REVIEW

Recent advances in osteoclast biology

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Abstract

The bone is an essential organ for locomotion and protection of the body, as well as hematopoiesis and mineral homeostasis. In order to exert these functions throughout life, bone tissue undergoes a repeating cycle of osteoclastic bone resorption and osteoblastic bone formation. The osteoclast is a large, multinucleated cell that is differentiated from monocyte/macrophage lineage cells by macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL). RANKL transduces its signal through the signaling receptor, RANK. RANKL/RANK signaling activates NFATc1, the master regulator of osteoclastogenesis, to induce osteoclastogenic gene expression. Many types of cells express RANKL to support osteoclastogenesis depending on the biological context and the dysregulation of RANKL signaling leads to bone diseases such as osteoporosis and osteopetrosis. This review outlines the findings on osteoclast and RANKL/RANK signaling that have accumulated to date.

Keywords Osteoclast · RANKL · RANK · Bone diseases

Introduction

Bone is a unique organ in vertebrates acquired during the course of evolution. Its development is considered to be related to the migration of animals out of the sea onto land. Terrestrial animals need to withstand the more powerful effects of gravity on the ground. Moreover, they need to store minerals, which aquatic animals take up from sea water. Bone tissue consisting of hydroxyapatite has evolved in terrestrial animals to meet these demands (Ahlberg et al. 2005, 2008).

Bone tissue undergoes a continuous cycle of osteoclastic bone resorption and osteoblastic bone formation in order to be able to function properly over a lifetime. The balance between bone resorption and formation is influenced by mechanical loading on the musculoskeletal system, as well as interaction with other biological systems such as the endocrine, nervous and immune systems (Okamoto et al. 2017). With the integrative activity of this complex regulatory machinery, the shape, volume and density of the bone are maintained. There is vigorous bone formation in the growth period to increase the body size (Bassett and Williams 2016). In response to mechanical demands, bone remodeling tips toward formation, whereas unloading leads to decreased bone mineral density (Turner et al. 2009). In case of bone fracture healing, osteoblasts synthesize bone matrix for the fixation of the bone fragments, whereas osteoclasts resorb small bone fragments in the period just after fracture and immature bone matrix in the late phase of the healing (Einhorn and Gerstenfeld 2015; Ono and Takayanagi 2017).

The osteoclast is a large, multinucleated cell with an ability to degrade bone tissue by secreting H^+, Cl^-, cathepsin K (CtsK) and matrix metalloproteinases (MMPs) in the resorption area. These cells differentiate from monocyte/macrophage lineage cells. M-CSF stimulation of myeloid cells expressing the corresponding receptor, c-Fms, supports the survival of these progenitors and induces the expression of RANK, the essential receptor for osteoclastogenesis (Edwards and Mundy 2011). Stimulation of this receptor

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by RANKL drives the downstream signal, leading to osteoclastogenesis. After the discovery of RANKL and NFATc1, numerous studies on the pathway of RANKL/RANK signaling have been carried out and revealed the complex mechanisms underlying osteoclastogenesis (Edwards and Mundy 2011; Okamoto et al. 2017).

In this review, we outline the accumulated findings on the mechanism underlying the differentiation and function of osteoclasts, with a particular focus on RANKL/RANK signaling.

Osteoclast differentiation from progenitor cells

All hematopoietic lineage cells, including osteoclasts, originate from hematopoietic stem cells (HSCs) in the bone marrow. HSCs undergo self-renewal and differentiation into each hematopoietic cell type. HSCs do not directly become mature cells; instead, they give rise to several types of oligopotent progenitor cells that further develop into lineage-restricted progenitor cells, forming a hierarchical differentiation tree (Seita and Weissman 2010) (summarized in Fig. 1). HSCs lose their self-renewal capacity and become multipotent progenitors (MPPs), which still retain pluripotency. MPPs differentiate into oligopotent progenitors, common myeloid progenitors (CMPs), megakaryocyte–erythrocyte progenitors (MEPs) and common lymphoid progenitors (CLPs). Each stage of hematopoietic cell differentiation has recently been defined by the expression patterns of cell surface markers: HSCs, $Lin^-Sca-1^+c-Kit^+CD34^-$; MPPs, $Lin^-Sca-1^+c-Kit^+CD34^+$; CMPs, $Lin^-Sca-1^{lo/-}c-Kit^+CD34^+IL-7R^-Fc\gamma R^{lo}$; MEPs, $Lin^-Sca-1^-c-Kit^+CD34^-IL-7R^-Fc\gamma R^-$; and CLPs, $Lin^-Sca-1^{lo}c-Kit^{lo}IL-7R^+CD27^+Flk2^+$ (Seita and Weissman 2010) (Fig. 1).

