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REVIEW

Osteoclasts: New Insights

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Osteoclasts, the bone-resorbing cells, play a pivotal role in skeletal development and adult bone remodeling. They also participate in the pathogenesis of various bone disorders. Osteoclasts differentiate from cells of the monocyte/macrophage lineage upon stimulation of two essential factors, the monocyte/ macrophage colony stimulating factor (M-CSF) and receptor activation of NF-κB ligand (RANKL). M-CSF binds to its receptor c-Fms to activate distinct signaling pathways to stimulate the proliferation and survival of osteoclast precursors and the mature cell. RANKL, however, is the primary osteoclast differentiation factor, and promotes osteoclast differentiation mainly through controlling gene expression by activating its receptor, RANK. Osteoclast function depends on polarization of the cell, induced by integrin αvβ3, to form the resorptive machinery characterized by the attachment to the bone matrix and the formation of the bone-apposed ruffled border. Recent studies have provided new insights into the mechanism of osteoclast differentiation and bone resorption. In particular, c-Fms and RANK signaling have been shown to regulate bone resorption by cross-talking with those activated by integrin αvβ3. This review discusses new advances in the understanding of the mechanisms of osteoclast differentiation and function.

***Keywords*:** osteoclast; bone remodeling; M-CSF; RANKL; integrin αvβ3

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Introduction

Bone is a connective tissue that serves important mechanical and metabolic functions. It provides mechanical support for locomotion, protects vital organs such as the bone marrow and brain, serves as a metabolic organ with major reserves of calcium and phosphate to maintain mineral homeostasis, and may function as an endocrine organ to regulate energy expenditure (1). An efficient execution of these functions is achieved through constant and lifelong bone remodeling.

Bone remodeling, a physiological process in which old bone is degraded and replaced, is carried out by a functional and anatomic structure known as 'basic multicellular unit' (BMU)(2). The human adult skeleton has about 1-2 million active BMUs at any given time, which are temporally and spatially separated from each other and function in an asynchronous fashion. The remodeling process involves coordinated action of four anatomically distinct types of bone cells: bone lining cells, osteocytes, osteoclasts and osteoblasts (2, 3). Bone lining cells form a monolayer covering the bone surface in a quiescent state (4, 5). Osteocytes, the most abundant bone cells, embedded within the bone during skeletal maturation or previous cycles of bone remodeling, serve as the primary mechanosensing cell and may control the initiation of bone remodeling (6). Osteoclasts and osteoblasts are the bone resorbing cells and bone-forming cells, respectively. Whereas multinucleated bone resorbing osteoclasts differentiate from mononuclear cells of the monocyte/macrophage lineage (7), osteoblasts are derived from mesenchymal stem cells (MSC) (8). The bone line cells and osteocytes also belong to the osteoblast lineage (5, 6).

Importantly, during normal bone remodeling bone formation is tightly coupled to bone resorption to ensure no net change in bone mass or quality. In many pathological conditions, bone resorption exceeds formation,

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leading to excessive skeletal destruction. The most prevalent bone disease, postmenopausal osteoporosis, is caused by increased osteoclast formation and activity due to the decline in estrogen resulting from the cessation of ovary function in postmenopausal women (9, 10). Enhanced osteoclast formation and function are also implicated in the bone erosions of rheumatoid arthritis (RA) (11, 12), and osteolysis associated with the osteolytic complications of metastatic tumors, such as breast cancers (13).

Studies in the 1990s established that osteoclastogenesis requires two essential factors: M-CSF and RANKL (7). M-CSF participates in the osteoclastogenic process mainly by promoting the proliferation and survival of osteoclast precursors. RANKL functions as the primary factor driving differentiation of osteoclast precursors into osteoclasts. Moreover, during the same decade, significant progress was made in the understanding of the molecular mechanism of bone resorption. For example, integrins, particularly avβ3, were shown to play an important function in bone resorption (14). In the ensuing decade, insights were gained into the signaling mechanisms by which the two essential cytokines and adhesion molecules mediate osteoclast differentiation and function, as well as bone remodeling. This review focuses on recent advances in the understanding of the mechanisms of osteoclast differentiation and function in the context of newly proposed models of bone remodeling.

Osteoclast differentiation

Recent studies propose that in the context of bone remodeling, osteoclastogenesis occurs within a closed space termed the "bone remodeling compartment (BRC)", which is highly vascular and characterized by the presence of a canopy formed by bone lining cells (4, 5, 15) (Figure 1). While bone remodeling is a dynamic and continuous process, it can be divided into four major stages (3, 16): Stage 1: initiation of bone remodeling at a specific site by recruitment of osteoclast precursors into the BRC; Stage 2: predominant bone resorption period, which involves osteoclast differentiation and function with concurrent recruitment of mesenchymal stem cells (MSC)/osteoprogenitors; Stage 3: predominant bone formation phase characterized by osteoblast differentiation and function (osteoid synthesis); and finally Stage 4: mineralization of osteoid and conclusion of bone remodeling (17). Thus, in the context of bone remodeling, osteoclast formation starts with recruitment of osteoclast precursors to the BRC by osteocytes, which are the major mechanosensing and RANKL-producing cells in the bone and detect skeletal microdamage. Within the BRC, M-CSF and RANKL stimulate osteoclast differentiation. In addition, osteoclast precursors must attach to the bone matrix to undergo differentiation into osteoclasts (Figure 1).

