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Osteoclastogenesis - Current knowledge and future perspectives

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Abstract

The strength and integrity of the human skeleton depends on a delicate equilibrium between bone resorption by osteoclasts and bone formation by osteoblasts. This equilibrium is continuously compromised by a variety of genetic, humoral, and mechanical alterations. In osteoporosis, this balance shifts in favor of osteoclasts, and bone resorption exceeds bone formation. More detailed knowledge of the biology of osteoclasts and osteoclastogenesis has shown that the involved procedures can provide opportunities for developing therapeutic agents. Osteoclastogenesis is a multi-complex procedure that includes many stages, and each one of them presents as a potential target for therapeutic intervention, except for the stage of commitment of pre-osteoclasts, at least for the time being. Because the osteoclast is derived from the pluripotent hematopoietic stem cell, any intervention in this stage could result in serious adverse effects from the hematopoietic system. On the contrary, intervention in the later stages of differentiation, multi-nucleation, and activation, has proved to be very promising in the development of novel potent anti-resorptive agents. In the present review we summarized the current knowledge related to osteoclast differentiation and the new developing targets of pharmacological intervention in each stage of this extremely complicated and not completely elucidated process.

Keywords: Osteoclasts, Osteoporosis, Treatment, Differentiation, Commitment

Introduction

As humans live longer, degenerative skeletal diseases, such as osteoporosis, become increasingly prevalent. Regardless of cause, osteoporosis reflects a relative preponderance of osteoclast activity. The osteoclast is a unique bone-resorbing cell deriving from the cells of monocyte-macrophage lineage. During the last decade, a more detailed knowledge of the molecular mechanisms involved in osteoclastogenesis has driven research effort in generating new pharmacological agents that selectively inhibit the differentiation or the activity of these cells. Osteoclastogenesis is a complicated procedure that includes many stages, such as commitment, differentiation, multinucleation, and activation of immature osteoclasts. A variety of both systemic hormones and cytokines locally produced in the bone microenvironment regulate osteoclast differentiation and function. In addition, the osteoclast itself is a secretory cell and can produce cytokines, which stimulate or inhibit its own activity. Finally, other cells in the bone marrow microenvironment, like T and B lymphocytes, marrow stromal cells, osteoblasts and osteocytes, can influence osteoclast differentiation. The present review is focused on the current knowledge of the molecular mechanisms regulating osteoclastogenesis, as well as on the creation of new potential drugs that intervene in different stages of osteoclastogenesis. For descriptive purposes this review is divided into 4 parts/stages; commitment, differentiation, multinucleation, and maturation.

Commitment

The osteoclast is derived from the pluripotent hematopoietic stem cell, which gives rise to a myeloid stem cell that can further differentiate into megakaryocytes, granulocytes, monocytes/macrophages and osteoclasts (Figure 1). The earliest identifiable hematopoietic precursor able to form osteoclasts is the granulocyte-macrophage colony forming unit (CFU-GM), while CFU-M, the more differentiated monocyte precursor, forms osteoclasts at a much lower efficiency[1]. At this stage the principal transcription factors that are involved are the PU.1, the MITF, and the c-FOS. The cytokine M-CSF, stimulates the proliferation and prevents the apoptosis of early osteoclast precursors.

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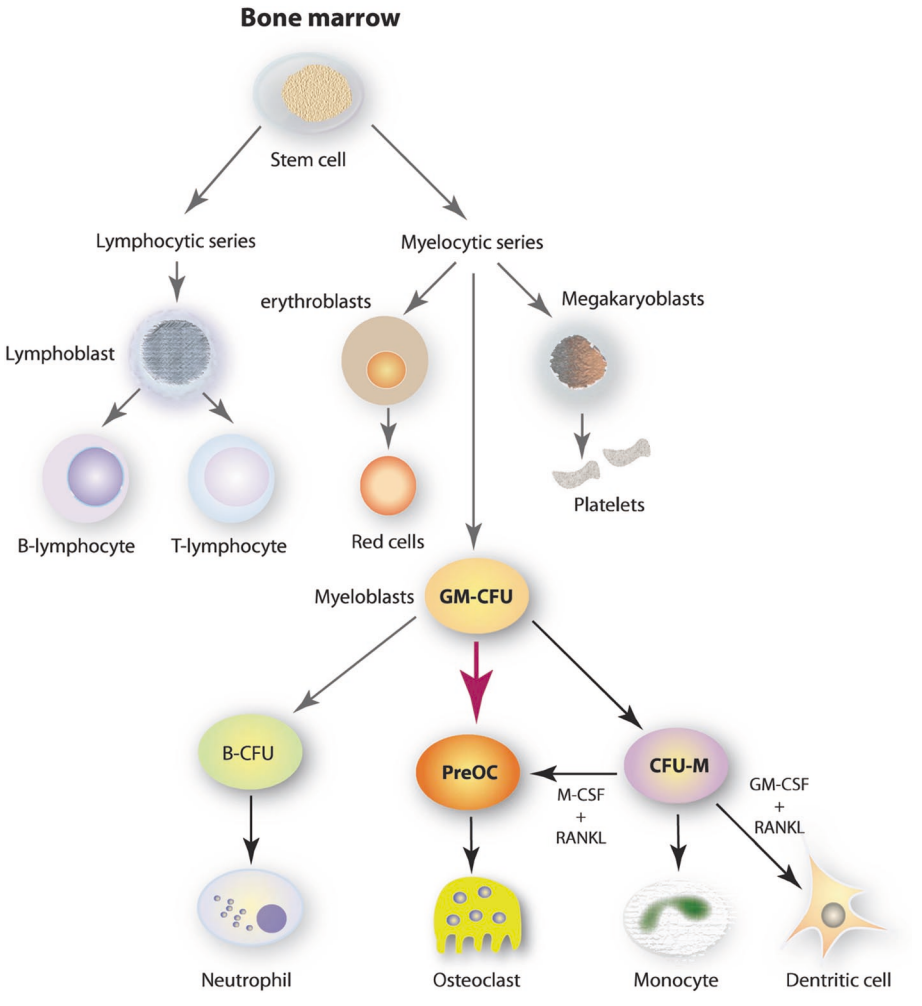

Figure 1. The cell fate of the pluripotent hematopoietic stem cell. The pluripotent stem cell gives rise to a myeloid stem cell which can further differentiate to megakaryocytes, granulocytes, monocytes/macrophages and osteoclasts.