Osteoclast progenitor cells originate from CMPs. It was reported in an early study that the $c-Kit^+CD11b^{lo/-}$ population in the bone marrow yields osteoclasts (Arai et al. 1999). The study also showed that $c-Kit^+CD11b^{lo/-}c-Fms^+$ cells

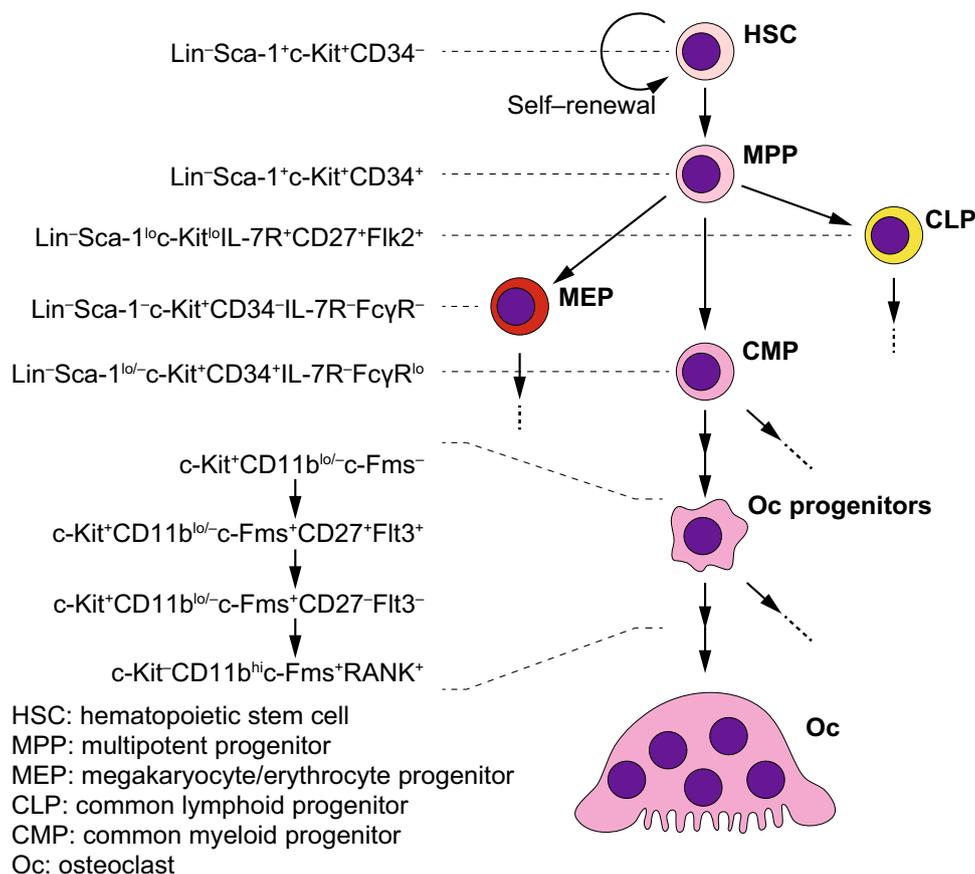Fig. 1 Osteoclast progenitor cells. $Lin^-Sca-1^+c-Kit^+CD34^-$ hematopoietic stem cells (HSCs) have a capacity for self-renewal and pluripotent ability to differentiate into all hematopoietic cell types. HSCs lose the self-renewal capacity upon differentiation and give rise to oligopotent progenitor cells. Among these progenitors, common myeloid progenitor cells (CMPs) are

the origin of osteoclasts. During osteoclastogenesis, osteoclast progenitors express c-Fms and RANK, receptors for M-CSF and RANKL, respectively. Stimulation by these cytokines generates osteoclasts

differentiate into macrophage-lineage cells more frequently than the c-Fms⁻ counterpart that has the capacity to produce granulocytes and erythrocytes as well, indicating that c-Fms expression specifies the fate of progenitor cells towards monocyte/macrophage-lineage cells.