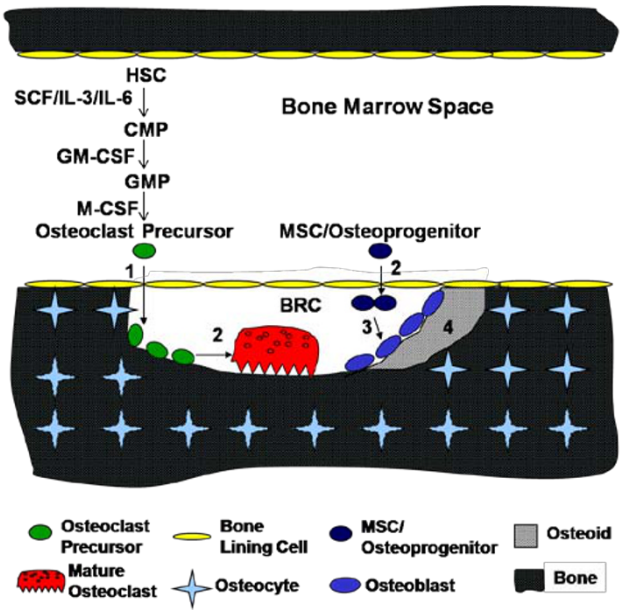

Figure 1: Proposed Model for Bone Remodeling. The remodeling process comprises four major distinct but overlapping stages: (1) initiation/activation of bone remodeling at a specific site; (2) predominant bone resorption phase with concurrent recruitment of MSC/osteoprogenitors; (3) predominant osteoblast differentiation and function (osteoid synthesis); and (4) mineralization of osteoid and completion of bone remodeling. HSC: hematopoietic stem cells; SCF: stem cell factor; CMP: common myeloid progenitor; GM-CSF: granulocyte-macrophage colony stimulating factor; GMP: granulocyte-macrophage progenitors; M-CSF: monocyte-macrophage colony stimulating factor; BRC: bone remodeling compartment; MSC: mesenchymal stem cells.

Normal bone remodeling requires coupling of bone formation to bone resorption to guarantee no change in bone mass after each remodeling cycle. The coupling may be controlled by several distinct mechanisms: 1) local and systemic factors regulate formation and function of both osteoblasts and osteoclasts (18, 19); 2) bone resorption releases growth factors, such as TGF-β and IGF-I from the bone matrix, which function to couple bone formation to bone resorption by promoting osteoblast differentiation and function (20, 21); and 3) osteoclasts produce factors that stimulate osteoblast differentiation and function, providing an additional coupling mechanism (22, 23). However, the BRC also serves as a crucial physical coupling mechanism by confining bone resorption and bone formation as well as putative coupling factors released from the bone matrix

and produced by osteoclasts in a closed compartment (24). Moreover, the BRC concept highlights the importance of cell type-specific and spatial expression of osteoclastogenic factors to osteoclast differentiation and function.

Origin of osteoclasts

Osteoclasts were initially thought to arise by fusion of osteoblasts (25). However, a number of animal model studies subsequently provided evidence that osteoclasts are hematopoietic in origin and unrelated to the osteoblast lineage. Gothlin *et al* used a parabiotic method to join the circulation of two rats together and found that osteoclasts migrating to a fracture in an irradiated rat were derived from the blood of its non-irradiated partner (26, 27). In addition, chick/quail chimera experiments and transplantation studies revealed that osteoclast precursors were present in hematopoietic tissues such as the bone marrow, spleen and peripheral blood (28-30). This notion was further substantiated by *in vitro* experiments. By co-culturing hematopoietic stem cells purified from mouse bone marrow and fetal bone rudiments, Scheven and co-workers reported that some of the stem cell populations differentiated into osteoclasts (31). Moreover, co-culturing experiments with mouse osteoblasts and spleen cells provided additional evidence that osteoclast progenitors were of hematopoietic origin (32). The most compelling and direct evidence supporting the hematopoietic origin of osteoclasts came from *in vitro* studies showing that mouse spleen cells or human peripheral blood monocytes differentiate into osteoclasts with RANKL and M-CSF treatment (33, 34).

Thus, within the bone marrow, the hematopoietic stem cells (HSC) give rise to common myeloid progenitors with stimulation of factors such as stem cell factor (SCF), interleukin-3 (IL-3) and IL-6. Granulocyte/macrophage colony stimulating factor (GM-CSF) promotes differentiation of common myeloid progenitors (CMP) into granulocyte/macrophage progenitors (GMP). With stimulation by M-CSF, GMP further differentiates into cells of the monocyte/macrophage lineage (35, 36), which are considered osteoclast precursors. In particular, committed osteoclast precursors express Mac-1, c-Fms and RANK, but not c-Kit (37). It is proposed that osteoclast precursors are recruited either from the bone marrow by crossing the bone lining cell monolayer or from capillaries that penetrate into the BRC (4, 24) (Figure 1).

M-CSF and c-Fms

M-CSF (also known as CSF-1), originally identified as a hematopoietic growth factor capable of selectively supporting *in vitro* macrophage colony formation from bone marrow progenitors in semisolid media (38), plays an important role in promoting the proliferation, differentiation and survival of cells of the monocyte lineage and regulating cells of the female reproductive tract (39). The essential role of M-CSF in osteoclastogenesis was revealed by studies showing that mice (op/op) and rats (tl/tl), which express non-functional M-CSF due to a point mutation in the Csf1 gene, completely lack osteoclasts and develop severe osteopetrotic phenotypes (40, 41). M-CSF is expressed by a variety of cell types including smooth muscle cells (42), vascular endothelial cells (43), hepatocytes (44), fibroblasts (45), T cells (46), bone marrow stromal cells (47) and osteoblasts (48). Bone marrow stromal cells and osteoblasts, however, are the major sources of M-CSF in the bone microenvironment (49). The new concept that bone remodeling occurs within the BRC may require reconsideration of this view (Figure 1). Given its location, the bone marrow stromal cell is an ideal source of M-CSF in the marrow space. Osteoblasts, however, are unlikely to serve as a source of M-CSF in the marrow space since they differentiate within the BCR (50, 51). Moreover, because osteoprogenitor recruitment and osteoblast differentiation occur after osteoclast differentiation, osteoblasts are also unlikely to produce M-CSF for initial phase of osteoclast differentiation in the BRC. Interestingly, osteocytes were shown to express M-CSF (52), suggesting that they may supply the cytokine in the BRC.

