negatively regulate PI3K signaling, thus reducing osteoclast differentiation (Takeshita et al., 2002; Sugatani et al., 2003). Mice lacking the SHIP-1 or tyrosine phosphatase SHP-1, both of which inhibit ITAM signaling, showed enhanced osteoclastogenesis and induced osteoporosis (Takeshita et al., 2002).

NF- κ B signaling

NF- κ B signaling activated by RANKL is important for osteoclast differentiation. NF- κ B is including five members: c-Rel (Rel), RelA (p65), RelB, NF- κ B1 (p105/p50), and NF- κ B2 (p100/p52). These NF- κ B proteins (p105 and p100) become shorter following post-translational processing into p50 and p52, respectively. Since p50 and p52 lack a transcription activation domain, they form dimers with Rel family members such as c-Rel (Rel), RelA (p65), RelB (Ghosh et al., 2002; Hayden et al., 2004). The NF- κ B p50/p52 double-knockout (dKO) mice develop severe osteopetrosis because of a total absence of osteoclasts and show growth retardation while single knockout of p50 or p52 failed to show developmental defects (Franzoso et al., 1997; Iotsova et al., 1997). Moreover, the dKO mice showed an increase in RANK-expressing splenocytes, which suggests that p50 and p52 are necessary for osteoclastogenesis but

not for RANK-expressing progenitor formation (Xing et al., 2002). Overexpression of c-Fos rescued the defect in osteoclast formation in the absence of RANKL and dKO splenocytes treated with RANKL or TNF failed to induce c-Fos, indicating that c-Fos are downstream of NF- κ B (Iotsova et al., 1997; Yamashita et al., 2007).

I κ B kinase (IKK) activation by RANKL stimulation induces phosphorylation and ubiquitin-dependent proteasomal degradation of I κ B. NF- κ B released from the NF- κ B/I κ B complex translocated into the nucleus and promotes transcription of target genes (Ghosh et al., 2002; Hayden et al., 2004). NF- κ B-inducing kinase (NIK) and IKK α are important for the RelB:p52 complex formation but NF- κ B activation via NIK is not necessary for osteoclast formation since NIK-deficient mice failed to show osteopetrosis phenotype (Ghosh et al., 2002; Novack et al., 2003; Hayden et al., 2004).

NFATc1

NFATc1 is a master transcription factor for the terminal differentiation of osteoclasts. Activated NFATc1 undergoes nuclear translocation and activates and induces osteoclast-specific genes such as tartrate-resistant acid phosphatase (TRAP), osteoclast-associated receptor (OSCAR), cathepsin K as well as NFATc1 itself (Takayanagi et al., 2002; Matsumoto et al., 2004; Asagiri et al., 2005). In addition, NFATc1 regulates cell-cell fusion of osteoclasts through up-regulation of the d2 isoform of vacuolar ATPase V0 domain (Atp6v0d2) and the dendritic cell-specific transmembrane protein (DC-STAMP) (Kim et al., 2008; Feng et al., 2009). The activation of NFATc1 is regulated by calcium/calmodulin signaling and RANK does not initiate calcium signaling directly in osteoclast precursors. Thus, the activation of NFATc1 is induced by RANKL, partially, and, for the robust activation of NFATc1, costimulatory signaling and RANKL signaling are collaborating (Takayanagi et al., 2002).

RANKL induces NFATc1 through NF- κ B and c-Fos activation and a deficiency of p50/p52 or c-fos causes failure of NFATc1 induction (Li et al., 2004; Matsuo et al., 2004; Asagiri et al., 2005). NFATc1-deficient embryonic stem cells failed to differentiate into osteoclasts, and the overexpression of constitutively active NFATc1 in osteoclast precursors

caused efficient osteoclast differentiation even in the absence of RANKL which suggests that NFATc1 is sufficient for osteoclastogenesis (Takayanagi et al., 2002).

CONCLUSION

The interest on the maintenance of bone homeostasis and bone remodeling occurring throughout adulthood as our life span is extended is increasing. Since 1990's, the finding of RANKL and RANK, and the studies using genetic engineered mouse models boosted significant progression in bone physiology. Especially, the importance of RANKL/RANK system in osteoclast biology has revealed critical signaling pathways and molecules regulating osteoclast differentiation. Recruitment of TRAF6 to RANK activates distinct signaling cascades and molecules as following: MAP kinases including JNK1/2, ERK1/2 and p38, Src/PI3K/Akt, Nuclear factor kappa B (NF κ B) and NFATc1. Thus, although key signaling pathways and molecules involved in osteoclastogenesis have already been identified, further investigations on the molecular mechanisms specific on differentiation stages, could be of interest. Especially, more studies on detailed molecular regulatory mechanism for osteoclast fusion and bone resorbing activity would help to develop effective therapeutic drugs for bone-related diseases.

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CONFLICT OF INTEREST

The author has no conflict of interest.

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