

Review

Regulatory roles and molecular signaling of TNF family members in osteoclasts

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Abstract

The tumor necrosis factor (TNF) family has been one of the most intensively studied families of proteins in the past two decades. The TNF family constitutes 19 members that mediate diverse biological functions in a variety of cellular systems. The TNF family members regulate cellular functions through binding to membrane-bound receptors belonging to the TNF receptor (TNFR) family. Members of the TNFR family lack intrinsic kinase activity and thus they initiate signaling by interacting intracellular signaling molecules such as TNFR associated factor (TRAF), TNFR associated death domain (TRADD) and Fas-associated death domain (FADD). In bone metabolism, it has been shown that numerous TNF family members including receptor activator of nuclear factor κ B ligand (RANKL), TNF- α , Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) play pivotal roles in the differentiation, function, survival and/or apoptosis of osteoclasts, the principal bone-resorbing cells. These TNF family members not only regulate physiological bone remodeling but they are also implicated in the pathogenesis of various bone diseases such as osteoporosis and bone loss in inflammatory conditions. This review will focus on our current understanding of the regulatory roles and molecular signaling of these TNF family members in osteoclasts.

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Keywords: Osteoclast; RANKL; TNF- α ; FasL; TRAIL; Bone remodeling

Abbreviations: AP-1, activator protein-1; ASK1, apoptosis signal-regulated kinase-1; CARD, caspase recruiting domain; CFU-GM, colony forming unit-granulocyte/macrophage; CRD, cysteine-rich repeat; CTR, calcitonin receptor; DD, death domain; DED, death effector domain; ERK, extracellular signal-regulated kinase; FasL, Fas ligand; FADD, Fas associated death domain; GCKR, germinal center kinase related; HSC, hematopoietic stem cell; IAP, inhibitor of apoptosis protein; I κ B, inhibitor of kappa B; IKK, I κ B kinase; IL, interleukin; JNK, Jun N-terminal kinase; LT- α , lymphotoxin α ; MAPK, mitogen-activated protein kinase; MAP3K, MAPK kinase kinase; MADD, MAP kinase-activating death domain; M-CSF, monocyte/macrophage-colony stimulating factor; MEKK, MAPK/ERK kinase kinase; MKK3, MAPK kinase 3; NF- κ B, nuclear factor kappa B; NFAT, nuclear factor of activated T cell; NIK, NF- κ B-inducing kinase; OPG, osteoprotegrin; OPGL, osteoprotegrin ligand; ODF, osteoclast differentiation factor; PI3-kinase, phosphoinositide-3OH kinase; PIP3, phosphatidylinositol-(3,4,5)-phosphate; PH, pleckstrin homology; PKB, protein kinase B; RA, rheumatoid arthritis; RANK, receptor activator of NF- κ B; RANKL, RANK ligand; RIP, receptor interacting protein; TAK1, TGF- β -activated kinase 1; TGF- β , transforming growth factor β ; THD, TNF homology domain; TNF, tumor necrosis factor; TNFR, TNF receptor; TRAF, TNFR associated factor; TRADD, TNFR associated death domain; TRAIL, TNF-related apoptosis-inducing ligand; TRAIL-R, TRAIL receptor; TRAP, tartrate-resistant acid phosphatase; TRANCE, TNF-related activation-induced cytokine; TLR, Toll-like receptor.

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1. Introduction

Since the molecular cloning of cDNAs for TNF- α and lymphotoxin α (LT- α , also named TNF- β), the first two members of the TNF family, in early 1980s (Gray et al., 1984; Pennica et al., 1984), the TNF family has been expanded to include 19 members (Bodmer et al., 2002; Locksley et al., 2001). The TNF family proteins are expressed as type II homo- or hetero-trimeric transmembrane proteins with exception of only one member VEGI, which lacks a predicted transmembrane domain and is therefore synthesized as a secreted soluble protein (Bodmer et al., 2002). Some of the membrane-bound TNF family members may be cleaved at membrane proximal residues to generate soluble forms. Structurally, these proteins are characterized by a conserved domain termed TNF homology domain (THD) in their C-terminal domains, which are the extracellular domains of the membrane-bound members (Pennica et al., 1984). Functionally, despite that the founding members TNF- α and LT- α were initially identified as proteins that possess tumor cytotoxicity, it has been now recognized that members of the TNF family regulate a variety of cellular functions such as cell differentiation, function, survival and/or apoptosis (Gaur and Aggarwal, 2003).

The TNF family members exert the diverse cellular functions by binding and activating their respective receptors belonging to the TNFR family (Bodmer et al., 2002; Locksley et al., 2001). This receptor family consists of 29 known members that are typically single-spanning type I transmembrane proteins with extracellular domains containing two to four homologous cysteine-rich repeats (CRD). However, a few TNFR members exist as secreted soluble proteins. For those members occurring as transmembrane proteins, they have a cytoplasmic domain of variable length bearing little sequence homology (Arch and Thompson, 1999; Bodmer et al., 2002; Darnay and Aggarwal, 1999). Moreover, these TNFR family members can be divided into two subfamilies based on the presence of a death domain (DD) in their cytoplasmic domains: (1) the DD-containing receptors such as TNFR1, Fas, TRAIL-R1 and TRAIL-R2; and (2) the receptors lacking a DD such as RANK, TNFR2, CD27 and CD40 (Arch and Thompson, 1999; Bodmer et al., 2002).

Members of the TNFR family lack intrinsic enzymatic activity in their intracellular domains. As a result, they transduce signaling by recruiting adapter proteins, primarily DD-containing proteins and members of the TRAF family. The DD-containing proteins include FADD and TRADD. These proteins link the DD-containing receptors to downstream proteases of the caspase family necessary for activation of apoptosis. The TRAF family contains six members (TRAFs 1, 2, 3, 4, 5 and 6), each containing a ring and zinc finger motif in their N-terminal and C-terminal domains that mediate self association and protein interaction (Inoue et al., 2000). The TRAFs link either the DD-containing receptors (via other adapter proteins) or the

receptor lacking a DD to activation of various signaling pathways such as NF- κ B, JNK, ERK and p38 (Baud and Karin, 2001; Locksley et al., 2001).

