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Review

The molecular understanding of osteoclast differentiation

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Abstract

Osteoclasts are multinucleated cells of monocyte/macrophage origin that degrade bone matrix. The differentiation of osteoclasts is dependent on a tumor necrosis factor (TNF) family cytokine, receptor activator of nuclear factor (NF)-κB ligand (RANKL), as well as macrophage colony-stimulating factor (M-CSF). Congenital lack of osteoclasts causes osteopetrosis, investigation of which has provided insights into the essential molecules for osteoclastogenesis, including TNF receptor-associated factor (TRAF) 6, NF-κB and c-Fos. In addition, genome-wide screening techniques have shed light on an additional set of gene products such as nuclear factor of activated T cells (NFAT) c1. Here we summarize the efforts to understand the sequential molecular events induced by RANKL during osteoclast differentiation. RANKL binds to its receptor RANK, which recruits adaptor molecules such as TRAF6. TRAF6 activates NF-κB, which is important for the initial induction of NFATc1. NFATc1 is activated by calcium signaling and binds to its own promoter, thus switching on an autoregulatory loop. An activator protein (AP)-1 complex containing c-Fos is required for the autoamplification of NFATc1, enabling the robust induction of NFATc1. Finally, NFATc1 cooperates with other transcriptional partners to activate osteoclast-specific genes. NFATc1 autoregulation is controlled by an epigenetic mechanism, which has profound implications for an understanding of the general mechanism of irreversible cell fate determination. From the clinical point of view, RANKL signaling pathway has promise as a strategy for suppressing the excessive osteoclast formation characteristic of a variety of bone diseases.

Keywords: Osteoclast; Osteoclastogenesis; Osteopetrosis; RANKL; NFATc1

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Introduction

From a biological point of view, it is astonishing how well-preserved the bones of our human ancestors are in prehistoric sites dating from ancient times [72]. Bone is obviously resistant to dissolution, at least outside the body. Inside the body's highly active milieu, however, bone is remodeled at such a high speed that approximately 10% of the total bone content is replaced per year in adult humans [1]. This striking contrast emphasizes what an extraordinary and specific role osteoclasts play in the active maintenance of the bony skeleton. These multinucleated cells help dynamically remodel bones in coordination with osteoblasts, which deposit bone matrix. The excessive osteoclastic bone resorption relative to osteoblastic bone formation is often associated with osteopenic diseases including osteoporosis and rheumatoid arthritis, so the mechanism of osteoclast differentiation also has great clinical implications.

The difficulty in isolating or culturing bone cells left the molecular analysis of osteoclastogenesis unexplored for a long period, but the past decade has witnessed great breakthroughs in this field [60,121]. In addition to identification of M-CSF as an essential survival and proliferation factor for osteoclast precursor cells, the cloning of osteoclast differentiation factor, RANKL, enabled the reconstitution of dynamic differentiation processes, including cell fusion, in a culture system [120,126]. Now, under direct observation, osteoclasts form from RANKL-stimulated bone marrow monocyte/macrophage lineage cells in the presence of M-CSF [76,136,143]. This osteoclast differentiation system has developed into one of the most sophisticated culture systems available, allowing a rough but extremely useful visual evaluation of the differentiation process, and the cells undergoing differentiation may then be subjected to extensive molecular analyses. Here we focus on the studies on intracellular signal transduction of RANKL and summarize recent progress in the understanding of the mechanisms of osteoclastogenesis, which may also provide insights into the general workings of cell differentiation.

Methodological advances in osteoclast biology

Osteopetrosis is a rare congenital disease characterized by high bone density and impaired formation of bone marrow cavities, which is attributed to a lack or dysfunction of osteoclasts [125,127]. In the 1980s, naturally occurring mouse models of osteopetrosis were the main source of information on osteoclastogenesis, although the responsible genes were not completely characterized in those days [32]. Importantly, based on the observation that osteopetrosis is cured by bone marrow transplantation in gray-lethal (*gl/gl*) or microphthalmic (*mi/mi*) mice [127,133], the origin of osteoclasts was revealed to be of hematopoietic lineage.

All types of osteopetrosis are not rescuable by bone marrow transplantation since the defect is not only dependent on osteoclast precursor cells *per se* but also on the specific microenvironments. As a factor provided from the microenvironments, M-CSF was revealed to be an essential factor for osteoclastogenesis: it was identified as the causal gene for osteopetrotic *op/op* mice [136,143]. In the early days of gene disruption studies in mice, unexpected bone phenotypes were found in the knockout mice of certain protooncogenes. These results provided further insight into osteoclast biology. For example, mice deficient in *c-Fos* [54,134] or *c-Src* [84,115] exhibit severe osteopetrosis. Subsequent analyses revealed these molecules to be crucial for the differentiation and function of osteoclasts, respectively.

An *in vitro* osteoclast formation system, a coculture system of bone marrow cells and osteoblasts, was established in the late 1980s [116,118]. Through this coculture system, the importance of cell–cell contact between precursor cells and osteoblasts came to be appreciated [116]. This system provided not only fundamental knowledge about the effects of osteoclastogenic factors such as $1\alpha,25\text{-}(OH)_2$ vitamin D_3, parathyroid hormone (PTH) and prostaglandin E_2 on osteoblasts but also the insight into the membrane-bound molecule provided by osteoblasts which supports osteoclastogenesis [116]. Several groups independently cloned the long-sought osteoclast differentiation factor in 1997–98 [2,75,139,141], which is now widely called RANKL. Recombinant RANKL and M-CSF efficiently induce osteoclastogenesis, which has allowed this process to be extensively investigated in a culture of a relatively pure population of osteoclast precursor cells.

Novel screening techniques were developed as the genome project progressed and the genetic information became available. Large-scale screening of RANKL-inducible genes by microarrays and other methods led bone biologists to previously unfamiliar gene products such as NFATc1 [52,122] and dendritic cell-specific transmembrane protein (DC-STAMP) [74,140]. We can also make use of novel *in vivo* techniques that provide genetic evidence even when conventional knockout mice are embryonically lethal. A conditional knockout strategy would be the most attractive approach to overcome this difficulty, but osteoclast-specific gene deletion, especially at the early stage of differentiation, has yet to be established despite an increasing number of reports on osteoclast-specific Cre mice or inducible Cre-expressing mice [16,23,33,61,73,99,110]. Furthermore, two lines of alternative genetic approaches have recently been established: adoptive transfer of hematopoietic stem cells to osteoclast-deficient mice and osteoclast-deficient blastocyst complementation (Fig. 1 and [8]). The importance of *in vivo* evidence for the essential role of each gene cannot be overemphasized: these novel *in vivo* techniques will surely provide pivotal evidence on the roles of many molecules which