c-Kit⁺CD11b$^{lo/-}$c-Fms⁺ cells can be divided according to the expression of CD27 and Flt3. The CD27⁺Flt3⁺ subset was shown to be a common progenitor of macrophages, dendritic cells (DCs) and osteoclasts, whereas CD27⁻Flt3⁻ progenitors differentiate into macrophages and osteoclasts, but not DCs (Xiao et al. 2017, 2013). Stimulation of c-Kit⁺CD11b$^{lo/-}$c-Fms⁺ cells by M-CSF induces RNAK on these cells, and RANK⁺ cells lose c-Kit expression (Arai et al. 1999). Together, these findings indicate that osteoclast progenitors undergo a stepwise differentiation and finally become c-Fms⁺RANK⁺ progenitors, which are ready for RANKL stimulation.

Some studies have reported the transdifferentiation of immature DCs into osteoclasts. These studies have also pointed out that the transdifferentiation was promoted under inflammatory conditions: T cell stimulation by bacterial componetns (Alnaeeli et al. 2006), IL-1β (Speziani et al. 2007), or tumor necrosis factor (TNF)-α (Speziani et al. 2007). These results suggest that immature DCs may play a role as osteoclast progenitors in inflammatory bone loss, but the physiological relevance of DC-derived osteoclasts at present is not fully understood.

RANKL: the cytokine essential for osteoclastogenesis

Discovery of RANKL

Although osteoclast progenitors, monocytes and macrophages, are found in most body tissues, osteoclasts are found only in bone. Therefore, it was speculated that cells specifically existing in the bone tissue would support osteoclastogenesis (Rodan and Martin 1981). Co-culture experiments of spleen cells and osteoblastic cells were conducted and successfully yielded osteoclasts, suggesting that osteoblasts express an osteoclast differentiation factor (ODF) on their cellular membrane (Suda et al. 1999).

As a result of the quest to identify the binding partners for a factor inhibiting osteoclastogenesis (osteoprotegerin: OPG or osteoclastogenesis inhibitory factor: OCIF), ODF and OPG ligand (OPGL) were found (Lacey et al. 1998; Simonet et al. 1997; Tsuda et al. 1997; Yasuda et al. 1998). ODF and OPGL subsequently turned out to be identical to the molecules reported as RANKL and TRANCE (Anderson et al. 1997; Fuller et al. 1998). The essential signaling receptor for this cytokine was reported as ODF receptor (ODFR), which was found to be identical to RANK (Nakagawa et al.

1998). OPG/OCIF appeared to serve as a decoy receptor for RANKL. Today, these cytokine, signaling receptor and decoy receptor are called RANKL (encoded by *Tnfsf11*), RANK (encoded by *Tnfrsf11a*) and OPG (encoded by *Tnfrsf11b*), respectively.

The structures of RANKL/RANK/OPG

RANKL is a type II transmembrane protein of the TNF superfamily, with its N terminus constituting the intracellular region and C terminus containing the receptor-binding domain (Nelson et al. 2012). Like most TNF-family cytokines, RANKL forms a homotrimer by hydrogen bond and hydrophobic interactions, the former of which is thought to be requisite for the binding selectivity and the latter for the association of the monomers (Lam et al. 2001). An intersubunit cleft that forms between the subunits functions as the receptor-binding site (Fig. 2) (Liu et al. 2010), the occupation of which using a TNF receptor loop peptide mimic leads to the inhibition of osteoclastogenesis and bone resorption (Aoki et al. 2006; Ozaki et al. 2017).