The TNF family regulates cellular differentiation, function, survival and/or apoptosis in a variety of cell types/tissues/organs. As such, the TNF family has been shown to play important roles in regulating the following key biological processes such as lymphoid organogenesis, acute immune response, inflammation, bone homeostasis, mammary gland development, hair follicle and sweat gland development, and neural development (Locksley et al., 2001). Given the diverse roles the TNF family plays, it could not be possible to discuss the actions of all the TNF family members with enough details in a single review. This review will focus on the regulatory roles of several TNF family members in osteoclast biology and the signaling pathways activated by their corresponding receptors to exert their effects on osteoclasts.

2. The TNF family and osteoclast biology

Osteoclasts are our body's principal bone-resorbing cells that not only play a critical role in skeleton development and maintenance but are also implicated in the pathogenesis of various bone diseases including menopausal osteoporosis (Manolagas, 1998; Pacifici, 2001; Ross and Teitelbaum, 2001). Osteoclasts are multinucleated giant cells that differentiate from cells of hematopoietic origin (Ross and Teitelbaum, 2001; Suda et al., 1992; Teitelbaum et al., 1997). The osteoclast differentiation involves several major stages outlined in Fig. 1. The hematopoietic stem cells (HSC) give rise to circulating mononuclear cells termed colony forming unit-granulocyte/macrophage (CFU-GM). Macrophage/monocyte-colony forming factor (M-CSF) stimulates the proliferation of CFU-GM to maintain a pool of mononuclear cells in monocyte/macrophage lineage, which are widely viewed as osteoclast precursors and characterized by lack of two osteoclast markers: tartrate-resistant acid phosphatase (TRAP) and calcitonin receptor (CTR). The mononuclear precursors are attracted to prospective resorption sites by an unknown mechanism (presumably by chemotaxis) and they will then attach onto bone matrix to differentiate into pre-fusion osteoclasts with the stimulation of M-CSF and RANKL. The pre-fusion cells become both TRAP- and CTR-positive. With continuous stimulation of M-CSF and RANKL, the pre-fusion osteoclasts will further differentiate by fusion to become multinucleated cells. The multinucleated osteoclasts are not functional since they lack the ruffled membrane that is critical for bone resorption. RANKL continue to play an important role in activating osteoclasts by stimulating formation of the ruffled membrane (Jilka et al., 1999; Lacey et al., 1998; Suda et al., 1999). In addition, RANKL also promotes the survival of mature osteoclasts (Fuller et al., 1998; Lum et al., 1999; Wong et al., 1999a).

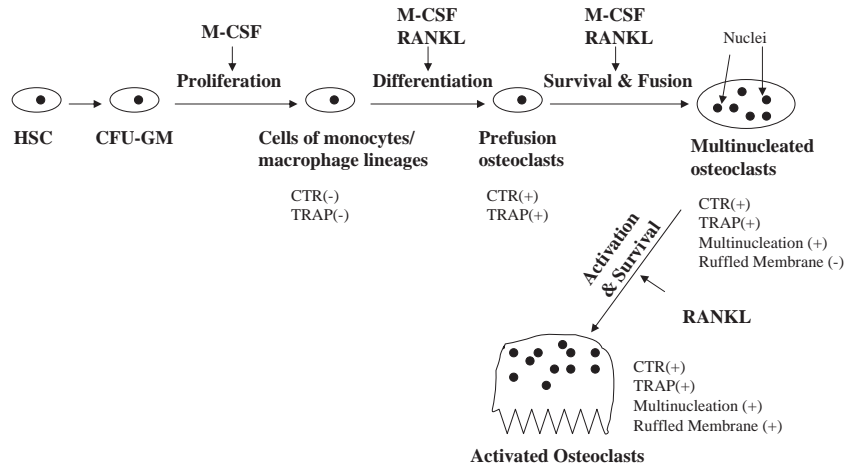


Fig. 1. Osteoclast differentiation pathway. HSC: hematopoietic stem cell, CFU-GM: colony forming unit granulocyte/macrophage, CTR: calcitonin receptor, TRAP: tartrate-resistant acid phosphatase.

Thus, the critical role for the TNF family in osteoclast biology can be easily appreciated given that RANKL, a member of the TNF family, has been recognized as one of the most prominent factors regulating osteoclast formation, activation and survival (Fig. 1). Moreover, the importance of the TNF family in osteoclast biology is further supported by the findings that a number of other TNF family members including TNF- α , FasL and TRAIL also play roles in modulating the differentiation, function, survival and/or apoptosis of osteoclasts. Together, these TNF family members not only regulate physiological bone remodeling but are also implicated in the pathogenesis of numerous bone disorders. In this review, I will first provide a concise and updated review of physiological and/or pathological roles of these TNF family members in bone metabolism. These TNF family proteins exert their effects on bone remodeling by activating distinct intracellular signaling pathways in osteoclasts. Enormous efforts have been devoted to elucidate the signaling pathways in the past several years. Therefore, as the second focus, I will review our current understanding of the signaling pathways activated by these factors in osteoclasts. A discussion on these TNF members in a single review may provide an opportunity for comparison and contrast of the roles and signaling mechanisms of these distinct TNF family members in osteoclasts.

3. RANKL

3.1. RANKL and its receptors RANK and OPG

RANKL, also known as OPLG, ODF and TRANCE, was identified independently by two bone groups (Lacey et al., 1998; Yasuda et al., 1998) and two immunology groups (Anderson et al., 1997; Wong et al., 1997) in the late 1990s. To date, RANKL has been shown to play pivotal roles in regulating various biological processes

such as bone homeostasis (Teitelbaum, 2000; Yasuda et al., 1998), immune function (Anderson et al., 1997; Wong et al., 1999b) and mammary gland development (Fata et al., 2000). RANKL is involved in bone metabolism by mediating osteoclast differentiation, function and survival (Lacey et al., 1998; Yasuda et al., 1998; Hsu et al., 1999).