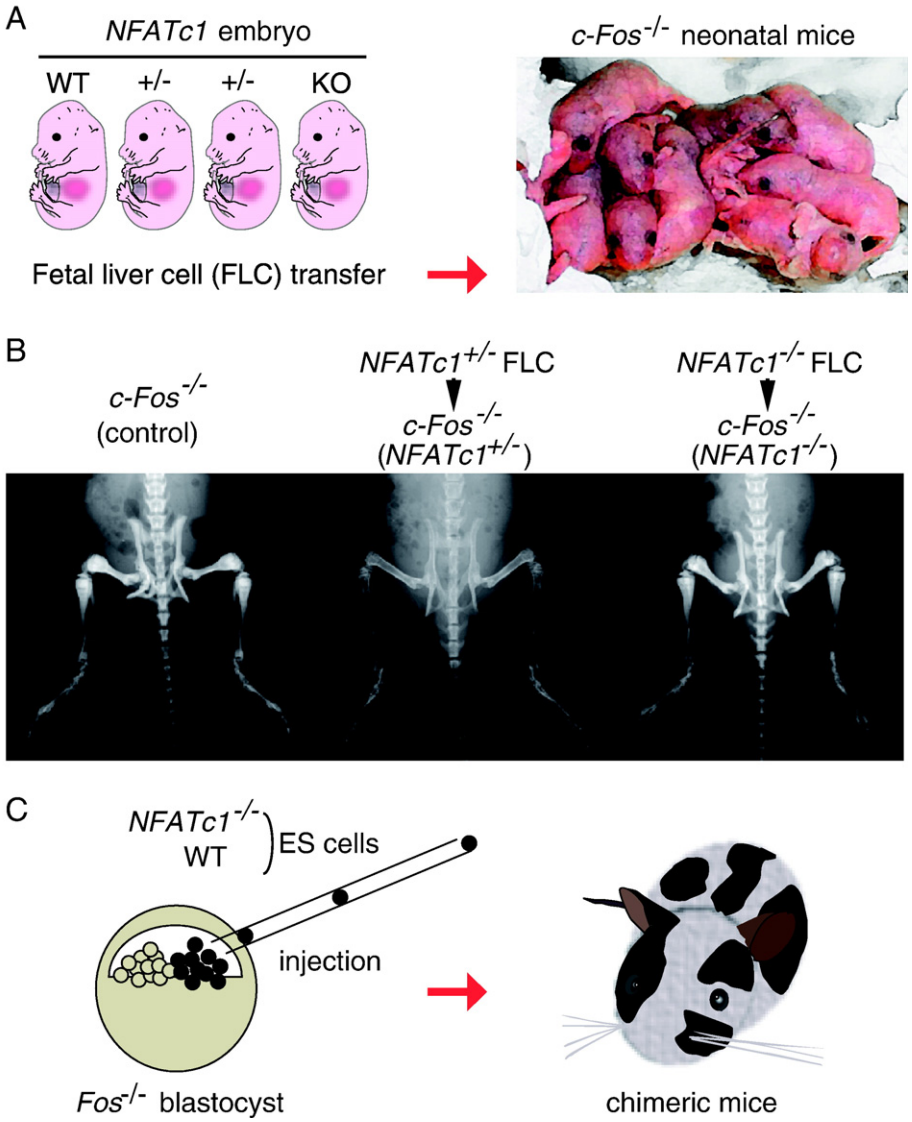

Fig. 1. *In vivo* evidence for the essential role of NFATc1 in osteoclastogenesis was obtained by applying the genetic approach developed in immunology to the field of bone biology. (A) Adoptive transfer of hematopoietic stem cells (HSCs) to osteoclast-deficient *c-Fos*$^{-/-}$ mice, the osteopetrosis of which is due to a defect intrinsic to hematopoietic cells [42]. In order to analyze the role of an embryonically lethal gene when deleted *in vivo*, fetal liver cells (FLCs) containing HSCs derived from homozygous or heterozygous mutant mice were injected to *c-Fos*$^{-/-}$ mice. Before the transfer, the hematopoietic system of neonatal *c-Fos*$^{-/-}$ mice has been destroyed by lethal irradiation or by administration of an anticancer drug such as busulfan. (B) Radiographic analysis of *c-Fos*$^{-/-}$ mice transferred with *NFATc1*$^{+/-}$ or *NFATc1*$^{-/-}$ FLCs. Severe osteopetrosis was observed after the transfer of *NFATc1*$^{-/-}$ FLCs, while normal bone marrow cavity was formed after the transfer of *NFATc1*$^{+/-}$ FLCs. Modified from Asagiri et al. [8]. (C) *c-Fos*$^{-/-}$ blastocyst complementation. *c-Fos*$^{-/-}$ blastocysts generate mice with no mature osteoclasts following implantation into foster mothers. Injection of normal embryonic stem (ES) cells into *c-Fos*$^{-/-}$ blastocysts leads to the generation of somatic chimeras with mature osteoclasts, which are derived from the injected ES cells. Chimeric mice obtained by complementation of *c-Fos*$^{-/-}$ blastocysts with *NFATc1*$^{-/-}$ ES cells completely lack osteoclasts and display severe osteopetrosis (see [8] for the detail).

to date have been only suggested by *in vitro* or inhibitor experiments.

List of players: lessons from osteopetrosis

As mentioned above, the naturally occurring and genetically modified osteopetrotic mice revealed a number of essential genes for osteoclast differentiation and activation. Characterization of the osteoclast-lineage cells in these mice provided important information on the stage of differentiation affected (Fig. 2 and see below).

The first group of genes includes *M-CSF* [76,136,143], *Csf1r/c-Fms* (encoding M-CSF receptor) [20] and the transcription factor *PU.1* [92,128], which are involved in the generation of common progenitors for macrophages and osteoclasts. The deletion of these genes results in the lack of both macrophages and osteoclasts. M-CSF induces the proliferation of osteoclast precursor cells, supports their survival and upregulates the RANK expression, which is a prerequisite for osteoclast precursor cells [6,109]. The transcription factor PU.1 binds to the promoter region of *Csf1r* and positively regulates transcription [144], and mice deficient in *PU.1* display an osteopetrotic