The PU.1 transcription factor

The PU.1 belongs to the Ets family of transcription factors and is responsible for the earliest established event in osteoclastogenesis. PU.1 null mice lack not only osteoclasts, but also macrophages, while preserving the ability to produce early monocytic cells[2]. Macrophage and neutrophil cell fate specification require the primary cell fate determinants PU.1 and C/EBPa, respectively[3]. Both factors are highly expressed in macrophages and neutrophils. Sub-threshold levels of PU.1 activate a mixed lineage pattern of gene expression within individual myeloid progenitors. An increase in PU.1 activity beyond the threshold induces resolution of the mixed lineage pattern leading to an overt differentiation into macrophages. The repression of neutrophil genes during macrophage differentiation is indirectly mediated by PU.1 via the induction of negative regulators (Figure 2). The zinc finger family transcription factors Egr-1 and Erg-2 have been implicated in regulating macrophage differentiation[4]. PU.1 initially activates a

Abbreviations	
CFU-GM	granulocyte-macrophage colony forming unit
M-CSF	macrophage colony stimulating factor
PI3K	phosphoinositol 3 kinase
ERK	extracellular signal-regulated kinases
PLCγ	phospholipase γ
GSK3β	glycogen synthase kinase 3β
CtBP	C-terminal binding protein
MAPK	mitogen activated protein kinase
Ostm	osteopetrosis-associated transmembrane protein 1
JNK	janus like kinase
TRAF 6	TNF receptor-associated factor 6
PTEN	phosphatase and tensin homologue deleted from chromosome 10
SHIP	Src homology (SH) 2-containing inositol-5-phosphatase
ICAM-1	intercellular adhesion molecule-1
LFA-1	Lymphocyte function-associated antigen-1
NFAT	nuclear factor of activated T-cells
MCP-1	monocyte chemotactic protein-1

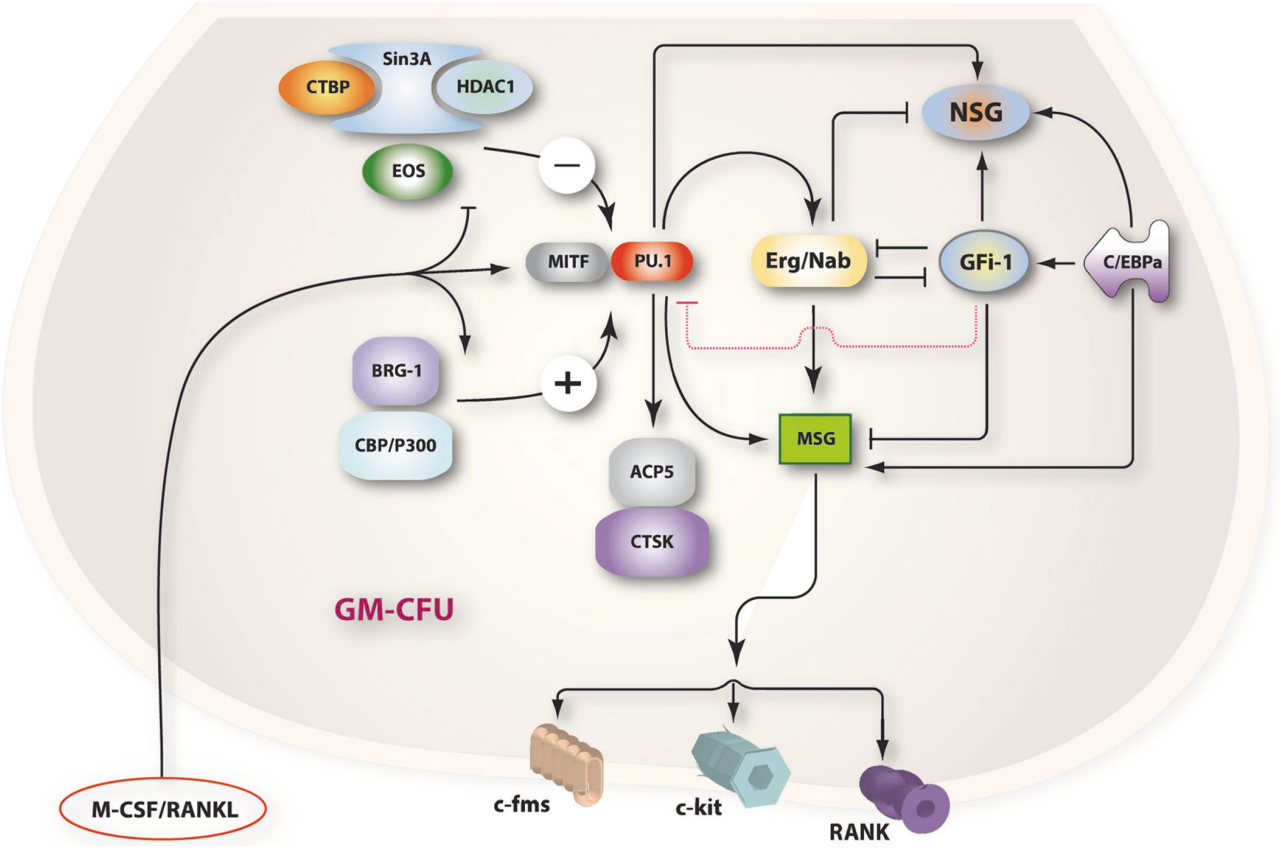

Figure 2. The commitment of pre-osteoclasts. Macrophage and neutrophil cell fate specification requires the primary cell fate determinants PU.1 and C/EBPa, respectively. Egr-1,2/Nab-2 and Gfi-1 represent lineage specific counteracting repressors. Gfi-1 is up-regulated by C/EBPa, represses PU.1, and counter-represses the Egr-1 and Egr-2. Interaction of PU.1 and MITF with the zinc finger protein Eos results in repression of the transcription of specific macrophage genes through recruitment of the co-repressors Sin3A and CtBP. M-CSF-/RANKL signaling results in recruitment of co-activators, CBP/p300 and BRG1 and thus activation of transcription of the macrophage genes cathepsin K (Ctsk) and acid phosphatase 5 (Acp5), MSG - Macrophage Specific Genes.

mixed lineage pattern of gene expression in myeloid progenitors, and then utilizes Egr-2 to resolve this pattern into a macrophage-specific output[5]. Egr-2, in collaboration with the co-repressor Nab-2, reinforces macrophage gene activity while antagonizing neutrophil-specific gene expression.