The discovery of RANKL helped establish that osteoblasts/stromal cells support osteoclast differentiation primarily by serving as a source of RANKL as well as M-CSF (Suda et al., 1999) (Fig. 2). Osteoblasts/stromal cells express both M-CSF and RANKL (membrane-bound RANKL and soluble RANKL). M-CSF and RANKL will bind to their respective receptor c-fms and RANK expressed on osteoclast precursors to stimulate osteoclast formation. In vitro, M-CSF and RANKL have been shown to be sufficient for osteoclastogenesis (Quinn et al., 1998). In mature osteoclasts, RANKL mediates osteoclast activation and survival (Lacey et al., 1998; Suda et al., 1999; Lum et al., 1999; Wong et al., 1999a). In addition, osteoblasts/stromal cells also produce a factor called OPG, which is decoy receptor for RANKL. OPG inhibits RANKL function by competing

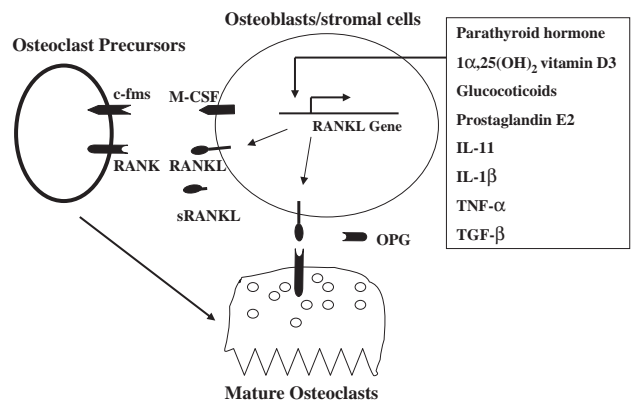


Fig. 2. Model on the action of the RANKL/RANK system in osteoclasts. sRANKL: soluble RANKL.

with RANK for RANKL (Suda et al., 1999; Teitelbaum, 2000).

Moreover, the unraveling of the RANKL/RANK system has also helped reveal that many osteotropic hormones and cytokines regulate osteoclast formation and function through modulating RANKL expression by osteoblasts/stromal cells (Hofbauer, 1999; Hofbauer et al., 2000; Ross, 2000) (Fig. 2). For instance, it has been known for a quite long time that in vitro generation of osteoclasts by coculturing osteoblasts/stromal cells and osteoclast precursors requires $1\alpha,25\text{-(OH)}_2$ vitamin D₃ and dexamethasone. However, it was not clear until the discovery of the RANKL/RANK system that $1\alpha,25\text{(OH)}_2$ vitamin D₃ and dexamethasone stimulate osteoclast formation in the coculture system by up-regulating RANKL production by osteoblasts/stromal cells (Kitazawa et al., 1999; Yasuda et al., 1998). In addition, other osteotropic hormones and cytokines such as IL-1, TNF- α , prostaglandin E₂, IL-11 and parathyroid hormone have also been shown to stimulate RANKL gene expression in osteoblasts and stromal cells (Hofbauer et al., 1999; Lee and Lorenzo, 1999; Yasuda et al., 1998). In contrast, transforming growth factor β (TGF- β) suppresses RANKL gene expression (Takai et al., 1998).

Both RANKL and RANK are essential for the osteoclastogenic process since mice lacking the gene for either protein developed osteopetrosis due to failure to form osteoclasts (Dougall et al., 1999; Kong et al., 1999a,b; Li et al., 2000). Consistently, knockout mice deficient for OPG developed early onset of osteoporosis due to elevated osteoclast differentiation (Bucay et al., 1998), whereas transgenic mice over-expressing OPG exhibited osteopetrosis, resulting from a decrease in late stages of osteoclast differentiation (Simonet et al., 1997). Taken together, these data indicate that the RANKL/RANK system plays an essential role in skeletal development and bone remodeling.

On the other hand, the RANKL/RANK system is also implicated in the pathogenesis of various bone diseases such as postmenopausal osteoporosis, bone loss in rheumatoid arthritis (RA) and tumor-induced osteolysis. A recent study showed that RANKL plays a pathological role in postmenopausal osteoporosis (Eghbali-Fatourehchi et al., 2003). Specifically, estrogen deficiency leads to the elevated expression of RANKL on both osteoblasts and lymphocytes. Moreover, RANKL expressed on activated T cells is also implicated in inducing bone loss and joint destruction in RA (Kong et al., 1999a). Finally, RANKL has been shown to contribute to bone metastasis and/or osteolysis in breast and prostate cancers (Kitazawa and Kitazawa, 2002; Zhang et al., 2001a).

3.2. RANK signaling in osteoclasts

Since the cloning of RANKL and RANK, enormous efforts have been undertaken to elucidate RANK-initiated intracellular signaling in osteoclast differentiation, function and survival. RANK was identified as a TNFR family

member lacking a DD (Anderson et al., 1997). Thus, presumably RANK transduces intracellular signals by utilizing TRAF proteins. Indeed, numerous studies showed that RANK directly interacts with TRAF proteins and these interactions may be responsible for activating the NF- κ B complex and JNK (Darnay et al., 1998, 1999; Galibert et al., 1998; Hsu et al., 1999; Kim et al., 1999; Wong et al., 1998). Collectively, TRAF1, 2, 3, 5 and 6 were shown to be able to bind to RANK in in vitro binding assays and/or in transformed cells in context of over-expression. TRAF4 appears to be a nuclear protein (Inoue et al., 2000), which does not interact with RANK. These early RANK-TRAF interaction studies suggested that RANK may contain multiple TRAF-binding motifs that regulate osteoclast differentiation, function and/or survival. Subsequently, recent functional studies indicated that RANK indeed contains multiple domains that are able to mediate osteoclast formation and function (Armstrong et al., 2002; Liu et al., 2004). More specifically, three RANK cytoplasmic motifs, PFQEP^{369–373}, PVQEET^{559–564} and PVQEQQ^{604–609}, are capable of independently mediating osteoclast formation and function (Liu et al., 2004) (Fig. 3). In addition, PVQEET^{559–564} and PVQEQQ^{604–609} are more potent than PFQEP^{369–373} in mediating osteoclast formation (Liu et al., 2004). The functional identification of these RANK motifs has not only revealed the complexity of the RANK signaling but also laid a foundation for further elucidation of RANK-initiated signaling in osteoclasts. Just for convenience of discussion below, PFQEP^{369–373}, PVQEET^{559–564} and PVQEQQ^{604–609} are designated as Motif 1, Motif 2 and Motif 3, respectively.

3.2.1. Signaling initiated by RANK cytoplasmic motif PFQEP^{369–373} (Motif 1)

Motif 1 has been previously shown to be a TRAF6-binding motif (Ye et al., 2002). A cell-permeable decoy peptide derived from this motif blocked osteoclast formation (Ye et al., 2002), establishing the functional relevance of this binding to osteoclast formation. Moreover, the functional involvement of TRAF6 in RANK signaling was further substantiated by the finding that TRAF6^{-/-} mice exhibited defect in osteoclast differentiation and/or function (Lomaga et al., 1999; Naito et al., 1999).