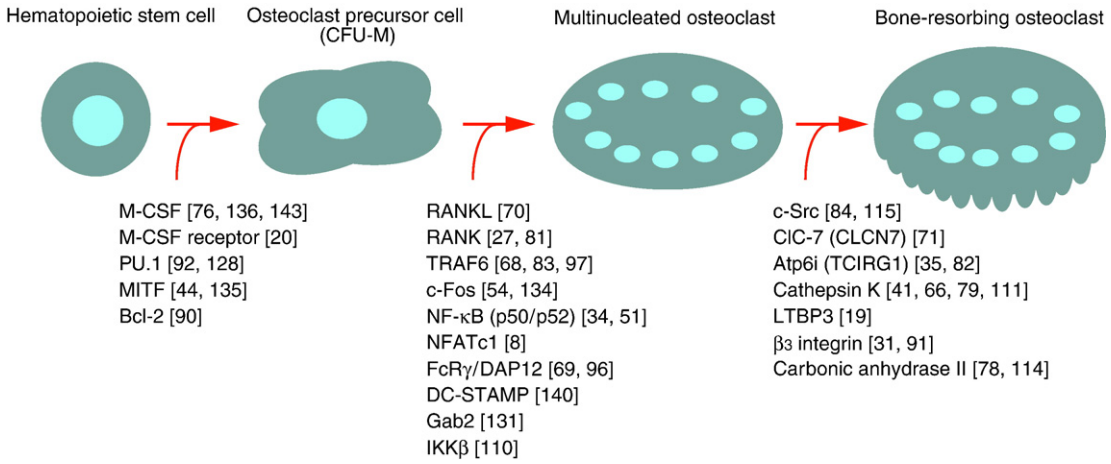

Fig. 2. Stage-specific key molecules for osteoclast differentiation and function. In response to M-CSF, hematopoietic stem cells undergo differentiation into macrophage colony-forming units (CFU-M), which are the common precursor cells of macrophages and osteoclasts. The defect in the molecules involved in this stage results in a lack of both macrophages and osteoclasts. The differentiation step from CFU-M to multinucleated osteoclasts is characterized by cell–cell fusion, which is mainly induced by RANKL and its downstream molecules. In the activation (maturation) stage, osteoclasts acquire bone resorbing activity. Note that the deficiency of *carbonic anhydrase* (*CA*) *II* in humans causes severe osteopetrosis while the deficiency in mice does not [78,114]. Although M-CSF is also important in the differentiation and maturation stages, the main stage to which it contributes is depicted based on the results of mouse genetic analysis.

phenotype similar to the *op/op* mice [128]. MITF is activated by M-CSF signaling [135] and binds to the *Bcl-2* promoter [90]. MITF regulates the expression of the anti-apoptotic protein Bcl-2 in the osteoclast lineage [90], and both *mi/mi* mice (carrying a mutation at the *MITF* locus) and *Bcl-2*-deficient mice exhibit osteopetrosis [44,90].

The second group includes *RANKL* [70], *RANK* [27,81], *TRAF6* [68,83,97], *c-Fos* [54,134], *NF-κB* (*p50/p52*) [34,51], *NFATc1* [8] and *Fc receptor common γ subunit* (*FcRγ*)/*DNAX-activating protein 12* (*DAP12*) [69,96], the deficiency of which leads to the loss of multinucleated osteoclasts causing severe osteopetrosis despite a normal or increased number of monocyte/macrophages. *DC-STAMP* [140], *Gab2* [131] and *IKKβ* [110] are also included in this group, but the osteopetrotic phenotype with a deficiency of these genes is less severe. In a culture system, *Syk* [69,96], *NIK* [100], and *IKKα* [110] have also been shown to be important for osteoclast differentiation.

The third group, which is beyond the scope of this review, is comprised of *c-Src* [84,115], *ClC-7* (*CLCN7*) [71], *Atp6i* (*TCIRG1*) [35,82] and *cathepsin K* [41,66,79,111] and *LTBP3* [19]. Mice deficient in these molecules have osteoclasts with no or very little bone resorbing activity, indicating these molecules to be crucial for osteoclast function. While ClC-7, Atp6i and cathepsin K are directly involved in bone resorbing process, the role of c-Src and its downstream signaling pathway is still under intensive investigation [46]. Despite a wealth of data showing the promotive role of TGF-β in *in vitro* osteoclast differentiation [37,59,105], there is no *in vivo* genetic evidence for it: $LTBP3^{-/-}$ mice will provide a clue to its function in osteoclast activity but further detailed analyses will be required in the future. Although the mutant mice do not exhibit a complete osteopetrosis but an osteosclerotic phenotype, *β_3 integrin* plays an important role in the activity (and differentiation) of osteoclasts in concert with M-CSF [31,91,109].

The above is a rough sketch of the current working pieces of the molecular puzzle. However, in fact, this is just the beginning of puzzling out the mysteries of osteoclast differentiation. It is still required the pieces be put in the context of RANKL signaling to understand the differentiation process in its sequential steps at the molecular level.

TRAF6: the multifunctional second messenger activated by RANKL

Using a series of genetically modified mice, it has been clearly demonstrated that RANKL and its receptor RANK are indispensable for osteoclastogenesis [126]. Since another essential cytokine for osteoclastogenesis, M-CSF [136,143], is basically important for the proliferation and survival of the osteoclast precursor cells [76], the factor that directly controls the differentiation process is now assumed to be RANKL: molecular events during osteoclastogenesis have thus come to be understood in the context of RANKL–RANK signaling. Although M-CSF also participates in the later differentiation stage through activating Akt, c-Fos and extracellular signal-regulated kinase (ERK) pathways that may interact with RANKL signals [109], this review mainly focused on the RANKL-induced signaling events due to article length constraints.

RANK lacks intrinsic enzymatic activity in its intracellular domain, and the analyses of molecules associating with the cytoplasmic domain of RANK revealed that it transduces signaling by recruiting adaptor molecules such as the TRAF family of proteins [21,38,137,138]. The TRAF family contains seven members (TRAFs 1, 2, 3, 4, 5, 6 and 7) and mainly mediates signals induced by TNF family cytokines and pathogen-associated molecular patterns (PAMPs) [10,50]. The cytoplasmic tail of RANK contains three TRAF6-binding sites and two sites for the binding of other TRAF family members including TRAF2, TRAF3 and TRAF5 [22,38,40,48,55,138,142]. TRAF1 has been shown to have the ability to bind to RANK in an *in vitro* binding assay and an overexpression study [38,138].

Despite these findings, the phenotype of knockout mice identified TRAF6 as the major adaptor molecule linking RANK to osteoclastogenesis. Two groups independently showed that *TRAF6*$^{-/-}$ mice developed severe osteopetrosis due to impaired bone resorption [83,97]. However, it was controversial whether TRAF6 is essential for the differentiation of osteoclasts since two groups attributed the osteopetrotic phenotype to different types of defect in the osteoclast lineage: the absence of osteoclasts [97] vs. osteoclasts without function [83]. A third mutant line was generated [68], and the mice also exhibited an osteopetrotic phenotype due to the severe impairment of osteoclast differentiation [63]. Additional *in vitro* experiments further support the importance of TRAF6 in osteoclast differentiation [40,55,67,123], which is coming to be generally accepted (reviewed in [60,125]). In contrast to the essential role of TRAF6 in osteoclastogenesis, the contributions of TRAF2, TRAF3 and TRAF5 seem to be relatively limited. For instance, the binding sites for TRAF2, TRAF3 and TRAF5 are dispensable for osteoclast formation [7]. In addition, osteoclast formation from *TRAF2*-deficient or *TRAF5*-deficient osteoclast progenitor cells is only minimally reduced [57,58].