As one of the two essential osteoclastogenic factors, M-CSF primarily promotes the proliferation and survival of osteoclast precursors. M-CSF exerts its effects by binding and activating its cognate receptor, c-Fms, which is the cellular homolog of the feline transforming virus v-Fms (53). c-Fms, a tyrosine kinase receptor belonging to the type III protein tyrosine kinase family, contains an extracellular ligand-binding, a single transmembrane and a cytoplasmic tyrosine kinase domain (54)(Figure 2). c-Fms is activated by either M-CSF-induced dimerization of the receptor monomers or the ligand-induced conformational change of a pre-assembled dimer. The ligand-induced activation of c-Fms results in enhanced activity of its tyrosine kinase domain, which trans-phosphorylates specific tyrosine residues in the cytoplasmic tail. These residues then serve as high-affinity binding sites for SH2 domain-containing signaling molecules (54). Early studies using transformed cells, which do not naturally express c-Fms, demonstrated that the receptor contains 8 tyrosine (Y) residues within the cytoplasmic tail (Y 544, Y559, Y697, Y706, Y721, Y807, Y921 and Y974) that can be trans-phosphorylated to serve as docking

sites for signaling molecules (54, 55). However, the data from these studies about the role of these tyrosine residues in cell proliferation, differentiation and survival were conflicting, raising concern about the functional significance of the findings (56).

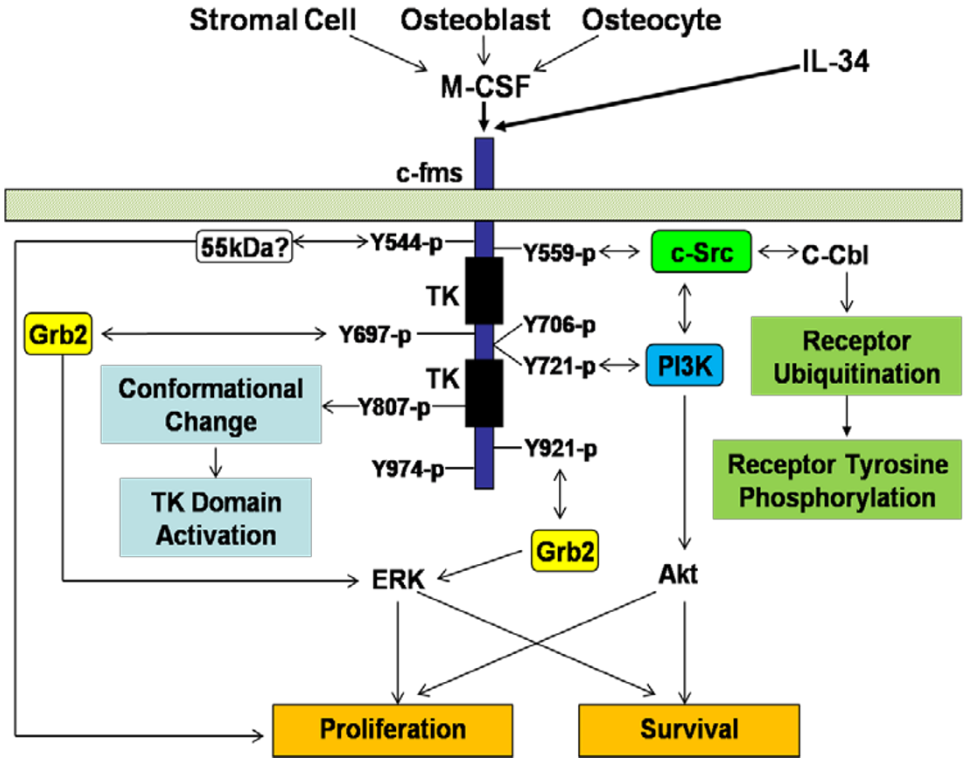

Figure 2: Known Signaling Pathways of c-Fms in the Proliferation and Survival of Osteoclast Precursors and their Differentiation into Osteoclasts. TK: kinase domain. Question marks (?) denote that the signaling molecule has not been fully identified and characterized.

Functional assays using a chimeric receptor approach or GM-CSF-dependent macrophages lacking c-Fms demonstrated that six (Y544, Y559, Y697, Y721, Y807 and Y921) of the eight tyrosine residues are functionally involved in regulating the proliferation of osteoclast precursors (57-60) (Figure 2). While phospho-Y544 was shown to bind a 55kd protein (55), its identity has not been fully characterized to date. Thus, although this juxtamembrane domain tyrosine residue is required for osteoclast proliferation (60), the precise signaling mechanism remains elusive. No signaling protein has been shown to bind to phosphor-Y807 in the activation loop, and phosphorylation of this tyrosine residue is proposed to cause a conformational change of the c-Fms intracellular domain to increase the activity of its kinase domain (61). Phospho-Y559 interacts with c-Src (62), which, in turn recruits PI3K to activate the Akt pathway (63) (Figure 2). A recent study demonstrated that the phosphor-Y559/c-Src also recruits c-Cbl complex, resulting in receptor ubiquitination (64). The c-Cbl-mediated c-Fms ubiquitination causes a conformational change in the kinase domain that allows amplification of receptor tyrosine phosphorylation and full receptor activation. In contrast, phospho-Y721 directly interacts with PI3K to mediate activation of Akt (63, 65). Phospho-Y697 and phospho-Y974 recruits Grb2 (66), which then interacts with Sos to subsequently activate the Ras/Raf/MEK/ERK cascades. Thus, phospho-Y559, phospho-Y697, phospho-Y721 and phospho-Y974 promote osteoclast precursor proliferation in part through the ERK and PI3K/Akt pathways (Figure 2).