Motif 1 activates NF- κ B and three mitogen-activated protein kinase (MAPK) pathways (JNK, ERK and p38) in response to RANKL stimulation (Liu et al., 2004). Activation of these pathways by Motif 1 involves the formation of a protein complex containing TRAF6, TGF- β -activated kinase 1 (TAK1) and an adaptor protein TAB2 (Fig. 3). TAK1 is a member of MAPK kinase kinase (MAP3K) family that is activated by various cytokines (Yamaguchi et al., 1995). In RANK signaling, it was shown that the RANKL-induced formation of the complex containing TRAF6, TAK1 and TAB2 leads to the activation of TAK1 (Mizukami et al., 2002). TAB2 facilitates the formation of the complex by linking TAK1 to TRAF6

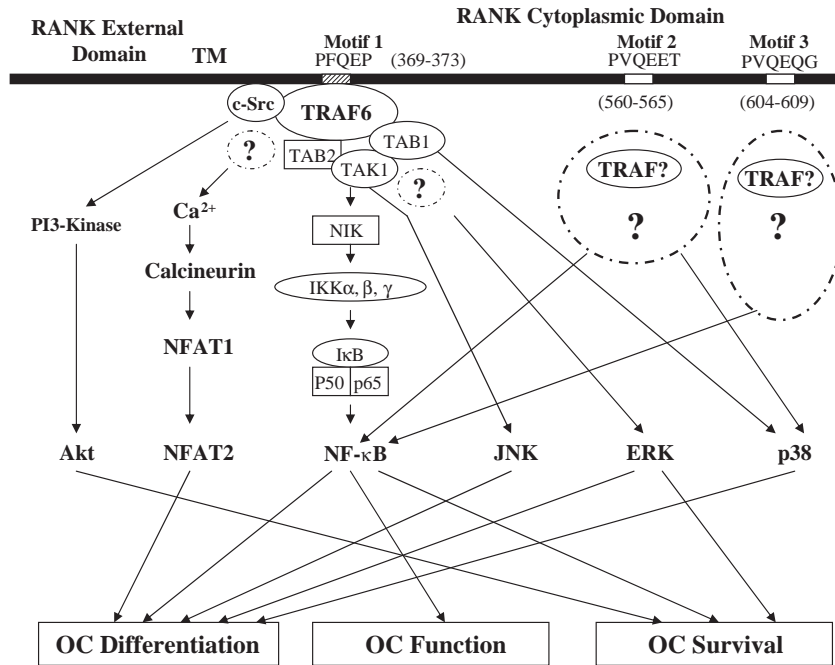


Fig. 3. RANK signaling in osteoclast differentiation, function and survival. OC: osteoclast, TM: transmembrane domain.

(Mizukami et al., 2002). Activated TAK1 phosphorylates NF- κ B-inducing kinase (NIK) to activate the I κ B kinase (IKK) $\alpha\beta\gamma$ complex (Ninomiya-Tsuji et al., 1999), leading to the activation of NF- κ B pathway (Fig. 3). In addition, activated TAK1 also activates JNK pathway (Lee et al., 2002b; Shirakabe et al., 1997). Activation of p38 is mediated by TAB1 which has been shown to be able to bind and recruit p38 to the TRAF6-TAK1 complex (Ge et al., 2002). However, how Motif 1 activates ERK pathway remains unclear but it is likely that TRAF6 is also involved in the process (Fig. 3).

RANKL activates Akt/protein kinase B (PKB) pathway through TRAF6 (Wong et al., 1999a) and Motif 1 was shown to be a TRAF6 binding site. Thus, although it has not been experimentally confirmed, Motif 1 may also initiate downstream signaling leading to the activation of Akt/PKB pathway. This is achieved by RANKL-induced formation of a signaling complex containing both c-Src and TRAF6 at the site (Fig. 3). In the signaling complex, TRAF6 enhances the kinase activity of c-Src, leading to the activation of phosphoinositide-3-OH kinase (PI3-kinase) (Wong et al., 1999a). Activated PI3-kinase in turn stimulates the formation of phosphatidylinositol-(3,4,5)-phosphate (PIP3) at the plasma membrane, which results in the recruitment of Akt/PKB via its pleckstrin homology (PH) domain. Akt/PKB is then activated at the membrane (Wong et al., 1999a) (Fig. 3).

Recent studies revealed that RANKL activates transcription factor, nuclear factor of activated T-cell 2 (NFAT2, also known as NFATc1), which plays an important role in osteoclastogenesis (Ishida et al., 2002; Takayanagi et al., 2002). Moreover, the activation of NFAT2 is in part mediated by TRAF6 (Takayanagi et al., 2002). RANKL-

induced recruitment of TRAF6 mobilizes intracellular calcium, which results in the activation of calcineurin (Takayanagi et al., 2002). Activated calcineurin in turn dephosphorylates and activates NFAT1, which will translocate into nuclei to form a ternary complex with c-Fos and c-Jun at the promoter for NFAT2 gene to stimulates the expression of NFAT2 (Ikeda et al., 2004) (Fig. 3). But, how TRAF6 mobilizes intracellular Ca^{2+} remains unknown.

3.2.2. Signaling initiated by RANK cytoplasmic motifs PVQEET⁵⁵⁹⁻⁵⁶⁴ (Motif 2) and PVQEQQ⁶⁰⁴⁻⁶⁰⁹ (Motif 3)

While the signaling pathways initiated by Motif 1 have been largely elucidated, those activated by Motif 2 and Motif 3 still remain obscure. The first issue would be which TRAF protein functionally binds to each of the motifs (Fig. 3). Previous in vitro data suggested that Motif 2 and Motif 3 may bind TRAF proteins other than TRAF6 (Galibert et al., 1998). In line with this finding, Galibert et al. (1998) showed that Motif 2 interacts with TRAF3 and Motif 3 is capable of binding TRAF1, TRAF2 and TRAF5. But, another in vitro study demonstrated that neither TRAF1 nor TRAF3 interacts with RANK (Hsu et al., 1999). Given the uncertainty, additional studies are needed to functionally identify TRAF proteins that specifically bind to these RANK motifs. Nevertheless, it has been shown that Motif 2 activates NF- κ B and p38 pathways in osteoclast precursors, whereas Motif 3 activates only NF- κ B pathway (Liu et al., 2004). Future functional identification of TRAF proteins bind to Motif 2 and Motif 3 will facilitate the elucidation of downstream signaling pathways leading to the activation of NF- κ B and/or p38 pathway by these two motifs.