By an as yet unknown mechanism, the binding of TRAF6 to RANK induces the trimerization of TRAF6, leading to the activation of NF-κB and mitogen-activated kinases (MAPKs), including Jun N-terminal kinase (JNK) and p38 [67,83,97,138]. TRAF6 contains an N-terminal RING finger domain and a stretch of predicted zinc finger domains [67]. The ubiquitin ligase activity mediated by the RING finger motif of TRAF6 has been shown to be important for NF-κB activation in other cell types [26]. However, deletion analysis indicated that the RING finger domain of TRAF6 is dispensable for the formation of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells [67]. Since this appears to be inconsistent with the essential role of NF-κB in osteoclastogenesis, the importance of the ubiquitin ligase activity of TRAF6 in osteoclastogenesis is yet to be established. It has been recently shown in the context of osteoclastogenesis that TRAF6 forms a signaling complex containing RANK and TAK-1-binding protein (TAB) 2, which results in TGF-β-activated kinase (TAK)1 activation [95]. Dominant negative forms of TAK1 and TAB2 inhibit the NF-κB activation induced by RANKL, and endogenous TAK1 has been shown to be activated in response to RANKL stimulation in a mouse macrophagic cell line RAW 264.7 [95]. Thus, *in vitro* experiments suggest that TAK1 is involved in the TRAF6-mediated activation of NF-κB and MAPKs, but the physiological relevance and precise molecular mechanism remain to be elucidated.

It is noteworthy that RANK has three TRAF6-binding sites, which is three times more than CD40. The explicit number of TRAF6-binding sites (i.e., the quantitative difference) may be a key to the specific nature of RANK for osteoclastogenesis [55], but it has been shown that the osteoclastogenic activity of RANK is one hundred times higher than CD40, not three times higher as might be expected [40]. Therefore, there appear to exist qualitative differences between RANK and other receptor signaling events. It is not still clear how RANK alone among the TRAF6-binding receptors is able to stimulate osteoclastogenesis so powerfully. One possible explanation is that RANK has specific adaptor protein(s) not associated with other TRAF6-binding receptors. For example, Gab2 has been shown to be associated with RANK and to play an important role in its signal transduction [131]. Since toll-like receptors and IL-1 also utilize TRAF6 as a critical signal transducer, it will be necessary in the future to identify how the TRAF6 signaling is specified in the context of RANKL signaling. It is also an issue of interest to determine how TRAF6 function is regulated through interaction with other proteins. Atypical PKC (aPKC)-interacting protein p62 is one of the candidate molecules involved in the regulation of TRAF6 ([28] and see the chapter Other signaling molecules).

NF-κB: an essential complex regulated by complex mechanisms

As RANK was named after its ability to activate NF-κB, it is well recognized that NF-κB activation is among the very early molecular events induced by RANK [2]. Although TRAF6 is critical for the RANK-induced activation of NF-κB, it is unlikely that NF-κB is the only downstream molecule mediating the complex functions of TRAF6. However, among the molecules immediately activated by TRAF6, genetic evidence supports the essential role of NF-κB, but not that of other molecules, such as MAPKs, in osteoclastogenesis.

NF-κB is a family of dimeric transcription factors that recognize a common DNA sequence called the κB site. In mammals, there are five NF-κB proteins: Rel (cRel), RelA (p65), RelB, NF-kappaB1 (p50) and NF-kappaB2 (p52, which is processed from its precursor, p100). While the Rel proteins contain transcriptional activation domains, such domains are absent in p50 or p52, whose activation function depends on heterodimerization with any of the three Rel proteins [39,43]. Although *p50*- or *p52*-deficient mice have no obvious bone disorder, mice doubly deficient in *p50* and *p52* developed osteopetrosis due to a defect in osteoclast differentiation, indicating a critical function of p50 and p52 in osteoclastogenesis [34,51]. The differentiation blockade in the *p50/p52*-deficient mice may result from the impaired DNA binding activity of the remaining Rel proteins, but the transcriptional targets and precise molecular mechanisms underlying the overlapping functions of p50 and p52 at present are not well understood.

NF-κB proteins reside in the cytoplasm of non-stimulated cells but rapidly enter the nucleus upon cell stimulation with a variety of agonists, including RANKL [2,11,123]. NF-κB activation depends on two pathways (Fig. 3). The classical NF-κB signaling pathway involves activation of the inhibitor of the κB (IκB) kinase (IKK) complex that phosphorylates the IκBs and targets them for ubiquitin-dependent degradation. The IκBs retain most of the NF-κB dimers, with the exception of p52:RelB, in the cytoplasm [39,43]. The alternative pathway is responsible for activation of the p52:RelB dimers, which are generated by the processing of the cytoplasmic p100:RelB complex [39,43,100,110]. If p50 and p52 are exclusively involved in the classical and alternative pathways, respectively, the phenotype of *p50/p52*-deficient mice suggests that both

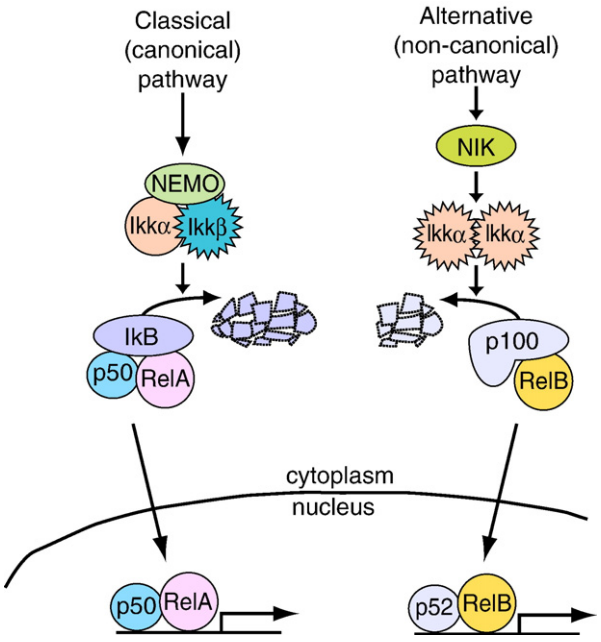

Fig. 3. Classical and alternative pathways of nuclear factor (NF)-κB activation. The classical (canonical) pathway is activated by tumor necrosis factor-α receptor 1/2, interleukin-1 receptor/toll-like receptor, B-cell receptor and T-cell receptor. Activation of this pathway is mediated by a trimeric complex containing NF-κB essential modulator (NEMO), inhibitor of NF-κB kinase (IKK) α and IKKβ. IKKβ phosphorylation of IκB leads to a proteasome-mediated degradation of IκB, which induces the translocation of p50:RelA to the cytoplasm. The alternative (non-canonical) pathway is activated by the B-cell-activating factor receptor, as well as the lymphotoxin β receptors and CD40. This pathway involves NF-κB-inducing kinase (NIK) activation of IKKα, which induces the processing of p52 from p100, resulting in dimerization and nuclear translocation of the p52:Rel B heterodimer.