M-CSF also plays a role in mediating survival of osteoclast precursors (67), although the specific tyrosine residues regulating this event are unknown. However, given that numerous studies have shown that the ERK and PI3K/Akt pathways are involved in mediating survival of osteoclast precursors, it is likely that phospho-Y559, phospho-Y697, phospho-Y721 and phosphor-Y974 also play a role in prolonging osteoclast precursor survival (Figure 2).

Interestingly, a new cytokine, which shares no sequence homology with M-CSF (designated IL-34), has been identified as another ligand of c-Fms (68). IL-34 and M-CSF differ in their structure and bind to distinct Fms

domains. They exhibit different bioactivities and activate signal pathways with distinct kinetics/strength. *In vitro*, IL-34 induces phosphorylation of ERK 1/2 and Akt through the activation of c-Fms, and promotes the adhesion and proliferation of osteoclast progenitors. More importantly, IL-34 is able to support osteoclastogenesis with RANKL in the absence of M-CSF, indicating that IL-34 can entirely substitute for M-CSF in RANKL-induced osteoclastogenesis. Nonetheless, given the different spatiotemporal expression of M-CSF and IL-34 (69), and a complete lack of osteoclasts in mice (op/op) and rats (tl/tl) (40, 41), this new cytokine may not be important to normal bone remodeling. It is likely that IL-34 may be involved in promoting osteoclastogenesis in certain pathological conditions. A recent study has implicated IL-34 in osteoclastogenesis in RA (70). Moreover, a number of other growth factors such as VEGF, FLT3 ligand, PIGF and HGF may substitute M-CSF to stimulate osteoclastogenesis (71, 72). These factors may also play a role in osteoclastogenesis in bone disorders.

RANKL, RANK and osteoprotegrin (OPG)

RANKL (also known as OPGL, ODF and TRANCE), another key osteoclastogenic factor, was discovered independently by several groups in the late 1990s (33, 73-75). RANKL, a member of the tumor necrosis factor (TNF) superfamily, exerts its functions by activating its cognate receptor RANK, which is a member of the TNF receptor (TNFR) superfamily (74). OPG is a soluble decoy receptor for RANKL and inhibits RANKL functions by competing with RANK for RANKL (76, 77). Initial *in vitro* studies implicated RANKL in regulating osteoclast formation and function (33, 73), immune cell survival and activation such as dendritic cell (DC) survival and activation (78-80), and T-cell activation (81, 82). However, RANKL and RANK knockout models further revealed that the RANKL/RANK/OPG system also plays a role in lymph node organogenesis (81, 83, 84), B-cell differentiation (81, 83), development of medullary thymic epithelial cells (85, 86), mammary gland development (87), and thermoregulation in females, as well as fever response in inflammation (88).

In vitro, M-CSF and RANKL have been shown to be sufficient for osteoclastogenesis (89). In mature osteoclasts, RANKL mediates osteoclast activation and survival (49, 73, 90, 91). 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 (81, 83, 92). Consistently, knockout mice deficient for OPG developed early onset of osteoporosis due to elevated osteoclast differentiation (76), whereas transgenic mice over-expressing OPG exhibited osteopetrosis, resulting from a decrease in late stages of osteoclast differentiation (77). Taken together, these data indicate that the RANKL/RANK system plays an essential role in skeletal development and bone remodeling.

RANKL is expressed in a number of cell types including bone marrow stromal cells (33, 73), osteoblasts (33, 93), B cells (74) and T cells (74) (Figure 3). The long-held view holds that while RANKL derived from bone marrow stromal cells and osteoblasts plays a role in normal bone remodeling (49), the elevated RANKL expression in B cells and T-cells and bone marrow stromal cells may contribute to bone loss in postmenopausal women (94, 95). However, recent studies have revealed that osteocytes also express RANKL (96, 97). Given the cell's abundance, osteocyte-derived RANKL plays an important role in the control of bone remodeling (96, 97).

RANK recruits TNF receptor associated factors (TRAFs) to activate various signaling pathways to promote osteoclastogenesis, as TRAFs are established signaling molecules shared by members of the TNFR family (98, 99). Early *in vitro* binding assays and over-expression systems in transformed cells demonstrated that five TRAF proteins (TRAF 1, 2, 3, 5 and 6) interact with RANK (100-105). Later studies revealed that RANK contains only three functional TRAF-binding motifs: $PFQEP^{369-373}$ (Motif 1), $PVQEET^{560-565}$ (Motif 2) and $PVQEQG^{604-609}$ (Motif 3), which mediate osteoclast formation and function (106, 107) (Figure 3). Moreover, these RANK motifs collectively activate six major signaling pathways (NF-κB, JNK, ERK, p38, NFATc1 and Akt) to regulate osteoclast formation, function and/or survival (93, 108)(Figure 3).