The RANKL expressed on the cell surface can be cleaved by MMPs, generating a soluble form (Hikita et al. 2006; Lum et al. 1999). The soluble form was reported to retain cytokine activity, which is enhanced by polymerization (Lum et al. 1999; Nakashima et al. 2000). Inhibition of RNAKL shedding by the knockdown of *Mmp14* in an osteoblast–osteoclast co-culture system resulted in increased osteoclastogenesis, indicating that membrane-bound RANKL is more effective than its soluble form (Hikita et al. 2006). To elucidate the physiological relevance of the membrane-bound and soluble forms of RANKL, a genetically modified mouse strain was recently generated in which the RANKL cleavage site is trimmed and the soluble form cannot be produced (the *Tnfsf11*$^{ΔS/ΔS}$ mouse) (Nagashima et al. 2017). Using these mice, the soluble form of RANKL was shown to be dispensable for the regulation of micro-fold cells (M cells), cells that transport antigens in the intestine to immune cells (Nagashima et al. 2017). The *Tnfsf11*$^{ΔS/ΔS}$ mouse and its counterpart, a mouse unable to produce membrane-bound RANKL (if generated), would be promising tools for the elucidation of the roles of the two types of RANKL in osteoclast differentiation, bone development and bone disease.

The signaling receptor of RNAKL, RANK, is a type I transmembrane protein and belongs to the TNF receptor (TNFR) superfamily of molecules, all of which consist of four cysteine-rich domains (CRDs). Of these, the middle two domains can directly bind to the corresponding ligands. As is for most of the other TNFR molecules, RANK rotates around the hinge region between CRD2 and CRD3, which contributes to the close contact to RANKL (Liu et al. 2010). Upon binding of RANKL, RANK
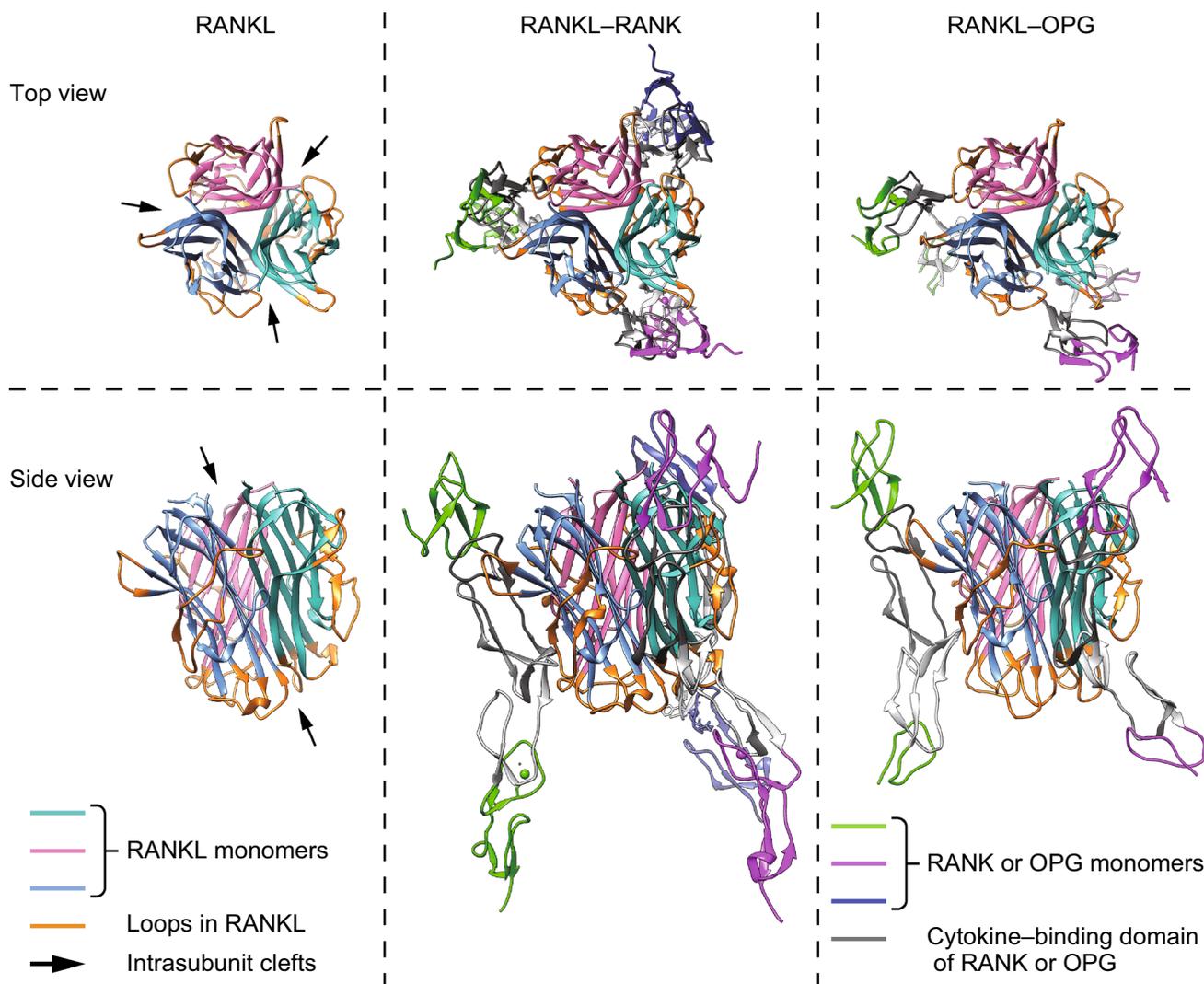
Fig. 2 Molecular structures of RANKL/RANK/OPG. Molecular structure images of the RANKL trimer (left), RANKL–RANK complex (center) and RANKL–OPG complex (right). The pictures in the top row are top view images and ones in the bottom row are side view images. Polymerization of the RANKL monomers forms a cleft between the monomers (arrows). The cleft and adjacent loops (orange lines) interact with the cytokine-binding domains of RANK or OPG (gray lines). RANKL and RANK bind with a stoichiometry of 3:3, whereas RANKL and OPG bind with a stoichiometry of 3:2. The structure files for the RANKL trimer, RANKL–RANK complex and RANKL–OPG complex were obtained from the RCSB PDB website repository (http://www.rcsb.org/pdb/home/home.do) (Lam et al. 2001; Liu et al. 2010; Nelson et al. 2012). Molecular graphics were composed using the UCSF Chimera package (Pettersen et al. 2004)