pathways are important for osteoclastogenesis and they regulate the common target gene(s) critical for osteoclastogenesis. However, recent reports have suggested that more complex mechanisms may be involved. NF-κB-inducing kinase (NIK) controls the processing of p100 to generate p52 and is therefore important for the alternative pathway [39,43]. *NIK*-deficient osteoclast precursors do not respond to RANKL in an *in vitro* differentiation system, but *NIK*-deficient mice do not exhibit osteopetrosis, suggesting that the alternative pathway is not essential [100]. Targeted disruption of *IKKα*, which is an essential catalytic IKK in the alternative pathway, results in an impairment of osteoclastogenesis only *in vitro* but not *in vivo*, while targeted disruption of *IKKβ*, which is an essential catalytic IKK in the classical pathway results in impairment of osteoclastogenesis both *in vitro* and *in vivo* [110]. Furthermore, a cell permeable peptide which blocks the classical pathway has been shown to efficiently inhibit osteoclast formation *in vitro* and inflammatory bone destruction *in vivo* [53]. These results suggest that the classical, not the alternative pathway, is indispensable for osteoclastogenesis; however, this is not completely consistent with the findings in the *p50*- and *p50/p52*-deficient mice since the former exhibit no bone abnormality.

Thus, the precise roles of the two NF-κB activation pathways and the function of each NF-κB component in osteoclastogenesis are not entirely clear. Crosstalk between the classic and alternative pathways through the inhibition of p50:RelA dimers by p100 is also proposed [100]. Further studies will be needed to obtain a complete and accurate understanding of the complex regulatory mechanisms involved.

Initial induction of NFATc1

What is the critical target gene of NF-κB in the early phase of osteoclastogenesis? The induction of NFATc1 is a hallmark event in the cell fate determination of osteoclasts [8,122], which will be discussed in the chapters below. Since NFATc1 induction was shown to be impaired in *TRAF6*$^{-/-}$ cells [122], it has been suggested that *NFATc1* is one of the key target genes of NF-κB in the early phase of osteoclastogenesis. This idea was also supported by the observation that an NF-κB inhibitor suppressed RANKL-stimulated induction of NFATc1 [119] and that NFATc1 induction is also impaired in *p50/p52*-deficient cells [80]. It is interesting that the effect of the NF-κB inhibitor on osteoclastogenesis is more profound when it is administered in the early phase of osteoclastogenesis than in the late phase, suggesting that NF-κB is involved in the activation of immediate-early responsive genes to RANKL [119]. Finally, chromatin immunoprecipitation (ChIP) experiments have revealed that NF-κB is recruited to the *NFATc1* promoter immediately after RANKL stimulation [8]. Consistent with this, the *NFATc1* promoter contains κB sites and NF-κB over-expression activated the *NFATc1* promoter in a luciferase reporter gene assay [8]. Another molecule that is recruited to the *NFATc1* promoter is NFATc2, a member of the NFAT family of transcription factors. NFATc2 preexists before RANKL stimulation and is recruited to the *NFATc1* promoter at the same time as NF-κB. NFATc2 and NF-κB cooperatively activate the *NFATc1* promoter within minutes of RANKL stimulation [8]. This is an important step toward the robust induction of NFATc1 at the next stage of differentiation. It should be noted that the loss of NFATc2 might be compensated for by other NFATs as *NFATc2*$^{-/-}$ mice display no defect in osteoclastogenesis [8].

c-Fos and other AP-1 proteins

RANK activates the transcription factor complex AP-1 partly through an induction of its critical component, c-Fos [122,132]. The AP-1 transcription factor is a dimeric complex composed of the Fos (c-Fos, FosB, Fra-1, Fra-2), Jun (c-Jun, JunB, JunD) and ATF (ATFa, ATF2, ATF3, ATF4, B-ATF) proteins [30,132]. Mice lacking c-Fos develop severe osteopetrosis due to a complete block of osteoclast differentiation [54,134]. Another member of the Fos family Fra-1, which is a transcriptional target of c-Fos during osteoclast differentiation, compensates for the loss of *c-Fos* both *in vivo* and *in vitro*, but *Fra-1*-deficient mice do not exhibit osteopetrosis [29]. This suggests that Fra-1 has an ability to compensate for the loss of *c-Fos* but is not the exclusive downstream molecule of c-Fos. FosB or Fra-2 also rescues the differentiation blockade of *c-Fos*-deficient osteoclast precursor cells *in vitro*, but their abilities to compensate are relatively weak [88]. Importantly,

it is reported that transgenic mice expressing dominant negative c-Jun under the control of the *TRAP* promoter exhibit osteopetrosis [49]. Since dominant negative c-Jun inhibits AP-1 activity by binding to the Fos, Jun and ATF families of proteins, this transgenic mouse confirms that AP-1 activity is critical for osteoclastogenesis [49]. It should be noted, however, that the results based on the dominant negative c-Jun provide no definitive information on the selective role of c-Jun itself because it inhibits all of the AP-1 proteins.

In contrast to the critical role of c-Fos, the Jun family of proteins, which are partners of the Fos family of proteins in the context of AP-1, plays a redundant role. Mice lacking Jun family proteins such as c-Jun and JunB were embryonically lethal, but conditional knockout mice showed that a deficiency in JunB or c-Jun leads to a considerable decrease in osteoclast formation, but not to the complete blockade of this process, suggesting that Jun members can substitute for each other during osteoclastogenesis [23,61,132].

It is unclear how c-Fos plays such an exclusive role among the AP-1 proteins. One possibility is that, although AP-1 complexes bind to similar DNA sequences, an AP-1 complex containing c-Fos may have a selective affinity to some of the target genes. It is alternatively possible that c-Fos is crucial for AP-1 interaction with its specific transcriptional partner that is required in osteoclastogenesis. Whatever the detailed mechanisms, c-Fos is clearly required for osteoclastogenesis and the next question which arises is the transcriptional target of c-Fos. Interestingly, NFATc1 induction by RANKL is completely abrogated in *c-Fos*-deficient cells [87,122]. ChIP experiments have shown that c-Fos is recruited to the *NFATc1* promoter 24 h after RANKL stimulation [8]. At this time, the main NFAT member recruited to the *NFATc1* promoter is NFATc1 itself [8], and an AP-1 complex containing c-Fos may cooperate with NFATc1 to enable the robust induction of *NFATc1* (i.e., autoamplification of NFATc1, see below). Thus, *NFATc1* is a common target gene of both of the essential transcription factors NF-κB and AP-1 during osteoclastogenesis.