Egr-1,2/Nab-2 and Gfi-1 represent lineage specific counteracting repressors. Induction of Egr-2 and Nab-2 by PU.1 results in their cross-linking to the Gfi-1 promoter region, and in the direct repression of the Gfi-1 gene. In turn Gfi-1, which is up-regulated by C/EBPa, physically interacts with PU.1, repressing PU.1-dependent transcription[6] and also counter-repressing the Egr-1 and Egr-2 genes in two different cellular contexts[4]. PU.1 also regulates the RANK gene transcription in myeloid progenitors, the receptor for RANKL, which is the key osteoclastogenic cytokine in osteoclast differentiation[7], as described below.

The M-CSF signaling

Transcription of the macrophage c-FMS gene, the sole receptor of M-CSF, is dependent on both PU.1 and Egr-1, 2[4] (Figure 3). M-CSF can induce its own receptor expression forming an autocrine loop to amplify M-CSF-mediated signals, while it has also been reported to stimulate PU.1[8]. Activation of c-FMS by M-CSF is necessary for the proliferation and survival of macrophage/osteoclast progenitor cells. Loss of function mutation in the M-CSF gene (*op*/*op* mice) results in a marked decrease of tissue macrophage and osteopetrotic phenotype due to the lack of osteoclasts[9]. M-CSF is produced constitutively in the bone microenviroment by a range of cells, including stromal cells/osteoblasts, and T-lymphocytes, in response to elevated serum PTH levels and inflammatory molecules such as TNF-α and IL-1[10]. Recent evidence suggests that TNF-α can also directly induce c-FMS expression through both M-CSF-dependent and -independent mechanisms[8]. Binding of M-CSF to c-FMS results in dimerization and hence activation of the receptor tyrosine kinase, leading to auto-phosphorylation on selected tyrosine residues. Available data indicate that the downstream signals PI3K, p42/44 ERK, the proto-oncogene c-Cb1 and PLCγ are the key transducers of M-CSF signaling[11] (Figure 3).

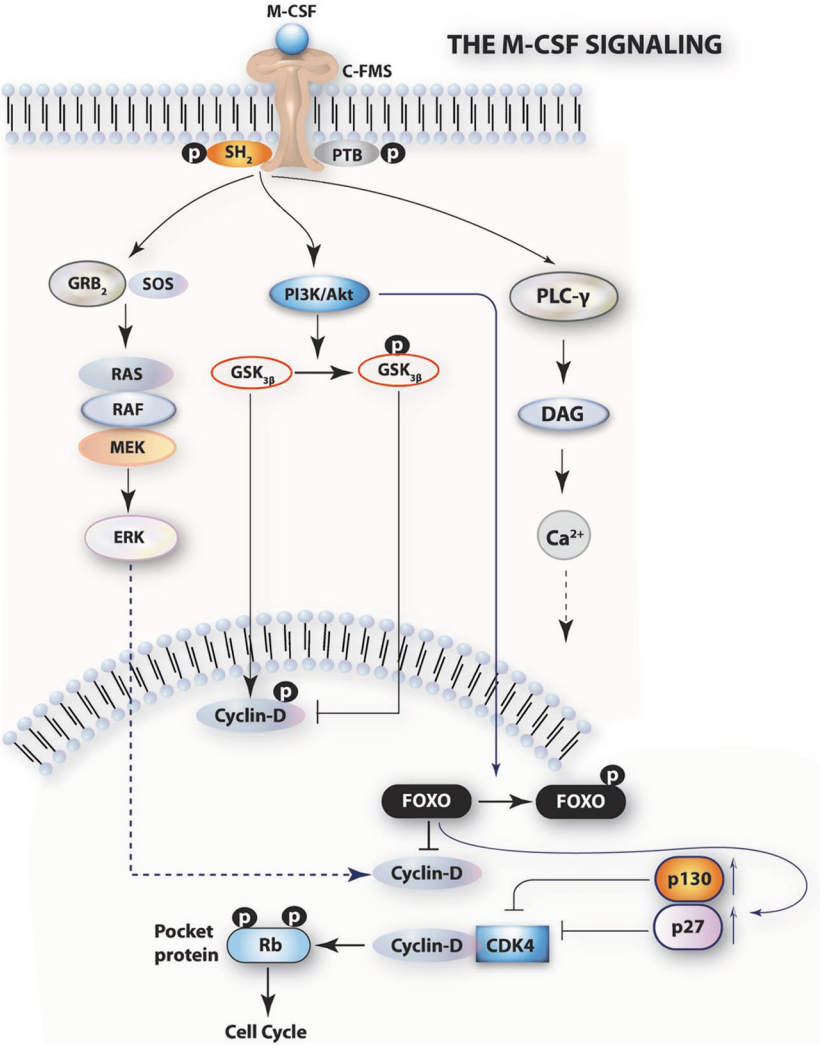

Figure 3. The M-CSF-signaling. Binding of M-CSF results in the dimerization and phosphorylation of the receptor c-fms on selected tyrosine residues. Each phosphorylated residue acts as a binding site for SH-2 and PTB domain-containing proteins. The adaptor protein complex Grb2/Sos is recruited to the receptor and stimulates the Ras/Raf/ERK/MEK pathway. PI3K/Akt phosphorylates and inactivates GSK3β and FOXO. GSK3β phosphorylates cyclin-D1. FOXO also phosphorylates cyclin-D1 and increases the cell cycle inhibitors p27 and p130. Upon binding of M-CSF, D-type cyclins form a cyclin D/cdk4 complex, which hyperphosphorylates the pocket protein Rb2, allowing entry to the cell cycle.

PI3K/AKT regulates cell proliferation via GSK3β and FOXO. The phosphorylation of GSK3β and FOXO suppresses their capacity to inhibit entry into the cell cycle.