Motif 1 recruits TRAF6 to form a signaling complex containing c-Src, TAB2, TAK1 and TAB1, which subsequently activates Akt, NF-κB, JNK, p38 and ERK pathways (91, 108-110)(Figure 3). In addition, the TRAF6-mediated signaling complex is also involved in RANKL-induced activation of NFATc1 expression, which is a master regulator of osteoclastogenesis (111, 112) (Figure 3). Several previous *in vitro* assays demonstrated that Motif 2 interacts with TRAF3, and Motif 3 binds TRAF2 or TRAF5 (100, 104), but the functional relevance of these *in vitro* to osteoclastogenesis remains to be determined. In osteoclast precursors, Motif 2 initiates signaling pathways leading to the activation of NF-κB and p38 pathways, whereas Motif 3 mediates only the activation of the NF-κB pathway (107). However, the molecular components of the signaling complexes formed upon the recruitment of TRAF proteins at these two RANK motifs have not been elucidated.

On the other hand, shortly after the discovery of RANKL/ RANK/OPG axis, it was proposed that RANK may

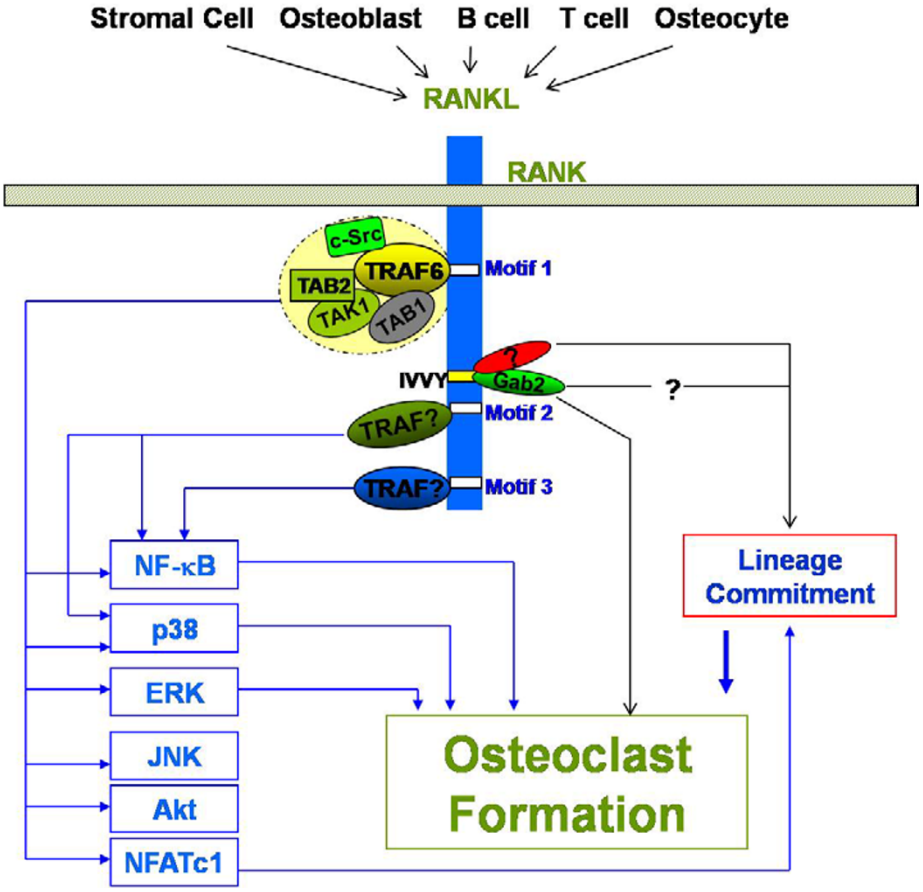

Figure 3: Major RANK Signaling Pathways in Osteoclastogenesis. Question marks (?) denote that these signaling molecule or pathways have not been functionally identified.

activate an unidentified and unique signaling pathway(s) essential for osteoclastogenesis (113). This idea was later supported by the comparison of the osteoclast generation potential of RANKL and IL-1. TRAF6 is an established downstream signaling molecule for both RANK and IL-1R (114) and is involved in osteoclastogenesis (115, 116). In fact, a single TRAF6-binding motif is capable of promoting osteoclastogenesis (107, 108). Though, administration of IL-1 to RANK knockout mice failed to generate any osteoclast *in vivo* (92). These observations indicate that RANK activates a TRAF6-independent signaling pathway(s) which is essential for osteoclastogenesis. Moreover, *in vitro* studies also showed that IL-1 failed to stimulate osteoclast formation in the presence of M-CSF (117, 118). Thirdly, RANK cytoplasmic domain shares no homology with any known members of the TNFR family (74), further suggesting that it may activate downstream signals different from those arising from other members of the TNFR family. A systematic structure/function study of the RANK cytoplasmic domain has identified a specific 4-a.a. RANK motif (IVVY535-538), which function to mediate commitment of macrophages to the osteoclast lineage in osteoclastogenesis (119). This finding is consistent with the previous study that RANKL plays an essential role in committing macrophages to the osteoclast lineage (120). Interestingly, while this study demonstrated that early commitment of macrophages to the osteoclast lineage requires the IVVY motif (119), a subsequent study showed that the IVVY motif also regulates the late stage (24-48 hours post-stimulation) of osteoclast formation by recruiting Gab2 (121) (Figure 3). It remains to be determined whether Gab2 is also involved in the IVVY motif-mediated osteoclast lineage commitment, or the IVVY motif interacts with a different signaling molecule to do so. Consistent with these findings obtained with mouse cells, truncating mutations leading to loss of a human RANK region containing the IVVY motif are implicated in causing osteopetrosis in humans (122).