3.2.3. Role of distinct pathways activated by RANK in osteoclast formation, function and survival

As summarized in Fig. 3, RANK initiates 6 major known signaling pathways through different signaling cascades in response to RANKL. These pathways play distinct roles in osteoclast differentiation, function and survival. NF- κ B and JNK (leading to AP-1 activation) pathways are essential for osteoclast differentiation (Franzoso et al., 1997; Grigoriadis et al., 1994). Mice lacking both p50 and p52, members of NF- κ B, develop osteopetrosis due to complete lack of osteoclasts (Franzoso et al., 1997). Similarly, mice lacking c-fos, a component of AP-1, do not form osteoclasts (Grigoriadis et al., 1994), leading to osteopetrosis. Moreover, NF- κ B also plays a critical role in osteoclastic bone resorption (Miyazaki et al., 2000) and osteoclast survival (Jimi et al., 1998). Akt primarily plays a role in promoting osteoclast survival (Lee et al., 2001; Wong et al., 1999a), while NFAT2 has been recently shown to be a critical transcription factor for osteoclastogenesis (Ishida et al., 2002; Takayanagi et al., 2002) (Fig. 3). Other two MAPK pathways (ERK and p38) are also involved in osteoclast differentiation and/or survival. ERK plays a functional role not only in osteoclast differentiation but also in survival (Lee et al., 2001, 2002a). In contrast, p38 was shown to be only involved in mediating osteoclastogenesis (Lee et al., 2002a; Li et al., 2002, 2003; Matsumoto et al., 2000b) (Fig. 3).

3.3. Perspectives and future directions

The unraveling of the critical role of the RANKL/RANK system in osteoclast differentiation, function and survival represents a major milestone in the understanding of osteoclast biology. In the past 6 years, we have witnessed many important advances in the investigation of physiological and pathological roles of the RANKL/RANK system in bone remodeling. However, the RANK-initiated intracellular signaling pathways have not been completely elucidated. Notably, despite numerous early studies indicated that RANK may contain multiple motifs that are able to mediate osteoclast differentiation and function by recruiting different TRAF proteins (Darnay et al., 1998, 1999; Galibert et al., 1998; Hsu et al., 1999; Kim et al., 1999; Wong et al., 1998), the prevailing view has still been that RANK-initiated signaling is primarily mediated through TRAF6 (Boyle et al., 2003). The recent demonstration of functional involvement of three different RANK motifs in osteoclast formation and function has pointed to additional complexity of RANK signaling that has not yet been fully recognized (Armstrong et al., 2002; Liu et al., 2004).

Especially, the three motifs activate different sets of signaling pathways (Liu et al., 2004), suggesting that these motifs do not utilize the same TRAF protein to transduce downstream signaling (Fig. 3). Since Motif 1 has been previously shown to recruit TRAF6 to activate various pathways (Ye et al., 2002), Motif 2 and Motif 3 may bind

TRAF proteins other than TRAF6. This is consistent with the previous data that RANK region containing Motif 2 and Motif 3 does not interact with TRAF6 (Galibert et al., 1998). The functional identification of TRAF proteins binding to these two motifs in the future will be a key step in elucidation of signaling pathways activated by these two motifs (Fig. 3). Moreover, although Motif 1 is capable of activating more signaling pathways (NF- κ B, JNK, ERK and p38) than Motif 2 (NF- κ B and p38) and Motif 3 (NF- κ B only), Motif 2 and Motif 3 are more potent in stimulating osteoclast differentiation (Liu et al., 2004) (Fig. 3). Future studies directed to address this difference may reveal more insights into the mechanism underlying RANKL-mediated osteoclast differentiation.

The previous investigation of RANK signaling in osteoclasts also generated a few controversies. The most notable one is regarding the precise role of TRAF6 in osteoclast biology. Two TRAF6 knockout mice were generated by different laboratories (Lomaga et al., 1999; Naito et al., 1999). Both groups showed that their TRAF6^{-/-} mice developed osteopetrosis due to impaired bone resorption, supporting an important role for TRAF6 in bone remodeling. However, one group showed that deletion of TRAF6 blocked osteoclast differentiation while the other group demonstrated that the absence of TRAF6 impaired only osteoclast function without affecting osteoclast formation. The functional identification of three RANK motifs capable of independently mediating osteoclast formation supports that TRAF6 is not essential for osteoclast formation, because only one of the three functional motifs has been shown to utilize TRAF6 (Fig. 3). Mutation of the TRAF6-binding RANK motif did not affect osteoclast formation in vitro (Liu et al., 2004). Nonetheless, additional studies, better by independent laboratories, are needed to further clarify the role of TRAF6 in osteoclast differentiation. A definitive establishment of role of TRAF6 in osteoclast differentiation is essential for further elucidation of RANK signaling, and probably for understanding osteoclast biology as a whole, especially given that several cytokines have been shown to modulate osteoclast formation and function through TRAF6. For instance, it was shown that INF- γ inhibits osteoclast differentiation by inducing degradation of TRAF6 (Takayanagi et al., 2000), which is based on the premise that TRAF6 is essential for osteoclast formation.

4. TNF- α

4.1. TNF- α and its receptors TNFR1 and TNFR2

TNF- α represents another important member of the TNF family that modulates osteoclast formation and function (Lam et al., 2002; Nanes, 2003; Romas et al., 2002). TNF- α exerts its function via two receptors, TNFR1 (also known as p55), which contains a DD (Himmeler et al., 1990; Loetscher

et al., 1990; Nophar et al., 1990), and TNFR2 (also known as p75), which lacks a DD (Gray et al., 1990; Schall et al., 1990; Smith et al., 1990). As discussed above, RANKL and its receptor RANK are essential for osteoclastogenesis since the mice deficient for either RANKL or RANK completely lack osteoclasts (Dougall et al., 1999; Kong et al., 1999a,b; Li et al., 2000). In contrast, the mice lacking TNF- α or its receptors do not exhibit any bone defects (Marino et al., 1997; Peschon et al., 1998), indicating that TNF- α -mediated signaling are not essential for skeletal development and physiological bone remodeling.