trimerizes and transduces the signal via adaptor molecules. The RANKL signaling pathway is discussed in the latter part of this manuscript.

The decoy receptor for RANKL, OPG, belongs to the TNFR superfamily as well. It contains four CRDs and bears a hinge region between CRD2 and CRD3 in order to bind to RANKL. Because of conformational difference from RANK in the hinge region, OPG rotates more than RANK and binds to RANKL in a different manner (Fig. 2). Such differences are considered to result in the higher affinity of OPG to RANKL than RANK.

The RANKL/RANK/OPG system in bone

Studies on genetically modified mice have demonstrated the essentiality of RANKL/RANK signaling in bone metabolism. *Tnfsf11*$^{-/-}$ mice, *Tnfrsf11a*$^{-/-}$ mice and mice overexpressing the rat *Tnfrsf11b* gene exhibit severe osteopetrosis (Dougall et al. 1999; Kong et al. 1999; Li et al. 2000; Min et al. 2000). In contrast, *Tnfrsf11b*$^{-/-}$ mice develop osteoporosis (Bucay et al. 1998; Mizuno et al. 1998). The bone phenotypes of these mice clearly demonstrate the significance of RANKL signaling in osteoclastogenesis. In human patients

with bone diseases, mutations in RANKL, RANK and OPG have all been reported. Familial expansile osteolysis (FEO), expansile skeletal hyperphosphatasia (ESH) and the familial form of early-onset Paget's disease of bone (PDB2) are rare bone diseases with constitutive activation of RANK owing to in-frame duplication of exon 1. Juvenile Paget's disease (JPD) is another type of Paget's disease that arises because of mutations in OPG (Walsh and Choi 2014).

FEO is an autosomal dominant disease characterized by deafness and loss of adult dentition in early life due to excessive bone resorption and resulting deformity. From the late juvenile period, osteolytic lesions appear in the tibia, ulna, humerus and femur, with bone thinning, pain and fracture (Whyte and Mumm 2004; Wright et al. 2009). Constitutive activation of RANK in FEO is caused by the mutations 84dup18 and 83dup18.

ESH is an autosomal dominant disease of accelerated bone remodeling, the patients of which suffer deafness, tooth loss, hypercalcemia and widening of the long bones accompanied by pain (Whyte and Mumm 2004; Wright et al. 2009). ESH is caused by a *TNFRSF11A* mutation, which is similar to that found in FEO (84dup15). However, unlike FEO, the long bones affected by ESH exhibit hyperostosis.