While RANKL is essential and sufficient for osteoclastogenesis in the presence of M-CSF, a number of other cytokines can also exert stimulatory or inhibitory effects on osteoclastogenic process. TNF-α and IL-1 are two proinflammatory factors which promote osteoclasto-

genesis and are implicated in bone loss in postmenopausal osteoporosis (123), and inflammatory conditions such as RA (12). However, while both IL-1 and TNF-α can activate TRAF-dependent signaling pathways (124), the two cytokines cannot promote osteoclastogenesis independently of RANKL (120, 125-127). Recent findings have revealed that TNF-α and IL-1 cannot stimulate osteoclastogenesis, in part due to their inability to activate the expression of osteoclast genes (128, 129). Moreover, RANKL plays a crucial role in TNF-α- and IL-1-induced osteoclastogenesis by priming osteoclast genes, including the gene coding for NFATc1, responsive to these proinflammatory cytokines, and the RANK IVVY motif is specifically involved in the priming process. Conversely, while interferon-γ (IFN-γ) potently inhibits osteoclastogenesis (130-133), the suppressive effect is significantly reduced when osteoclast precursors are pre-exposed to RANKL (134). IFN-γ inhibits the RANKL-induced expression of osteoclast genes, but RANKL pretreatment reprograms the process into a state in which they can no longer be suppressed by the immune cytokine (135). Thus, IFN-γ inhibits osteoclastogenesis by blocking the expression of osteoclast genes. Moreover, the IVVY535–538 motif in the cytoplasmic domain of RANK is responsible for rendering BMMs refractory to the inhibitory effect of IFN-γ. These findings provide new insights into the molecular basis of the requirement of RANK signaling for osteoclastogenesis induced by other cytokines.

Mechanism of bone resorption

Bone matrix consists of both inorganic and organic components (136). While the inorganic constituent of bone matrix is primarily crystalline hydroxyapatite-$[Ca_3(PO_4)_2]_3Ca(OH)_2$, the organic component contains roughly 20 proteins with type I collagen as the most abundant (>90%) (137). Thus, bone resorption involves dissolution of crystalline hydroxyapatite followed by proteolytic cleavage of the organic component of bone matrix (138). The osteoclast's resorptive machinery consists of two important structural and functional features, namely, the bone-apposed ruffled border and an isolated resorption compartment (Figure 4). The resorption compartment is formed by attachment of osteoclasts to bone matrix through the unique structure termed the sealing zone. The ruffled border transports protons and proteolytic enzymes into the resorption compartment to dissolve minerals and degrade bone matrix proteins, respectively.

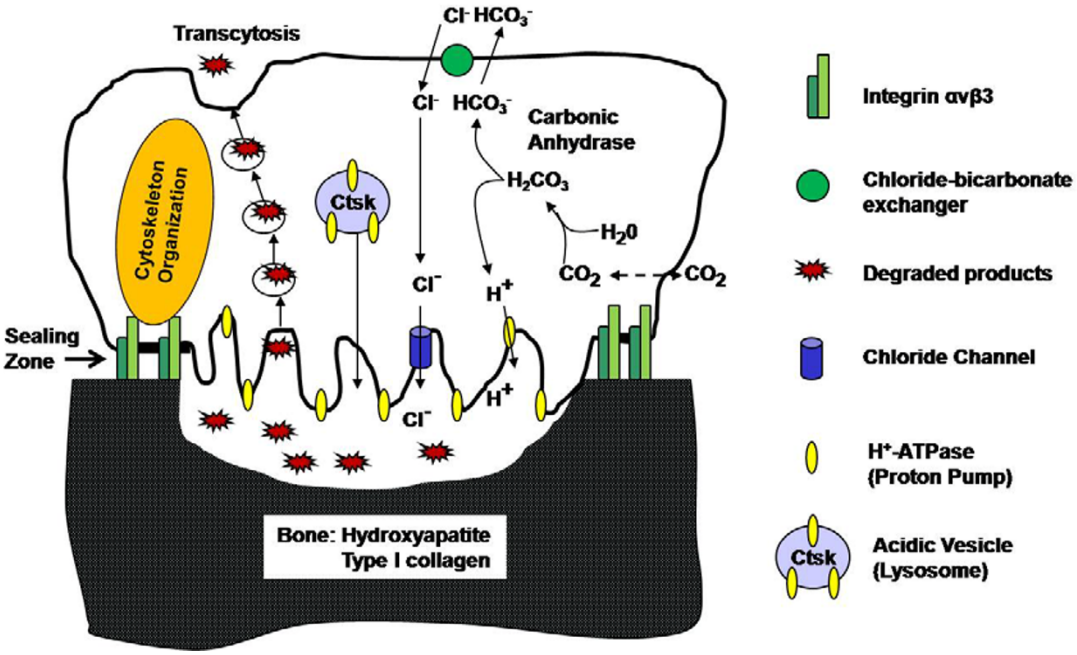

Figure 4: Mechanism of Bone Resorption. Ctsk: cathepsin K.

Vacuolar H^+-adenosine triphosphatase (H^+-ATPase), which is abundantly present in the ruffled border membrane (139, 140), transports protons to acidify the resorption compartment (~4.5)(141). The high concentrations of acid on a basic mineral liberates calcium: $[Ca_3(PO4)_2]_3Ca(OH)_2 + 8H^+ \leftrightarrow 10Ca_3 + 6HPO_4^{2-} + 2H_2O$. The source of the cytoplasmic protons is carbonic acid, which is generated by cytoplasmic carbonic anhydrase from carbon dioxide and water ($CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$)(142) (Figure 4). To maintain elcetroneutrality,

Cl^- are also transported into the resorption compartment via Cl^- channels which are charge-coupled to the H^+-ATPase and present in the ruffled border membrane (143, 144). The secretion of protons across the ruffled border membrane into the extracellular resorbing compartment leaves the conjugate base (HCO_3^-) inside the osteoclast, which must be removed from the cell. Furthermore, the osteoclast also must continuously supply Cl^- ions for secretion into the resorption compartment. These two tasks are accomplished by a passive chloride-bicarbonate exchanger in the basolateral membrane (145) (Figure 4).