Nonetheless, TNF- α has been shown to be implicated in the pathogenesis of postmenopausal osteoporosis (Nanes, 2003; Pacifici, 1996, 1998, 2001). The pathological role for TNF- α in postmenopausal osteoporosis was initially proposed based on the early observations that TNF- α is able to promote osteoclastogenesis in vitro and estrogen inhibits TNF- α production by cells such as human osteoblasts and peripheral blood mononuclear cells in vitro (Manolagas and Jilka, 1995; Pacifici et al., 1991; Pacifici, 1996). Recent studies demonstrated that knockout mice deficient for either TNF- α or TNFR1 are resistant to ovariectomy-induced bone loss (Roggia et al., 2001), confirming the pathological role of TNF- α in postmenopausal osteoporosis. Moreover, it was also shown that nude mice, which lack T lymphocytes, are protected from ovariectomy-induced bone loss (Cenci et al., 2000), revealing that circulating T cells are the major source of estrogen-regulated TNF- α . As a key pro-inflammatory and potent osteoclastogenic cytokine, TNF- α is also involved in bone loss in various inflammatory conditions such as rheumatoid arthritis and periodontitis (Assuma et al., 1998; Lam et al., 2002; Nanes, 2003; Romas et al., 2002). Activated macrophages are believed to be the major source

of TNF- α in these inflammatory conditions (Nishimura et al., 2003; Romas et al., 2002).

4.2. TNFR1 and TNFR2 signaling in osteoclasts

Both TNFR1 and TNFR2 are implicated in osteoclast formation and function. TNFR1 positively regulates osteoclast formation and function (Abu-Amer et al., 2000; Roggia et al., 2001). In contrast, TNFR2 exerts an inhibitory effect on osteoclast formation and function (Abu-Amer et al., 2000). Moreover, it has been established that TNF- α exerts its effect on osteoclast differentiation and function by activating various signaling pathways including NF- κ B (Wei et al., 2002), JNK (Wei et al., 2002), p38 (Li et al., 2003; Matsumoto et al., 2000a; Wei et al., 2002), ERK (Lee et al., 2001; Wei et al., 2002) and Akt (Wei et al., 2002) in osteoclast precursors/osteoclasts (Fig. 4). The specific roles of these pathways in osteoclast biology have been discussed above and summarized in Fig. 3. Although it is now clear that TNF- α is capable of activating NF- κ B, JNK, p38, ERK and Akt pathways in osteoclast precursors/osteoclasts, the precise signaling cascades leading to the activation of these pathways have largely not been functionally established in osteoclast precursors/osteoclasts.

TNFR1- and TNFR2-activated signaling pathways have been intensively investigated in a variety of other cell types. Fig. 4 summarizes the current understanding of the signaling pathways initiated by TNFR1 and TNFR2 primarily based on the studies involving cells other than osteoclast precursors/osteoclasts. It is worthwhile to emphasize that many of the pathways described in Fig. 4 have been shown to be applicable to many different cell types (Dempsey et al., 2003; Locksley et al., 2001; Wajant et al., 2003). Thus, it is

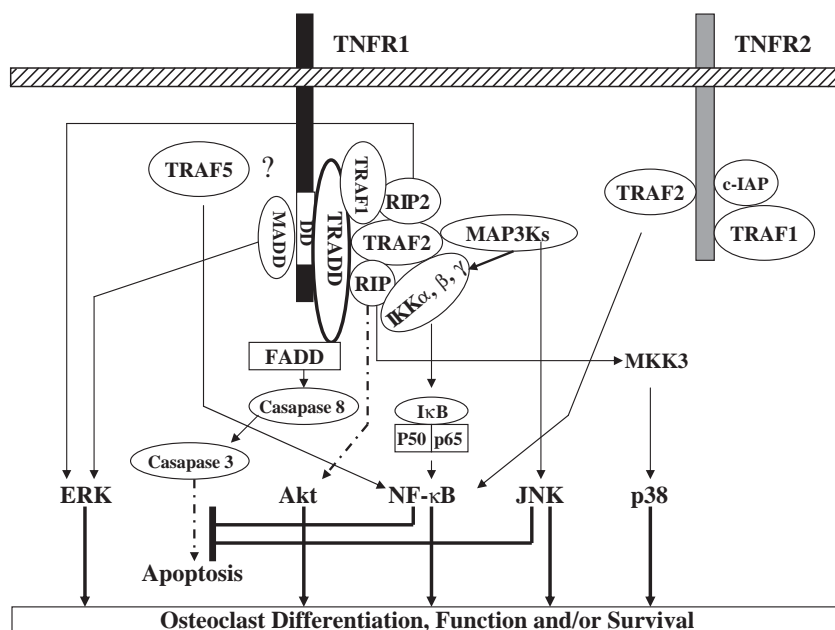


Fig. 4. TNFR1 and TNFR2 signaling in osteoclast differentiation, function and/or survival.

reasonable to assume that a considerable portion of the signaling cascades described in Fig. 4 may hold true for osteoclast precursors/osteoclasts, too. Nonetheless, future studies are needed to evaluate whether these signaling cascades are indeed employed by osteoclast precursors/osteoclasts to mediate TNF- α -dependent activation of the NF- κ B, JNK, p38, ERK and Akt pathways.

Whereas TNFR2 binds TNF- α more tightly, TNFR1 is believed to be the predominant receptor mediating intracellular signaling in most cell types (Dempsey et al., 2003; Locksley et al., 2001; Wajant et al., 2003). TNFR1 is one of the TNFR family members possessing a DD (Himmler et al., 1990; Loetscher et al., 1990; Nophar et al., 1990). Consequently, TNFR1 signaling is primarily initiated by the DD in its cytoplasmic domain (Dempsey et al., 2003; Wajant et al., 2003) (Fig. 4). Upon ligand binding, TNFR1 trimerizes and recruits TRADD, which in turn interacts with FADD to activate the caspase cascade leading to apoptosis. Both TRADD and FADD contain DD and their interaction is mediated by their DDs. The associated FADD then recruits pro-caspase-8 to the complex to activate caspase-8 by removal of the pro-domain (Dempsey et al., 2003). Both FADD and pro-caspase-8 contain death effector domain (DED) and the interaction between FADD and pro-caspase-8 is modulated through their DEDs. Then, activated procaspase-8 cleaves and activates caspase 3 (Dempsey et al., 2003) (Fig. 4). Only in very few cases, TNFR1 transduces apoptotic signal. In most circumstances, TNFR1 initiates signals leading to the activation of gene transcription and cellular functions and the pro-apoptotic signaling is suppressed by a simultaneous activation of NF- κ B and JNK (see below), which protect cells from apoptosis by blocking the activated apoptotic pathway (Dempsey et al., 2003) (Fig. 4). Given that TNFR1 was shown to primarily promote osteoclastogenesis and it does not induce apoptosis in osteoclasts, the TNFR1-initiated apoptotic pathway in osteoclasts is likely to be suppressed by the same mechanism.