The MITF transcription factor

MITF (microphthalmia-associated transcription factor) is a basic helix-loop-helix-leucine zipper protein, which has been implicated in the differentiation and survival of developmentally unrelated cell types, including osteoclasts[12]. MITF, like PU.1, is expressed in macrophages, osteoclasts and in the common mononuclear precursor of these cell types[12]. Interaction with PU.1, at least partly, account for the ability of MITF to selectively regulate target genes, like cathepsin K (Ctsk), acid phosphatase 5 (Acp5), and osteoclast-associated receptor (OSCAR) during osteoclast differentiation[13-14]. In cells deprived of M-CSF, MITF is sequestered to the cell cytoplasm through interactions with 14-3-3 proteins[15]. Recent evidence demonstrated that interaction of PU.1 and MITF with the zinc finger protein Eos results in repressing transcription from specific promoters, through recruitment of the co-repressors Sin3A and CtBP[13] (Figure 2). M-CSF-/RANKL signaling activate expression of osteoclast specific target genes by two mechanisms: 1) Down-regulates Eos expression at the level of mRNA and protein, leading to dissociation of the co-repressors from target genes, and 2) Phosphorylates and activates MITF by both ERK and p38 MAPK pathways, leading to recruitment of the co-activators CBP/p300 and BRG1[13-16].

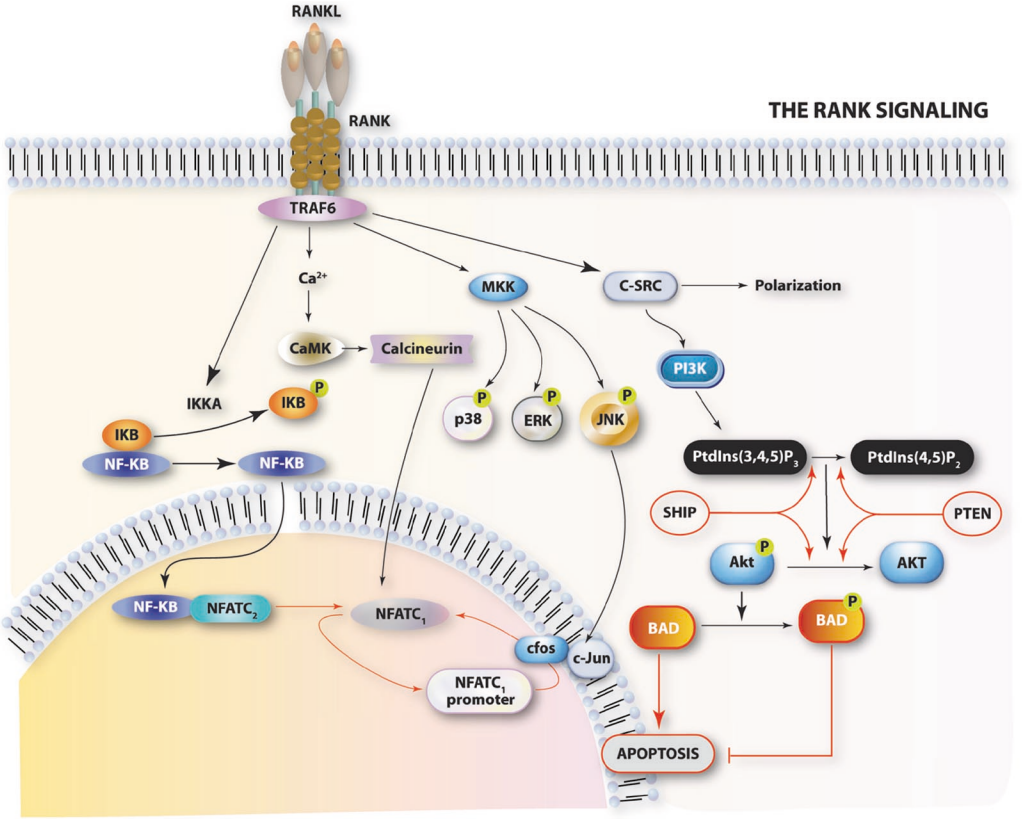

Figure 4. The RANKL/RANK signaling. RANKL activates the receptor RANK in a trimeric symmetric complex with TRAF6. Downstream signaling includes activation of IKK and NF- B, and MKK and subsequent stimulation of p38 MAPK, ERK, and JNK. Activation of the MAPKs leads to activation and nuclear translocation of the transcription factors, c-Fos, c-Jun, and NFATc1, The c-src-dependent activation of PI3K results in increased PtdIns(3,4,5)P3 and subsequent activation of Akt, which then phosphorylates and inactivates the pro-apoptotic protein Bad. This pathway can be inactivated by PTEN and SHIP, expressed by osteoclasts and their progenitor cells.

An important distinction is that the effect of Eos is specific for the MITF/PU.1 complex. Other PU.1 and MITF target genes, in both macrophages and osteoclasts, like c-FMS, RANK, and Bcl-2, are not affected by Eos over-expression[12]. Thus, Eos does not regulate the entire osteoclast gene expression. On the other hand, the presence of MITF and PU.1 at promoters of target genes allows committed precursors to respond rapidly to M-CSF/RANKL signaling and to reprogram gene expression. Recently, novel MITF target genes have been identified, including chloride channel 7 (Clcn7), which is necessary for the acidification and bone resorbing activity of osteoclasts and Ostm1, a membrane protein of unknown function important for Clcn7 protein stability[17]. Mutations in Clcn-7 or in the Ostm1 gene cause osteopetrosis in humans[18].

The c-FOS transcription factor

The transcripton factor AP-1 is a heterodimeric protein consisting of FOS proteins (c-FOS, FOS-B, FRA-1, FRA-2) and JUN proteins (c-JUN, JUN-B, JUN-D). Mice deficient in c-FOS exhibit osteopetrosis due to an osteoclast differentiation defect, while the number of macrophages increases[19], indicating that inhibition of differentiation occurs later than in PU.1-deficient mice. RANKL and M-CSF signaling induce c-FOS-dependent transcription of FRA-1, which is a target of c-FOS[20]. Prolonged ERK activation by both M-CSF and αvβ3 integrin signaling is required for stable expression of c-FOS[21]. In physiological circumstances, where concentrations of M-CSF is limited, signals derived from the αvβ3 integrin are likely to be indispensable for transcriptional activation of c-FOS[22]. Previous studies have shown that c-FOS is a key mediator of the lineage commitment between osteoclasts and dendritic cells, which are also derived from monocyte/macrophage precursor cells[23]. The differentiation into an osteoclast or a dendritic cell lineage is reciprocally inhibited by GM-CSF and M-CSF respectively. While M-CSF and sRANKL induce osteoclastogenesis, GM-CSF and sRANKL induce dendritic cell differentiation from single common precursors and RANKL has proved to be an activating factor of dendritic cells[24]. However, after the transduction of the differentiation signal by M-CSF and RANKL, and c-FOS expression, cells are no longer competent to respond to GM-CSF[23].