MAPKs awaiting *in vivo* studies

A series of *in vitro* experiments have suggested that MAPKs play an important role in osteoclastogenesis, but *in vivo* evidence has yet to be obtained. MAPKs are involved in the activation of AP-1 components [14] and therefore may have a role in osteoclastogenesis by modulating AP-1 activity, but the molecular mechanisms of their functions are not well understood. Mammals express at least four distinctly regulated groups of MAPKs, p38-MAPKs (p38α/β/γ/δ), JNK1/2/3, ERK1/2 and ERK5 [14]. Many of the MAPKs have been shown to be activated downstream of RANK. Based on the effect of the specific inhibitor of p38α and β (SB203580) in RAW 264.7 cells, it was suggested that p38α and/or β are involved in osteoclast formation [86]. It has also been shown that p38 is important for the induction of the *cathepsin K* gene [85]. The functions of the other p38-MAPK isoforms p38γ and δ remain to be elucidated. MEKs (ERK1/2 kinases) are also activated by RANKL; however, inhibition of ERK activity by an MEK inhibitor does not suppress osteoclastogenesis [86] but rather potentiates it [47], suggesting that the ERK pathway negatively regulates osteoclastogenesis. It has been suggested that there is competitive crosstalk between the p38 and ERK pathways [47]. ERK is also suggested to be involved in osteoclast survival [94]. Although mice with *JNK1/2/3* genes individually inactivated are viable and do not show obvious bone abnormalities, *in vitro* study has indicated that at least JNK1 is involved, albeit partially, in osteoclastogenesis [23]. More detailed *in vivo* analyses are needed to obtain conclusive evidence on the role of the MAPKs in osteoclastogenesis.

An essential and integral role of NFATc1

RANKL signaling stimulates the activation of NF-κB, AP-1 and MAPKs, but similar pathways are also activated by cytokines such as IL-1 that do not induce osteoclastogenesis. In a genome-wide search for the RANKL-inducible genes specifically required for the terminal differentiation of osteoclasts, NFATc1 was shown to be strongly induced by RANKL [52,122]. As mentioned above, NFATc1 expression is dependent on the TRAF6-NF-κB and c-Fos pathways which are activated by RANKL, suggesting an integral role of NFATc1 in RANKL signaling.

The NFAT transcription factor family was originally identified in T cells and is now comprised of five members including NFATc1 (NFAT2), NFATc2 (NFAT1), NFATc3 (NFAT4), NFATc4 (NFAT3) and NFAT5. This family of transcription factors developed along with the evolution of the vertebrate, and they are involved in the regulation of a variety of biological systems such as cardiovascular and muscular systems in addition to the immune system [17,45]. The activation of NFATc1/c2/c3/c4 is mediated by a specific phosphatase, calcineurin, which is activated by calcium/calmodulin signaling. Consistent with this, calcineurin inhibitors such as FK506 and cyclosporin A strongly inhibit osteoclastogenesis [52,122]. The necessary and sufficient role of *NFATc1* in osteoclastogenesis was suggested by the *in vitro* observation that *NFATc1*$^{-/-}$ embryonic stem cells do not differentiate into osteoclasts and the finding that ectopic expression of NFATc1 causes bone marrow-derived precursor cells to undergo osteoclast differentiation in the absence of RANKL [122].

However, *in vivo* analysis of *NFATc1*-deficient mice has been hampered by embryonic lethality [24,106]. To overcome this problem, two novel techniques were invented: (1) adoptive transfer of hematopoietic stem cells to osteoclast-deficient *c-Fos*$^{-/-}$ mice and (2) *c-Fos*-deficient blastocyst complementation (Fig. 1). Ultimately, these techniques made it possible to fully demonstrate the critical function of NFATc1 in osteoclastogenesis *in vivo* (Fig. 1 and [8]).

Autoamplification of NFATc1 and epigenetic regulation

Since NFATc1 and NFATc2 play a redundant role in the immune system (e.g., cytokine production from lymphocytes is not affected unless both genes are disrupted [104]), the question arises as to how NFATc1 plays such an exclusive function in

osteoclastogenesis. An interesting observation was obtained from rescue experiments using *NFATc1*$^{-/-}$ osteoclast precursor cells: osteoclast formation in *NFATc1*$^{-/-}$ cells was recovered not only by forced expression of NFATc1 but also by that of NFATc2 [8]. How can we reconcile the indispensable *in vivo* role of NFATc1 in osteoclastogenesis with the observation that *NFATc1* deficiency is compensated for by forced expression of NFATc2? We considered the following explanation: NFATc1 and NFATc2 proteins have a similar function to induce osteoclastogenesis if and when they are ectopically expressed at a similarly high level. Therefore, the essential role of NFATc1 is not achieved by the unique function of the protein, but also by an NFATc1-specific gene regulatory mechanism.

Accordingly, we analyzed the mRNA expression of *NFATc1* and *NFATc2* genes during osteoclastogenesis. The mRNA of *NFATc1* is induced selectively and potently by RANKL, while *NFATc2* mRNA is expressed constitutively in precursor cells at a low level [8,122]. Importantly, FK506, which suppresses the activity of NFAT through an inactivation of calcineurin, downregulates the induction of *NFATc1*, but not *NFATc2*. This suggests that *NFATc1* is selectively autoregulated by NFAT during osteoclastogenesis. As expected, ChIP experiments revealed that NFATc1 is recruited to the *NFATc1* but not the *NFATc2* promoter 24 h after RANKL stimulation, and the occupancy persists during the terminal differentiation of osteoclasts, indicating that the autoamplification mechanism by NFATc1 is specifically operative in the *NFATc1* promoter. (The *NFATc1* promoter is mainly occupied by NFATc2 until 1 h after RANKL stimulation, but this is not mentioned here in detail to keep the story simple. See below.)

Why does the autoamplification occur only in the case of *NFATc1* gene regulation? If NFAT binding sites are only found in the *NFATc1* promoter, the selective recruitment would be easily explained. However, NFAT binding sites are in fact found in both the *NFATc1* and the *NFATc2* promoters: the promoter sequence thus cannot be the answer. Histone acetylation is a marker of the transcriptionally active chromatin structure, and transcriptional coactivators such as CBP and PCAF have histone acetylase activity [3,25]. Investigation of the recruitment of CBP and PCAF to the *NFATc1* promoter yielded positive results. The rate of histone acetylation in the *NFATc1* promoter increased gradually after RANKL stimulation, and methylation of histone H3 lysine 4, which is characteristic of a transcriptionally active locus, is also upregulated exclusively in the *NFATc1* promoter, however, this was not observed in the *NFATc2* promoter [8]. Conversely, the *NFATc2* promoter is constantly associated with methylated DNA binding proteins, such as methyl-CpG binding protein 2 (MeCP2), suggesting that epigenetic modification of the *NFATc2* promoter is responsible for the muted pattern of gene expression [8,108]. Thus, contrasting epigenetic modification of the *NFATc1* and the *NFATc2* promoters might explain their unique spatiotemporal induction pattern during osteoclastogenesis. In conclusion, the essential role of the *NFATc1* gene is determined not only by the function of the encoded protein but also by an NFATc1-specific gene regulatory mechanism. It remains an issue to be pursued in future work to determine how such a specific epigenetic regulation is at work only in osteoclasts.