Furthermore, TNFR1 also mediates the activation of NF- κ B by involving two adaptor proteins TRAF2 and/or RIP (receptor interacting protein). As shown in Fig. 4, in addition to FADD, TRADD can also interact with TRAF2 and RIP (Hsu et al., 1996a,b). Both RIP and TRAF2 are involved in activating NF- κ B. TRAF2 recruits the IKK complex into the TNFR1 signaling complex while RIP is responsible for activating the IKK complex (Devin et al., 2000), leading to the activation of NF- κ B pathway. Alternatively, TRAF2 may associate with several MAP3K including NIK, mitogen-activated protein kinase/ERK kinase (MEKK) 1 and MEKK2 to activate the IKK complex (Baud et al., 1999; Malinin et al., 1997; Song et al., 1997; Yang et al., 2001). The precise signaling cascade leading to the activation of Akt by TNFR1 still remains unknown. But, a recent study demonstrated that RIP is involved in mediating the activation of Akt in Toll-like receptor 4 (TLR4) in response to LPS stimulation (Vivarelli

et al., 2004), proposing a possibility that the activation of Akt by TNFR1 may also involve RIP (Vivarelli et al., 2004) (Fig. 4).

Finally, TNFR1-initiated signaling have been shown to be able to activate the three MAPK pathways: JNK, p38 and ERK (Luschen et al., 2000; Tran et al., 2001; Wajant et al., 2003) (Fig. 4). TNFR1-mediated JNK activation involves TRAF2. TRAF2 interacts with several MAP3Ks such as apoptosis signal-regulated kinase-1 (ASK1), MEKK1 and germinal center kinase related (GCKR) to activate JNK (Baud et al., 1999; Nishitoh et al., 1998). TNFR1-mediated p38 activation may involve RIP and MAPK kinase 3 (MKK3) since a deletion mutant of RIP led to reduction in p38 activation (Yuasa et al., 1998) and MKK3-deficient cells exhibited a dramatic reduction in TNF- α -induced activation of p38 (Wysk et al., 1999). It has been suggested that two possible pathways may be used to activate ERK pathways. First, a DD-containing protein termed MADD (MAP kinase-activating death domain) has been shown to be able to associate with TNFR1 via the DD of TNFR1, leading to activation of ERK (Suarez-Cuervo et al., 2003). Alternatively, the TNF- α -induced ERK activation may be mediated by a protein called RIP2 (Kakonen et al., 2002), which is a homolog of RIP. RIP2 is recruited into the signaling complex by TRAF1 and TRAF2 (McCarthy et al., 1998; Thome et al., 1998). RIP2 contains an N-terminal kinase domain and a C-terminal CARD (caspase recruiting domain) domain. The kinase domain of RIP2 is involved in activating ERK by directly phosphorylating ERK (Kakonen et al., 2002).

TRAF2 plays a central role in TNFR1 signaling. TRAF2 is involved in all four pathways except the apoptotic signal (Fig. 4). However, TRAF2-deficient mice showed that the lack of TRAF2 only led to a modest impairment in TNF- α -induced NF- κ B activation (Yeh et al., 1997), suggesting that an alternative signaling pathways may compensate the TRAF2-mediated NF- κ B activation. Subsequently, TRAF2 and TRAF5 double knockout mice demonstrated that TNF- α -induced NF- κ B activation is severely impaired in the double knockout mice, revealing that TRAF5 is also involved in TNF- α -induced NF- κ B activation (Nakano et al., 2000). It is worthwhile to point out that this important aspect of TNFR1 signaling involving TRAF5 has not received enough attention. It is still not clear which region of TNFR1 interacts with TRAF5 (Fig. 4).

As discussed above, although TNFR2 has higher affinity for TNF- α , TNFR1 is thought to be the predominant receptor that transduces intracellular signaling in most cell types. As a result, most efforts have been focused on delineating the signaling pathways activated by TNFR1 and our understanding of TNFR2-activated signaling pathways is unfortunately limited. Nevertheless, it has been shown that in response to TNF- α binding, TNFR2 trimerizes and the trimerization will lead to direct binding of TRAFs 1, 2 and c-IAP to the receptor. Interestingly, TRAF1 and TRAF2 were initially cloned as cytoplasmic factors capable of

directly interacting with a TNFR2 intracellular region, which mediates cell signaling (Rothe et al., 1994). Binding of TRAF2 to TNFR2 leads to the activation of NF- κ B (Rothe et al., 1995; Shu et al., 1996). It remains obscure why TNFR2 exerts inhibitory effect on osteoclast formation and function, despite the recruitment of TRAF2 by TNFR2 activates NF- κ B, a potent transcription factor involved in osteoclast formation, activation and survival (Franzoso et al., 1997; Jimi et al., 1998; Miyazaki et al., 2000).

4.3. Indirect effect of TNF- α on osteoclast differentiation and function

As highlighted in Fig. 5, the predominant action of TNF- α in osteoclast formation and function is likely mediated by directly targeting osteoclast precursors and mature osteoclasts, i.e., the binding of TNF- α to its receptors on osteoclast precursors/osteoclasts initiate various signaling cascades shown in Fig. 4, leading to the modulation of osteoclast formation, function and survival (Abu-Amer et al., 2000; Zhang et al., 2001b). Significantly, it has become clear that TNF- α can also modulate osteoclast formation and function by enhancing the expression of RANKL by osteoblasts and stromal cells (Hofbauer et al., 1999; Kitaura et al., 2004). Regulation of RANKL expression in osteoblasts/stromal cells by TNF- α is mediated via TNFR1 (Abu-Amer et al., 2004). However, the molecular mechanism by which TNF- α regulate RANKL gene expression in osteoblasts/stromal cells has still remained elusive. Presumably, some, if not all, of the signaling pathways depicted in Fig. 4 may occur in osteoblasts/stromal cells and, therefore, are also involved in the regulation of RANKL gene expression in osteoblasts/stromal cells by TNF- α . Future studies aimed at elucidating the molecular mechanism underlying the TNF- α -mediated RANKL gene expression in osteoblasts/stromal cells will provide a better understanding of role of TNF- α in the pathogenesis of various bone disorders.