Therapeutic interventions

Up to now, there are no research data available concerning any possible therapeutic intervention in this stage of osteoclast development. This is probably because the transcription factors and the cytokines involved are ubiquitously expressed and regulate many different cell lineages, and therefore any intervention could lead to unpredictable and probably serious side effects from other cell lines, mainly the hematopoetic lineage.

Differentiation

The RANK/RANKL signaling

RANKL, a member of the TNF superfamily, is a membrane-residing protein on osteoblasts and their precursors, which activates receptor RANK, on osteoclast precursors. In physiological conditions, RANKL is principally expressed by stromal cells in bone marrow and osteoblasts in the periosteum. However, in states of skeletal inflammation, such as rheumatoid arthritis, RANKL is produced in abundance by T-lymphocytes[25]. On this occasion, RANKL may be cleaved from the cell membrane and then interact with RANK as a soluble ligand. Deletions of the RANKL (*Tnfsf11*) or RANK (*Tnfrsf11a*) genes result in the absence of osteoclasts due to arrested differentiation of M-CSF expanded osteoclast progenitor cells. The decoy receptor, osteoprotegerin (OPG), is also produced by osteoblasts, and acts through binding to RANKL and preventing its interaction with RANK. Recent evidence demonstrates that osteoprotegerin is a critical regulator of postnatal skeletal development and homeostasis in humans. Homozygous deletion of the TNFRSF11B gene on chromosome 8q24.2 that encodes osteoprotegerin, is associated with juvenile Paget's disease[26]. RANKL expression is regulated by a variety of hormones such as PTH, PGE2, and forskolin, all acting via the cyclic AMP/protein kinase A (PKA) pathway, and by D3, acting via the VDR-mediated pathway[27]. In addition TNF-a, signaling through p38 MAPK, induces stromal cell expression of IL-1, which in turn up-regulates its own receptor and promotes RANKL production. However, whereas IL-1 is downstream of TNF in the osteoclastogenic process, the reciprocal does not occur[28]. RANKL activates the receptor RANK on osteoclast progenitor cells in a trimeric symmetric complex (Figure 4), interacting with an adaptor molecule TNF receptor-associated factor 6 (TRAF6). Six TRAFs (TRAF1–TRAF6) have been reported so far on the basis of their similarities in the carboxy-terminal TRAF domain but only TRAF6 seems to have a critical role in osteoclastogenesis. TRAF6-deficient mice show severe osteopetrosis due to impaired osteoclastogenesis[29] Despite the activation of apparently overlapping TRAF6-dependent signaling cascades by other receptors, such as CD40 and the IL-1R/Toll-like receptor family members (TLR), only RANKL can induce osteoclastogenesis[30-31]. Recent studies suggest that the quantitative difference in TRAF6-activation, manifested by the degree of its recruitment to the surface receptor and p38 kinase activation, is probably the key mechanism that distinguishes RANKL from other receptors in terms of osteoclastogenic potential[32]. The downstream intracellular signaling pathways include TRAF6-dependent activation of IkB kinases (IKK) a and b, and MAP kinases (MKK), p38 MAPK, ERK, and JNK. Activation of IkB kinase induces phosphorylation of the inhibitory protein IkB, which forms a complex with the inactive form of NF-kB in the cytoplasm. Once phosphorylation occurs the NF-kB/IkB complex dissociates. NF-kB is released and translocates to the nucleus where it binds to specific DNA sequences triggering transcription of specific genes. The IkB is tagged by ubiquitin and is degraded by the proteasome. The importance of the NF-κB pathway for osteoclast formation is demonstrated by the finding that mice deficient in both NF-κB subunits p50 and p52 are osteopetrotic, with marrow cavities filled with unremodeled osteocartilaginous matrix[33]. Nuclear translocation of NF-kB is also accelerated by RANK-induced elevations of intracellular Ca^{2+} via PLC[34].

The involvement of the JNK pathway in osteoclastogenesis is indicated by the observations that RANK over-expression leads to enhanced activation of JNK and NF-kB[35]. Phosphorylation and activation of the MAPKs by RANKL[36] leads to activation and nuclear translocation of the ATF2, c-FOS, c-JUN, and NFATc1, resulting in transcription of genes, mostly unknown, but of vital importance for osteoclast differentiation and activation. Signaling pathways downstream of RANK (similar to that of M-CSF) also include activation of PI3K, mediated by TRAF6/c-SRC[37]. PI3K generates the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P3] and subsequently activates the anti-apoptotic serine/threonine kinase AKT (PKB). In turn, AKT phosphorylates and inactivates BAD, a pro-apoptotic protein member of the BCL-2 family. The PI3K/AKT/Bad pathway is inhibited by the tumor suppressor gene PTEN by two mechanisms. PTEN dephosphorylates PtdIns(3,4,5)P3 to the inactive PtdIns(4,5)P2, and can also act as a protein phosphatase by dephosphorylating active AKT. In addition, it has been demonstrated that RANK may regulate the expression of PTEN in osteoclasts, indicating that PTEN plays a role in regulating the balance between active and non-active AKT[39]. Besides PTEN, SHIP, another 5-phosphatase can also dephosphorylate PtdIns(3,4,5)P3 and AKT. The phenotype of SHIP -/- mice, includes large hyper-resorptive osteoclasts, and is very similar to that of patients with Paget's disease[39]. Cell to cell contact between osteoblasts/stromal cells and pre-osteoclasts is necessary for the activation of RANK/RANKL signaling. It has been demonstrated that the eosinophil chemotactic factor-L (ECFL), an autocrine factor produced by osteoclast precursors, increases the expression of the cell adhesion molecules ICAM-1 and LFA-1 that are also expressed in osteoblasts[40]. ICAM-1 is one of the ligands for LFA-1, and interactions between them are involved in a cell to cell contact between osteoclast precursors and osteoblastic/stromal cells or between osteoclast precursors in the fusion stage of osteoclast formation[41].