As mentioned above, NFATc2 is present prior to RANKL stimulation and is involved in the initial induction of *NFATc1* in cooperation with NF-κB. In addition, NFATc2 has the capacity to induce osteoclastogenesis when overexpressed [8,49]. However, the physiological role of NFATc2 in osteoclastogenesis seems to be limited to the initial induction of *NFATc1* and can be substituted possibly by other NFATs since *NFATc2*-deficient mice have no obvious defect in osteoclast differentiation [8].

Transcriptional control governed by NFATc1

What are the transcriptional targets of NFATc1? Accumulating evidence suggests that a number of osteoclast-specific genes are directly regulated by NFATc1. Based on promoter analyses, the *TRAP* [65,87,122], *calcitonin receptor* [4,65,87,122], *cathepsin K* [65,85] and *β3 integrin* genes [18] are regulated by NFATc1, although the critical binding sites are not fully identified. The osteoclast-specific immunoreceptor osteoclast-associated receptor (OSCAR) is also regulated by NFATc1 [62,65]. Promoter deletion and ChIP studies in addition to the analyses using knockout cells convincingly showed that the *OSCAR* promoter is the direct target of NFATc1. It is not understood how the target genes of NFATc1 promote the differentiation process, but recent reports have provided a clue to this question. DC-STAMP, a putative seven-transmembrane spanning protein, is essential for the cell–cell fusion of osteoclasts [74,140]. Its expression is rapidly induced in osteoclast precursor cells by RANKL, and forced expression of DC-STAMP induces the formation of TRAP-positive multinucleated cells. Since the RANKL-mediated induction of *NFATc1* in *DC-STAMP*$^{-/-}$ cells is normal [140], DC-STAMP may work downstream of NFATc1 to promote multinucleation, although further analyses are still needed.

The AP-1 complex is known to be a transcriptional partner of NFAT in lymphocytes, and crystal structure analysis revealed the formation of the NFAT:AP-1 complex to be crucial for DNA binding [15]. Likewise, an NFAT:AP-1 complex is important for the induction of the *TRAP* and *calcitonin receptor* genes as well as the robust autoamplification of NFATc1 [122]. It has also been shown that NFATc1 cooperates with PU.1 and MITF on the *cathepsin K* and the *OSCAR* promoters [65,85]. It is noteworthy that both PU.1 and MITF, which are thought to be important for the survival of osteoclast precursor cells, also participate in osteoclast-specific gene induction at the terminal stage of differentiation. Thus, NFATc1 forms an osteoclast-specific transcriptional complex containing AP-1 (Fos/Jun), PU.1 and MITF for the efficient induction of osteoclast-specific genes [8]. It should also be noted that the components of the NFATc1 complex are not always the same: the cooperation between NFATc1 and PU.1/MITF was not observed on the *calcitonin receptor* promoter, suggesting that the differential composition of the transcriptional complex may contribute to the spatiotemporal expression of each gene during osteoclastogenesis [65].

Taken together, the process of osteoclast differentiation can be divided into three stages in the context of transcriptional control by NFATc1, as shown in Fig. 4. (i) The binding of RANKL to RANK results in the recruitment of TRAF6, leading to the activation of downstream molecules such as NF-κB. NFATc2 is recruited to the *NFATc1* promoter at a very early phase. Cooperation of NFATc2 and NF-κB initiates the induction of *NFATc1*. (ii) Stimulated by calcium signaling, NFATc1 is activated and binds its own promoter. This leads to the robust induction of *NFATc1* (i.e., autoamplification). AP-1 (containing c-Fos) is critical for this autoamplification. RANKL induces selective recruitment of NFATc1 to the promoter of *NFATc1*, but not to that of *NFATc2*, which is explained by epigenetic regulation. (iii) A number of osteoclast-specific genes such as *cathepsin K*, *TRAP*, *calcitonin receptor* and *OSCAR* are activated by a transcriptional complex containing NFATc1 and cooperators such as AP-1, PU.1 and MITF.

Costimulatory signals for RANK

As cultured bone marrow cells differentiate into osteoclasts in response to recombinant RANKL and M-CSF, it has been widely accepted that the RANK and M-CSF receptor together transmit signals which are sufficient for osteoclastogenesis [11]. Recently, the screening of osteoclast-specific genes has shed light on a novel type of receptor. OSCAR is an immunoglobulin-like receptor expressed by osteoclasts and is involved in the cell–cell interaction between osteoblasts and osteoclasts [64]. It was subsequently shown that OSCAR associates with an adaptor molecule, FcRγ [69,93]. FcRγ harbors an immunoreceptor tyrosine-based activation motif (ITAM), which is critical for the activation of calcium signaling in immune cells [107]. Another ITAM-harboring adaptor, DAP12, has been reported to be involved in the formation and function of osteoclasts [56].

Notably, mice doubly deficient in FcRγ and DAP12 exhibit severe osteopetrosis owing to the differentiation blockade of osteoclasts, demonstrating that the immunoglobulin-like receptors associated with FcRγ and DAP12 are essential for osteoclastogenesis [69,96]. These receptors include OSCAR, triggering receptor expressed in myeloid cells (TREM)-2, signal-regulatory protein (SIRP) β1, and paired immunoglobulin-like receptor (PIR)-A, although the ligands and exact function of each of these receptors remain to be elucidated. ITAM-mediated signals cooperate with RANK to stimulate calcium signaling through ITAM phosphorylation and the resulting activation of Syk and PLCγ. Therefore, these signals should properly be called costimulatory signals for RANK (Fig. 5). Initially, characterized in natural killer and myeloid cells, the immunoglobulin-like receptors associated with FcRγ or DAP12 are thus identified as unexpected but essential partners of RANK during osteoclastogenesis [9,69,121]. It is not fully understood how RANK can specifically induce osteoclastogenesis in cooperation with ITAM signaling, but it is partly explained by the observation that phosphorylation of ITAM is upregulated by RANKL [69]. In addition, RANKL stimulation results in an increased expression of immuno-

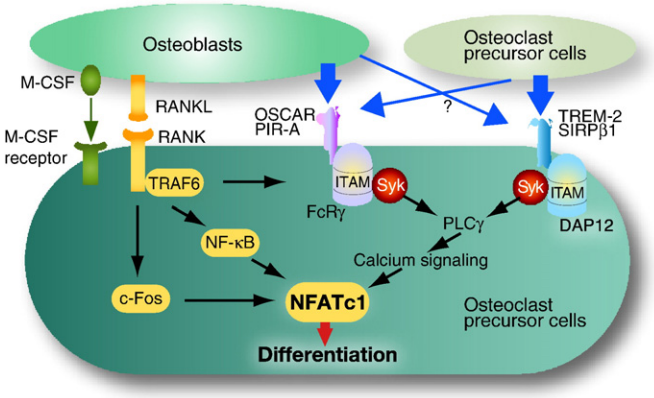

Fig. 5. Cooperation of RANKL and immunoreceptor tyrosine-based activation motif (ITAM) signals in osteoclastogenesis. Adapter proteins such as Fc receptor common γ subunit (FcRγ) and DNAX-activating protein 12 (DAP12) associate with immunoglobulin-like receptors including osteoclast-associated receptor (OSCAR), paired immunoglobulin-like receptor (PIR)-A, triggering receptor expressed on myeloid cells (TREM)-2 and signal-regulatory protein (SIRP) β1 via complementarily charged amino acid residues in the transmembrane domains of each protein. The RANKL–RANK signaling and the stimulation of the immunoglobulin-like receptors cooperatively phosphorylate ITAM. Then, the Syk family kinase is recruited to the phosphorylated tyrosine residues in ITAM via its SH2 domain. This leads to the activation of phospholipase Cγ (PLCγ) and calcium signaling, which is critical for *NFATc1* induction and activation. *NFATc1* induction is also dependent on c-Fos and TRAF6, both of which are activated by RANKL.