4.4. Perspectives and future directions

The recognition of TNF- α as an important factor implicated in postmenopausal osteoporosis and bone loss in various inflammatory conditions has prompted the development of therapeutic approach targeting the action

of TNF- α for treating these diseases (Romas et al., 2002). A promising strategy involves the use of soluble TNFR1 to neutralize the action of TNF- α (Feige et al., 2000; Romas et al., 2002). Alternatively, the targeting of TNFR signaling pathway may represent additional, if not better, therapeutic strategies for blocking the action of TNF- α . However, we have not fully elucidated TNFR1 and TNFR2 signaling pathways in osteoclasts. In particular, despite the revelation of the inhibitory role of TNFR2 in osteoclastogenesis, the molecular mechanism underlying the effect still remains unclear. Furthermore, most of the previous studies on TNFR1 and TNFR2 signaling were performed in cells other than osteoclasts or its precursors, supporting the necessity to further evaluate and confirm the key findings on TNFR1 and TNFR2 signaling using osteoclasts and/or its precursors. Finally, given the recent observations that TNF- α can also indirectly promote osteoclast formation and function by up-regulating RANKL expression in osteoblasts/stromal cells, the investigation of the molecular mechanism controlling TNF- α -induced RANKL expression represents an integral part of the future investigation of role of TNF- α in the pathogenesis of various bone diseases.

5. FasL/Fas and TRAIL

Although RANKL and TNF- α are two widely studied and well recognized members of the TNF family that play pivotal roles in physiological and/or pathological bone metabolism, emerging evidence indicate that several other TNF/TNFR family members, such as FasL/Fas and TRAIL, are also implicated modulating osteoclast formation and function. These new findings have further underscored an important role of the TNF family in bone metabolism.

5.1. FasL and its receptor Fas

The revelation of functional roles for FasL and its receptor Fas in osteoclasts was partially prompted by the recent recognition that control of osteoclast apoptosis is a critical regulatory factor in bone remodeling and alteration in osteoclast apoptosis is attributed to the pathogenesis of bone disorders including postmenopausal osteoporosis (Manolagas, 2000). The FasL/Fas system has been shown to regulate cellular apoptosis in a variety of cell types, especially those of the immune system (Nagata, 1999). A recent study showed that Fas is not only expressed in osteoclasts but also its expression is increased during osteoclast differentiation (Wu et al., 2003). In vitro, cross-linking of Fas by anti-Fas antibody promoted apoptosis of osteoclasts and the apoptotic pathway in osteoclasts is mediated by the classical Fas signaling seen in other cell systems, which include the activation of caspases 3 and 9 and the release of cytochrome *c* from mitochondria (Wu et al., 2003). In line with the in vitro findings, aged mice bearing mutation in FasL or Fas exhibited increased number

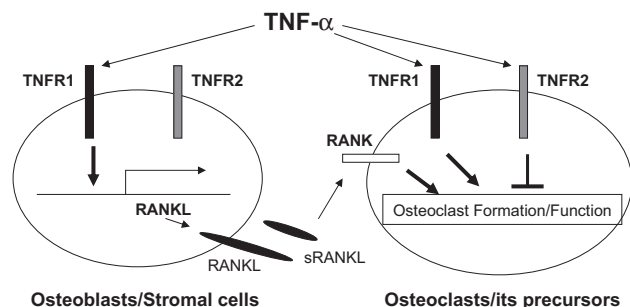


Fig. 5. Mode of the action of TNF- α in osteoclast formation and function.

of osteoclasts and/or decreased bone mineral densities (Wu et al., 2003). Interestingly, several agents including alendronate and sodium chloride promote osteoclast apoptosis by up-regulating Fas expression in osteoclasts (Sun et al., 2002; Wang et al., 2000).

5.2. TRAIL

TRAIL, also known as Apo2L, is a widely recognized TNF family member capable of inducing apoptosis through interaction with its receptors (Wang and El Deiry, 2003). TRAIL has multiple receptors: TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4 and OPG. TRAIL-R1 and TRAIL-R2 are transmembrane receptors containing a conserved DD in their cytoplasmic tails and they are involved in the activation of apoptosis. TRAIL-R4 is also a transmembrane protein with a short cytoplasmic domain lacking conserved DDs, while TRAIL-R3 lacks transmembrane and cytoplasmic regions and is anchored to the plasma membrane through a glycosphospholipid moiety (Wang and El Deiry, 2003). As discussed above, OPG was originally identified as a decoy receptor for RANKL (Lacey et al., 1998; Simonet et al., 1997). Interestingly, it was later shown that OPG is also able to bind TRAIL (Emery et al., 1998), raising the possibility that TRAIL may also play a role in bone metabolism. However, the mice deficient for TRAIL showed no bone phenotype (Sedger et al., 2002), ruling out that TRAIL plays a role in physiological bone remodeling. Intriguingly, a recent study demonstrated that TRAIL inhibited osteoclastogenesis *in vitro* (Zauli et al., 2004). Moreover, TRAIL exerts the inhibitory effect by blocking the activation of p38 pathway induced by RANKL and M-CSF rather than inducing cytotoxic effects or OPG expression (Zauli et al., 2004).

6. Conclusions

RANKL and TNF- α are two well recognized members of the TNF family that play important physiological and/or pathological roles in bone metabolism. The research in last several years have not only advanced our understanding of the regulatory roles of RANKL and TNF- α in osteoclast differentiation, function and survival but also provided many important insights into the signaling mechanisms of these two TNF family members in osteoclasts. Moreover, emerging evidence indicate that two additional members of the TNF family, FasL and TRAIL, are also implicated in regulating osteoclast differentiation or apoptosis, further supporting the importance of the TNF family to osteoclast biology.

Nevertheless, the signaling pathways activated by these TNF family members in osteoclasts and/or its precursors have been only partially elucidated whereas the regulatory roles of these TNF family members have largely been established. Thus, future efforts should be focused on

investigating signaling mechanism by the various factors. To this end, it will be important to further elucidate predicted downstream signaling pathways, to identify new signaling molecules, and/or to test novel modes of signaling by those proteins already shown to participate in the signaling process. Probably, it is equally important to evaluate many of the existing data using authentic osteoclasts and/or its precursors since a considerable portion of the previous data were obtained from studies with cells irrelevant to osteoclasts. More importantly, the key *in vitro* findings need to be further investigated and confirmed *in vivo*. Notably, as discussed above, the previous studies have also created several considerable controversies regarding the signaling by these TNF family members in osteoclasts. While some discrepancies may result from the different model systems used or from the experiments performed under different conditions, others may simply represent true biological differences that we have not appreciated. Therefore, future studies aimed at addressing these controversies will not only convincingly define the signaling pathways of these factors in osteoclasts but may also elucidate novel mechanisms implied by some of these controversies.

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