The transcription factor NFAT

The NFAT family of transcription factors includes five members. The necessary and sufficient role of NFATc1 in osteoclastogenesis was suggested by *in vitro* observations that NFATc1(-/-) embryonic stem cells do not differentiate into osteoclasts, and that ectopic expression of NFATc1 causes bone marrow-derived precursor cells to undergo osteoclast differentiation in the absence of RANKL[42]. Furthermore, loss of function mutation in the NFATc1 gene, leads to abolision of the capacity to form osteoclasts after RANKL stimulation, whereas M-CSF stimulation of monocyte/macrophage precursors is normal[42]. Pre-existing NFATc2 is recruited to the NFATc1 promoter at the very early phase, but this is not enough to activate the NFATc1 promoter. NFATc2 co-operates with NF-kB to activate the initial induction of NFATc1, followed by an auto-amplification phase of NFATc1[43], where c-FOS, plays a critical role as it is recruited selectively to the NFATc1 promoter[44]. Translocation of NFATc1 to the nucleus involves RANK-induced Ca^{2+} oscillations and activation of Ca^{2+}/calmodulin-dependent calcineurin, a serine/threonine phosphatase[45]. NFATc1 regulates many osteoclast-specific genes, such as cathepsin K, TRAP, calcitonin receptor (CTR) and osteoclast-associated receptor (OSCAR)[46-47], forming complexes with other transcription factors like PU.1, MITF and c-FOS, although the components of the transcriptional complex are not always the same.

Autocrine and paracrine signaling

The osteoclast, is itself a secretory cell. IL-6 was the first autocrine factor identified which stimulated osteoclast formation. Osteoclasts from patients from Paget's disease produce high levels of IL-6[48]. Annexin II is another heterotetramer autocrine factor produced by osteoclasts which stimulates osteoclast formation indirectly by increasing production of RANKL and GM-CSF on marrow stromal cells[49]. Recently the first surface receptor for annexin II was identified[50]. ADAM8 mediates its effects at late stages of osteoclast precursors via its receptor $\alpha_9\beta_1$ integrin[51]. Osteoclasts from $\alpha 9$ knockout mice are small and contracted, do not form actin rings and therefore resorb bone poorly, similar to osteoclasts that lack $\beta 3$ integrin[52]. C3 component of complement is produced by stromal cells in response to $1\alpha,25\text{-}(OH)_2D_3$ and is involved in osteoclast development by potentiating M-CSF-dependent proliferation of pre-osteoclasts[53]. Two novel autocrine-paracrine inhibitors of osteoclast formation have also been identified. The osteoclast inhibitory peptide-1 (OIP-1), a GPI-linked protein that can be cleaved from the cell surface to inhibit osteoclast formation[54] and the C-terminal peptide of OIP-2, which is cleaved by autocatalysis when the protein is secreted, and also inhibits osteclastogenesis[55].

Therapeutic interventions

Recombinant OPG was proven effective in preventing the bone loss resulting from a lack of estrogen[56]. However, the formation of significant antibody titers in a patient given OPG brought that development to an end. Recently, denosumab, a human monoclonal antibody that binds with high affinity and specificity to RANKL, has been tested in the treatment of osteoporosis in postmenopausal women[57]. Because the interactions between RANKL and OPG are also involved in immune regulations, blocking RANKL provides an additional risk for adverse systemic effects of the immune system[58]. Novel potent inhibitors of NF-Kb, like guggulsterone, used to treat osteoarthritis, are also under intense research for the development of new therapeutic agents against bone loss[59]. In addition, NFAT, which is a major osteoclastogenic transcription factor, also regulates negatively osteoblast differentiation by regulating FRA-2 expression. Recent analyses have demonstrated that inhibition of the calcineurin/NFAT signaling by cyclosporine increases the expression of alkaline phosphatase and osteocalcin[60]. These data provide the mechanism for the possible development of a novel anabolic therapeutic target for osteoporosis, aiming at NFAT signaling.

Multinucleation of pre-osteoclasts

Multi-nucleation is an essential step in the differentiation of osteoclasts, as mono-nucleated macrophages cannot resorb bone efficiently. Multi-nucleated osteoclasts are formed by the fusion of RANK+mononuclear precursors after contact with a cell expressing RANKL. An osteoclast precursor cell in contact with a RANKL presenting cell will receive the RANKL signal and initiate a cascade of gene expression that includes the production of the chemokines MCP-1, and RANTES, which are chemotactic signals for monocytes[61]. Chemokine-mediated fusion increases the size of the osteoclast, and also transfers the RANKL signal to the additional nuclei that are now in the multi-nucleated cell. As mentioned above, GM-CSF and RANKL represent two competing differentiation signals: RANKL to osteoclasts and GM-CSF to dendritic-like cells. MCP-1 overcomes GM-CSF mediated repression of osteoclast differentiation, permitting the cells to pass through multinucleation, to authentic bone resorbing osteoclasts[62]. RANKL also induces the MCP-1 receptors (CCR2 and CCR4), G protein-coupled receptors that stimulate the PI3K signaling pathways[63]. Thus, RANKL induction of MCP-1 sets up both autocrine, affecting the osteoclast producing MCP-1, and paracrine pathways, affecting cells destined to fuse with the RANKL-stimulated osteoclast. One of the reasons that inflammatory diseases, such as rheumatoid arthritis, are associated with increased osteoclast activity is because they display increased chemokine activity[64]. MCP-1 stimulates TRAP and CTR through induction of NFATc1[64]. However, these multinuclear cells cannot resorb bone efficiently, indicating that a different RANKL signaling pathway is required for activation of bone resorption even in the presence of nuclear NFATc1[64]. Furthermore, RANKL via NFATc1 induces the expression of fusion-mediating molecules such as the d2 isoform of vacuolar ATPase Vo domain