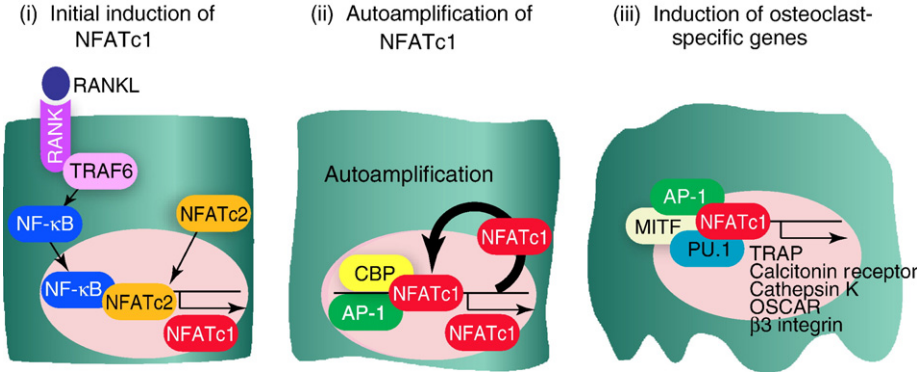

Fig. 4. A schematic diagram of the three stages of osteoclast differentiation governed by NFATc1 (see the text for details).

receptors such as OSCAR, thereby augmenting the ITAM signal [62,65]. It is also conceivable that RANK activates an as yet unknown pathway that specifically synergizes with or upregulates ITAM signaling, but further studies are necessary to better understand the specificity of RANKL.

It remains unknown whether inhibitory type receptors containing immunoreceptor tyrosine-based inhibitory motif (ITIM) contribute to negative regulation of the ITAM signal in osteoclast differentiation. However, osteoclastogenesis is reported to be enhanced in mice lacking phosphatases, such as Src homology (SH)-2-containing protein tyrosine phosphatase (SHP)-1 [5,129] or SH-2-containing inositol 5′-phosphatase (SHIP)-1 [124], which counterbalance the ITAM signal in the immune system.

The importance of the ITAM-harboring adaptors and the receptors associated with them in bone metabolism is also underscored by reports that mutations in the genes *DAP12* and *TREM-2* cause skeletal and psychotic abnormalities known as Nasu-Hakola disease [101,103]. It is reported that osteoclast differentiation and function are impaired in human cells with mutations in *DAP12* or *TREM-2* [13,102]. Although it is necessary to exercise great caution in linking the phenotype of mouse models and the clinical manifestation in humans, the therapeutic efficacy of the blockade of RANKL signaling has already been reported in human trials [89], indicating that the mouse genetic studies do indeed provide an important molecular basis for novel therapeutic strategies.

Other signaling molecules

Considering the importance of TRAF6 and calcium signaling, there are other candidate molecules related to these pathways and involved in osteoclastogenesis. For example, protein kinase C (PKC) is known to be activated by calcium signaling. It has been shown that the aPKC-interacting protein p62 is upregulated and binds to TRAF6 during osteoclastogenesis [28]. Null mutation of *p62* in osteoclast precursor cells causes severe impairment of osteoclast formation in a culture system. However, in contrast to the *in vitro* phenotype, *p62*-deficient mice exhibit no defects unless they are challenged by osteoclastogenic stimuli such as PTH-related peptide (PTHrP) [28]. On the other hand, *p62* mutation in human has been shown to be associated with 5q35-linked Paget's disease of bone (PDB), a genetic disorder characterized by focal and disorganized increase of bone turnover [77]. In the 5q35-linked PDB patients, mutant *p62* proteins which lack the ability to bind mono-ubiquitin, as well as K48-linked polyubiquitin chains, are expressed [12].

c-Src, which is required for osteoclastic bone resorption [84,115], has also been shown to bind TRAF6 [137], suggesting that c-Src may be involved in the differentiation of osteoclasts. However, it is well documented that there is no differentiation defect in *c-Src*$^{-/-}$ mice, indicating that the loss of c-Src is compensated for by other molecules during differentiation but not in bone resorption. Downstream of c-Src, the lipid kinase phosphatidylinositol (PI) 3-OH kinase (PI3K) and phosphoinositide-dependent protein kinase 1 (PDK1), activates Akt/PKB [130,137]. The role of Akt in osteoclastogenesis was suggested by a small interfering (si)RNA-mediated knockdown approach [117], and a potent PI3 kinase inhibitor, wortmannin, suppressed osteoclastic bone resorption *in vitro*, possibly through inhibition of ruffled border formation and reduced osteoclast survival [36,98], but further studies will be necessary to provide conclusive evidence for the *in vivo* function. The functions of mammalian target of rapamycin (mTOR), another downstream molecule of PI3K, are also controversial and the subject of active investigation [113,117].

Conclusion and future directions

After the discovery of RANKL, understanding of the mechanisms of osteoclast differentiation, activation and survival has been advanced considerably. Despite this progress, however, several important questions remain to be answered. Why does only RANK induce osteoclastogenesis? Why does NFATc1 autoamplify at such a high efficiency only in osteoclasts? What is the target molecule of NFATc1 that orchestrates the late phase of the differentiation process? Some researchers have expressed the opinion that the discovery of RANKL had brought about or at least signaled the end of osteoclast study, but in fact it has turned out to be a beginning of a new period of interesting and intense investigation. Furthermore, since the RANKL-induced osteoclast differentiation system is a very sophisticated culture system unusually suited to molecular analysis, it is possible that this system will also provide insights into the general mechanism of cell lineage commitment.

The efficacy of anti-RANKL antibody has been reported in several preliminary human trials, and its therapeutic utility will be eventually a reality in the clinic [89,112]. If we consider the limitations of this type of biological agent (e.g., the high cost, emergence of neutralizing antibodies and injection procedure), compounds with similar effects but reduced limitations are likely to be a matter of intense pursuit. A detailed molecular understanding of osteoclast differentiation is essential for the design of novel drugs that target osteoclastic bone resorption.

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