The degradation of the organic component of bone matrix is accomplished by the lysosomal proteolytic enzyme cathepsin K (146). In keeping with this notion, patients with a mutation in the cathepsin K gene develop pycnodystosis (147). Moreover, cathepsin K knockout mice exhibit osteopetrosis due to a defect in matrix degradation (148), further supporting a functional role for cathepsin K in bone resorption. The removal of the products is accomplished by transcytosis via a vesicular process (149, 150). The transcytosis process includes several steps. First, degraded products are endocytosed, then transported along a transcytotic vesicular pathway toward the anti-resorptive side of the cell, and finally released out of the cell at the anti-resorptive side (Figure 4).

Formation of ruffled border

The ruffled border membrane is not only a morphological characteristic of the osteoclast but it is also the cell's resorptive organelle. Formation of the ruffled border membrane probably results from the insertion of the proton pump, H-ATPase, and cathepsin K-bearing vesicles into the plasma membrane facing the bone (151). In vesicular exocytosis, eukaryotic cells release hydrophilic secretory products into the extracellular space or translocate specific functional proteins to the plasma membrane (152, 153). Similarly, in unattached osteoclasts, acidifying vesicles containing the proton pumps distribute diffusely throughout the cytoplasm. Upon attachment of cells to bone, matrix-derived signals prompt the acidifying vesicles to migrate and insert into the plasma membrane facing the bone.

Fusion of exocytotic vesicles with the target plasma membrane is mediated by v- (vesicular) and t- (target) soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNAREs)(154). SNARE function is further regulated by synaptotagmins (Syt), a family of vesicular trafficking proteins that uniquely link the vesicle and target (155, 156). Mammalian cells express fifteen Syt isoforms with different distributions and affinities for calcium- and phospholipids (157). Most Syt isoforms are ubiquitously expressed except for Syt I, II, III, V, and X, which appear primarily in the nervous system and neuroendocrine cells. Interestingly, Syt VII, which is broadly expressed and regulates calcium-dependent exocytosis of lysosomes in fibroblasts, macrophages and other cell types (158), plays an essential role in the formation of the ruffled border by mediating fusion of secretory lysosomal vesicles to the bone-apposed plasma membrane in osteoclasts (159).

Interestingly, emerging evidence indicates that proteins involved in regulating autophagy, including Atg5, Atg7, Atg4B, and LC3, play a role in the development of the osteoclast ruffled border (160). Moreover, given that Rab7, which is required for osteoclast function, localizes to the ruffled border in an Atg5-dependent manner, autophagy proteins participate in the polarized secretion of lysosomal contents into the extracellular space by directing lysosomes to fuse with the plasma membrane.

Establishment of the resorption compartment

Osteoclastogenesis also requires attachment of precursor cells to the bone matrix. The bone-cell interaction is mediated in part by integrin avβ3 (161) (Figure 4), which recognizes the RGD sequence present in various bone matrix proteins such as osteopontin, vitronectin, and bone sialoprotein (162-164). Interaction of integrin avβ3 with the bone matrix induces cytoskeleton organization that polarizes the osteoclast's resorptive machinery to the bone-cell interface, where it creates an isolated resorptive compartment consisting of an actin ring surrounding a ruffled border. This is reflected by the observation that integrin β3 knockout mice have increased bone mass due to a functional defect in osteoclasts (165).

Mechanistically, integrin avβ3 mediates cytoskeleton organization through the cytoplasmic domain of β3 subunit (166). Binding of integrin avβ3 to its ligand leads to phosphorylation and activation of c-Src, which is constitutively associated with the cytoplasmic domain of β3 subunit (Figure 5). Activated c-Src in turn phosphorylates Syk, whose SH2 domains bind the phosphotyrosines of the co-stimulatory ITAM protein and Dap12 (167). This avβ3-induced signaling complex then recruits Slp76 which functions as an adaptor for Vav3 (168). Recruitment of Vav3, a guanine nucleotide exchange factor, converts small GTPases of the Rho family from the inactive GDP form to the active GTP-bound form (169). Rac is a Rho family GTPase expressed in osteoclasts and participates in avβ3-mediated organization of the cell's cytoskeleton. Cdc42, another Rho GTPase, exerts only a mild cytoskeletal effect through atypical PKCs, and its

major impact is on proliferation of osteoclast precursors and survival of mature osteoclasts (170). In contrast, Rac1 and Rac2 function as two key effector molecules of the avβ3 integrin, as their deletion results in a severe compromise of the osteoclast cytoskeleton and osteopetrosis (171). Integrin avβ3 mediated cytoskeleton organization leads to the formation of the actin ring, which isolates the resorptive space from its surroundings (Figure 4).

Regulation of bone resorption

Net bone resorption is determined by the number of osteoclasts, and the capacity of the individual cell to migrate and degrade bone matrix (Figure 5). As discussed above, osteoclast activation is primarily characterized by the establishment of the bone resorption compartment and the formation of the ruffled border. This is achieved by migration and insertion of acidifying vesicles into the plasma membrane facing the bone, driven by matrix-derived signals. Osteoclasts migrate to new sites to initiate more cycles of bone resorption. Each such event requires the cell to reassemble its resorption compartment and develop the ruffled border after detachment from the bone.

M-CSF plays a role in regulating bone resorption by collaborating with avβ3 in osteoclast cytoskeleton re-arrangement, as both factors stimulate the same c-Src-initiated signaling complex (172, 173) (Figure 5). M-CSF alters the conformation of avβ3 from its low to high affinity state (174), and also activates Rac in an integrin avβ3-dependnet manner (172). Thus, M-CSF organizes the osteoclast cytoskeleton by inside-out activation of the integrin. Moreover, M-CSF can also directly exert an effect on cytoskeleton organization through forming a multimeric signaling protein containing proteins binding to Y559/697/721 of c-Fms cytoplasmic domain (175) (Figure 5).