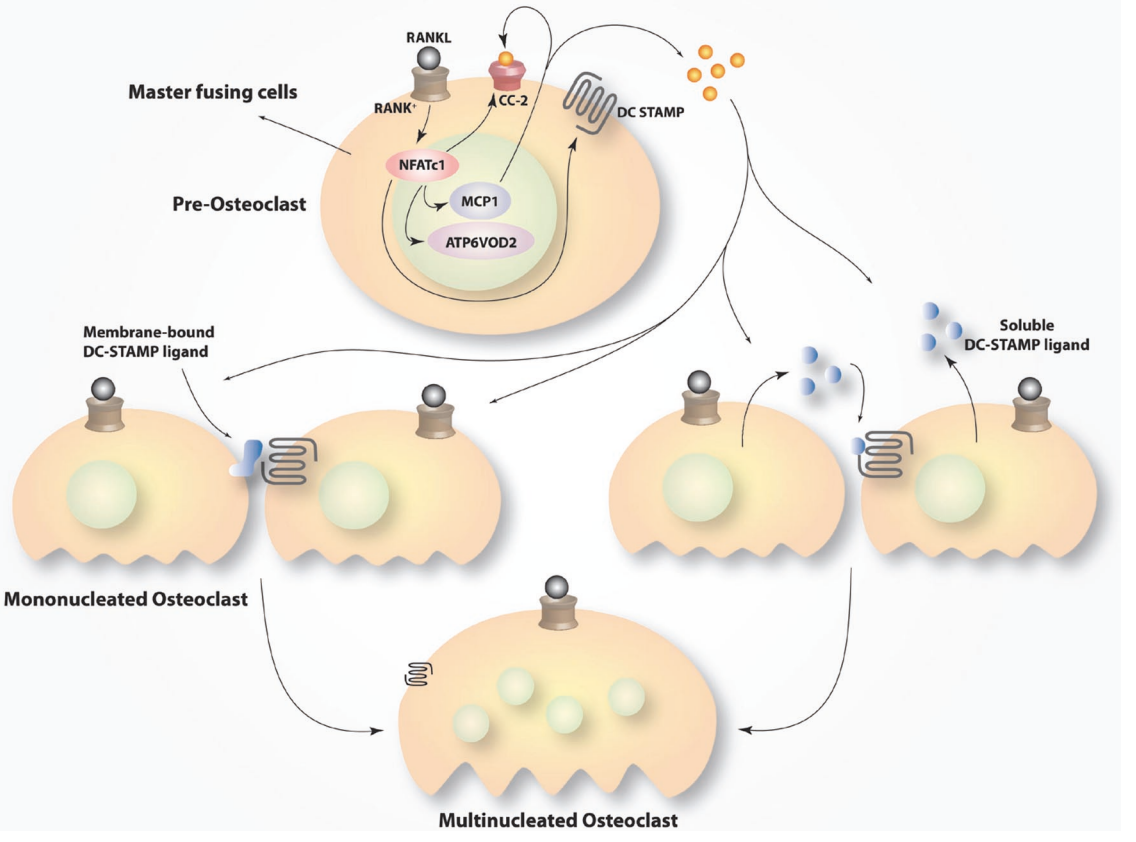

Figure 5. The fusion of mononuclear pre-osteoclasts. RANKL induces the expression of DC-STAMP and Atp6v0d$_2$. The DC-STAMP-expressing osteoclast becomes the master-fusing cell, which can fuse with a DC-STAMP-negative follower cell. The ligand for DC-STAMP may be membrane-bound or soluble.

(Atp6v0d2) and the dendritic cell-specific transmembrane protein (DC-STAMP), by binding directly to their promoter regions[65]. DC-STAMP-deficient mice developed mild osteopetrosis, attributed to the defect in the fusion of osteoclasts[65]. In the same way v-ATPase Vo subunit d2-deficient mice exhibit impaired osteoclast fusion and increased bone formation[66]. The DC-STAMP-expressing osteoclast becomes the master-fusing cell, which takes the lead in "cellocytosing" another one, and fuses with a DC-STAMP–negative follower cell. Once fusion of two cells is completed the two cells form a binucleated cell, which then becomes the "master" fuser and can fuse with other mono- or multinucleated cells[67] (Figure 5). The ligand for DC-STAMP may be membrane bound or soluble, which could be released by either of the fusion partners. It is possible that the induction of MFR (macrophage fusion receptor) is also involved in this process[68]. MFR belongs to the family of signal regulatory proteins (SIRPs), which have intrinsic signaling functions dictated mostly by a transmembrane region that associates with adaptor molecules containing an immunoreceptor activating motif (ITAM) DAP12, and FcRγ. Stimulation of these adaptor proteins have also been proposed to play a significant role in the formation of multinuclear osteoclasts[69].

Therapeutic interventions

It is known that calcitonin inhibits chemokine-stimulated cell fusion, presumably through the MCP-1-induced CTR, and blocks osteoclast bone resorption activity, acting at two stages of osteoclast differentiation. This explains the beneficial effect of calcitonin in inflammatory bone loss, where high chemokine production is observed. In addition, recent studies have demonstrated that novel V-ATPase inhibitors, which have inhibition selectivity, can be systemically administered to animals and proven to be highly efficacious against bone loss[70].

Activation of immature multi-nucleated osteoclasts

The final step to the mature bone-resorbing multinucleated osteoclast involves the polarization of the cell membrane with the generation of two polarized structures. A villous organelle unique to the resorbing osteoclast, known as the *ruffled membrane*, and an actin ring-like structure, the *sealing zone*, which isolates the resorptive microenvironment from the general extracellular space. Failure of polarization and cytoskeletal organization results in osteoclast dysfunction, and