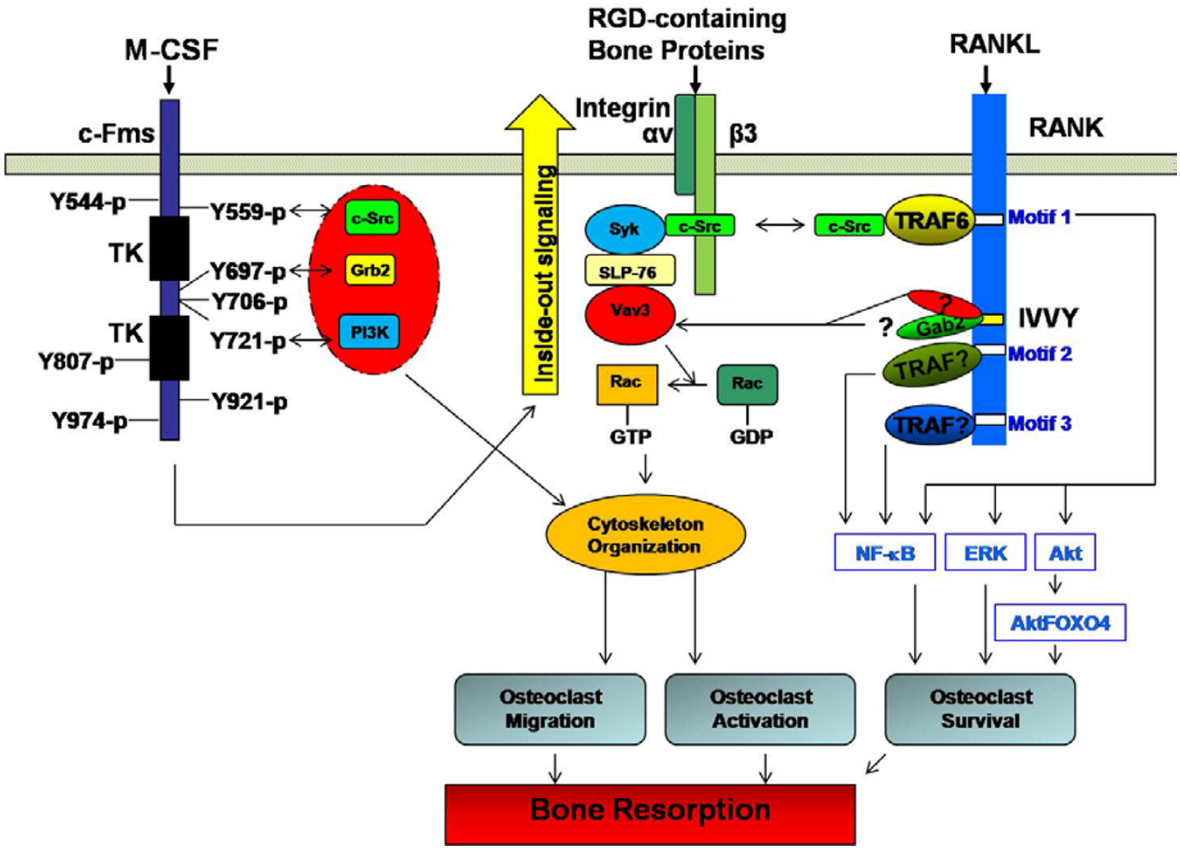

Figure 5: Integrin αvβ3 Signaling and their Crosstalk with RANK and c-Fms Signaling in Osteoclast Function. TK: kinase domain. Question marks (?) denote that the signaling molecule has not been fully identified and characterized.

RANKL promotes bone resorption by enhancing osteoclast survival and activation. Upon RANKL binding, RANK mediates osteoclast survival primarily via Motif 1, which activates Akt/PKB and its downstream effector AFX/FOXO4 (176). Moreover, Motif 1 can also activate NF-κB and ERK (107), both of which are mediators of osteoclast survival (177, 178) (Figure 5). Motif 2 and Motif 3 can also exert a small effect on osteoclast survival by activating

the NF-κB pathway (107). The IVVY motif is also implicated in mediating the function and survival of mature osteoclasts by indirectly interacting with Vav3 to modulate the osteoclast cytoskeleton (179). Furthermore, a recent study indicated that RANKL activates osteoclasts by inducing c-Src to associate with RANK Motif 1 in an avβ3-dependent manner (180). avβ3 and activated RANK form complexes in the presence of c-Src. c-Src binds activated RANK via its SH2 domain and avβ3 via its SH3 domain, suggesting the kinase links the two receptors.

Conclusions and perspectives

The large body of *in vitro* work and critical mouse model studies in the 1990s established the crucial role of M-CSF, integrin avβ3 and RANKL in osteoclast biology. The first decade of this century has witnessed significant advances in our understanding of the molecular mechanisms by which these factors regulate osteoclast differentiation and function. In particular, these studies have led to an appreciation that M-CSF and RANKL promote osteoclast activation through functional crosstalk with integrin avβ3. Moreover, the newly proposed model that bone remodeling operates within a closed system, combined with the new finding on the role of osteocyte-derived RANKL in bone remodeling, emphasize the importance of cell type-specific and spatial expression of osteoclastogenic factors including RANKL and M-CSF to normal, as well as abnormal bone remodeling. Finally, the discovery of IL-34 as a new ligand for c-Fms has raised the possibility that this cytokine may play an important role in the pathogenesis of bone disorders.

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