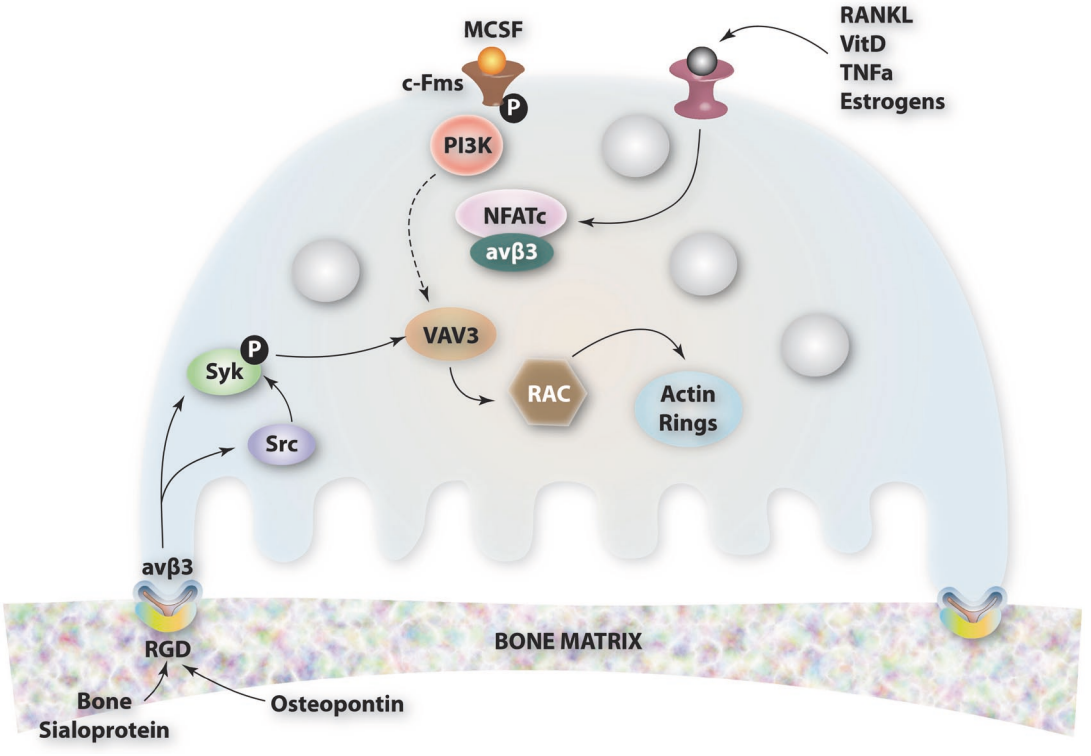

Figure 6. Activation of multinuclear immature osteoclast. Upon occupation of the avβ3 Syk and c-Src are recruited independently to its cytoplasmic domain. C-Src phosphorylates and activates Syk which in turn induces VaV_3. Both occupancy of M-CSF and avβ3 collaborate to phosphorylate VaV_3 which then activates Rac leading to cytoskeletal organization. NFAT binds to the promoter of avβ3, and increases its expression.

in varying degrees of osteopetrosis. Mineralized matrix recognition is mediated by the integrin αvβ3, the principal osteoclast integrin, and the RGD (arginine-glycine-aspartic acid) sequence in osteopontin and bone sialoprotein. Upon occupation of αvβ3, c-SRC kinase binds directly to the terminal three amino acids of the β3 subunit. The spleen tyrosine kinase SYK, which is also essential for cytoskeletal organization of the osteoclasts, binds to the cytoplasmic domain of β3 independently of c-SRC. Therefore, SYK, c-SRC, and αvβ3 form a ternary complex in the cell[71]. The ITAM-adapter proteins, Dap12 and FcRγ, associate with surface receptors present in osteoclasts such as the triggering receptor expressed in myeloid cells-2 and -3 (TREM2, 3)[72]. Activation of the adapter- associated receptor leads to phosphorylation of the tyrosines within the ITAM, probably by SRC-family kinases, which in turn recruit and activate SYK kinase[73]. However, the maintenance of ITAM-initiated SYK activity is under the aegis of the associated c-SRC[74]. Activated ITAM-bound SYK, targets the Vav family of guanine nucleotide exchange factors (GEFs), mainly Vav3 which predominates in osteoclasts, leading to induction of the Rho GTPase, Rac[75]. Both αvβ3 integrin signaling and M-CSF occupancy of c-FMS, collaborate to phosphorylate Vav3, which in turn activates Rac, leading to organization in the osteoclast cytoskeleton[76] (Figure 6).

Therapeutic interventions

αvβ3 integrin, presents as a potential anti-resorptive target for the treatment of osteoporosis. Small molecule inhibitors of the integrin, bone sparing in oophorectomized rats and in osteoporotic women are presently in clinical trials[77]. Moreover, small molecular weight compounds that mimic the tripeptide RGD sequence, recognized by the integrin, were shown to have similar effects[78]. Previous human and mouse studies have clearly demonstrated the role of TREM2 /DAP12 signaling in chemotaxis of both dendritic cells and osteoclasts, as well as in normal bone resorption by mature osteoclasts during *in vitro* conditions[72]. Recent evidence contends that TREM2 may be an attractive target for the pharmacologic modulation of bone remodeling in pathologic conditions since TREM2 blockade has been reported to prevent the functional resorption and regulate migration of osteoclasts[79]. The phenotype of c-SRC knock-out mice is consistent with osteopetrosis, which suggests that inhibitors against this enzyme may also be therapeutic for osteoporosis[80]. Finally, SYK, as a nexus of a novel signaling pathway regulating osteoclast function, is itself a candidate anti-resorptive therapeutic target[71].

Conclusion

Intense research effort during the last 10 years has proved that osteoclastogenesis is a multicomplex process involving many different stages with multiple interactions among them. Anti-resorptive drugs such as estrogen, raloxifene and biphosphonates, the mainstay of anti-osteoclastic therapy until recently, are known to increase the apoptosis of osteoclasts and inhibit their bone resorptive activity. However, due to the fact that bone formation is tightly coupled to bone resorption, the abrogation of osteoclasts resulted in reduced bone formation as well. The detailed knowledge of the molecular mechanisms and the downstream signaling pathways involved in osteoclastogenesis as well as the screening for the osteoclast specific genes induced by these pathways, provide a novel field for the generation of therapeutic agents that can manipulate osteoclast activity without interfering with bone formation. This possibility is illustrated by recent studies using inhibitors of c-SRC, the v-ATPase or chloride channel CLC-7 in osteoclasts, where bone resorption was decreased but bone formation was maintained[81]. However, whether these important findings in animals will translate into improved fracture efficacy in clinical trials remains to be seen. Even so, the prospect of using pharmaceutical intervention to inhibit bone resorption without inhibiting bone formation is now a distinct possibility[82]. Moreover there is also very little known about the role of canonical Wnt signaling in osteoclasts. Wnt3a regulates osteoclast differentiation via an indirect mechanism involving, through Runx2, the down-regulation of RANKL expression and induction[83]. These reciprocal changes in RANKL and OPG expression mediate the effects of Wnt signaling on osteoclast differentiation *in vivo*. Despite the major role of Wnt signaling in osteoblastogenesis[84], it is still largely unknown whether and to what extent it directly affects osteoclasts[85]. Finally, although the current knowledge holds that RANKL/RANK interaction and subsequent signaling via TRAF-6 are both essential and adequate for the generation of functional osteoclasts, recent data have suggested that alternative pathways independent of RANKL signaling may exist. Stimulation of TNFα, the dominant cytokine extant in inflammatory osteolysis, in the presence of co-factors such as TGF-b, has proved to provide adequately osteoclasts from hematopoietic precursors from TRANCE-, RANK-, or TRAF6-null mice[86].

Based on current knowledge, future interventions that will selectively alter a specific stage of the osteoclastogenesis or identification of distinct differentiation pathways may be able to shift back or even reverse bone loss in pathological conditions, such as the postmenopausal osteoporosis or chronic inflammatory bone diseases.

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