PDB is characterized by progressive osteoclast-mediated osteolysis and the subsequent excessive bone formation that lead to changes in the overall appearance of the bone (Whyte and Mumm 2004; Winn et al. 2017). Despite the fact that the causes of PDB and FEO both lie in the RANKL/RANK/OPG system, the affected bone is different; the skull, spine, pelvis and femur are the common sites affected in PDB (Winn et al. 2017; Wright et al. 2009). Bone deformity leads to fracture, osteoarthritis, nerve entrapment and pain. It is also reported that Pagetic bone frequently undergoes tumor transformation. PDB2 is caused by a mutation in *TNFRSF11A* (75dup27), while JPD is caused by mutations in *TNFRSF11B* (D182del, C65R, C87Y, F117L, V199RfsX5 and D323SfsX3).

Osteopetrosis (Albers–Schönberg disease) is another osteoclast-related bone disease characterized by an increased bone mass owing to dysfunction or loss of osteoclasts. The bone marrow in these patients is occupied by bone tissue, resulting in impaired hematopoiesis, which in turn leads to recurrent infections, anemia and hemorrhage. The affected bones easily undergo fracture. Nerves can be compressed by proliferating bone tissue, leading to blindness and deafness (Coudert et al. 2015). According to the inheritance pattern and the clinical features, osteopetrosis is divided into groups: adult-onset autosomal dominant osteopetrosis (ADO)1; ADO2; intermediate autosomal osteopetrosis (IAO); and autosomal recessive osteopetrosis (ARO). Mutations in the molecules related to osteoclast function are known to result in the differing types of osteopetrosis (LDL receptor-related protein 5: *LRP5*, ATPase A3 subunit: *TCIRG1*, chloride voltage-gated channel 7: *CLCN7*, osteoporosis-associated transmembrane protein 1: *OSTM1*, pleckstrin homology domain containing, family M member 1: *PLEKHM1*, sorting nexin 10: *SNX10* and carbonic anhydrase II: *CA2*) (Chen et al. 2016; Cleiren et al. 2001; Coudert et al. 2015; Sly et al. 1983). Several different mutations in *TNFRSF11A* and *TNFSF11* (A145delS177, M199K and V277WfsX5), in which the trimerization of RANKL is hampered, have been reported as the causes of ARO (Wright et al. 2009). These together indicate the clinical significance of the RANKL/RANK/OPG system for bone metabolism.

RANKL-independent osteoclastogenesis

The studies introduced above have clearly established the concept that RANKL is an indispensable and exclusive cytokine for osteoclastogenesis. However, a controversy over its exclusivity arose soon after the concept was put forward: factors other than RANKL [TNF-α, A proliferation-inducing ligand (April), B cell activating factor belonging to the tumor necrosis factor family (BAFF), nerve growth factor (NGF), Insulin-like growth factor (IGF-1), IGF-2, lymphotoxin-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator on T cells (LIGHT), transforming growth factor (TGF)-β, IL-6, IL-11, secreted osteoclastogenic factor of activated T cells (SOFAT) and ROS generated by lysyl oxidase (LOX)] were reported to induce osteoclastogenesis (Tanaka 2017). Because these factors are not always reported to induce osteoclastogenesis in the absence of RANKL, the controversy has yet to be brought to a close.

The inconsistency is derived, in part, from experimental difficulties. In most of the culture experiments, cells form murine bone marrow or human peripheral blood were used as the osteoclast progenitors. These cells contain not only osteoclast progenitors but also mesenchymal cells that can express RANKL, thus stimulating osteoclast progenitors. Cell sorting based on antigen–antibody reaction can be an effective means of collecting a "pure" population of osteoclast progenitors. However, the purity of the sorted cells is largely dependent on the affinity and the specificity of the antibodies employed. It is reported that anti-human CD14 antibodies, which are supposed to enrich myeloid cells, bind to mesenchymal cells owing to cross-reactivity (Pilz et al. 2011). The use of such antibodies for collecting osteoclast progenitors results in the contamination of mesenchymal cells that express RANKL, resulting in an ambiguity over whether the factors above induce osteoclastogenesis independently on RANKL (Cox et al. 2015; Tsukasaki et al. 2017).

Thus, in the studies on RANKL-independency, the use of RANK-deficient progenitor cells and the purification of
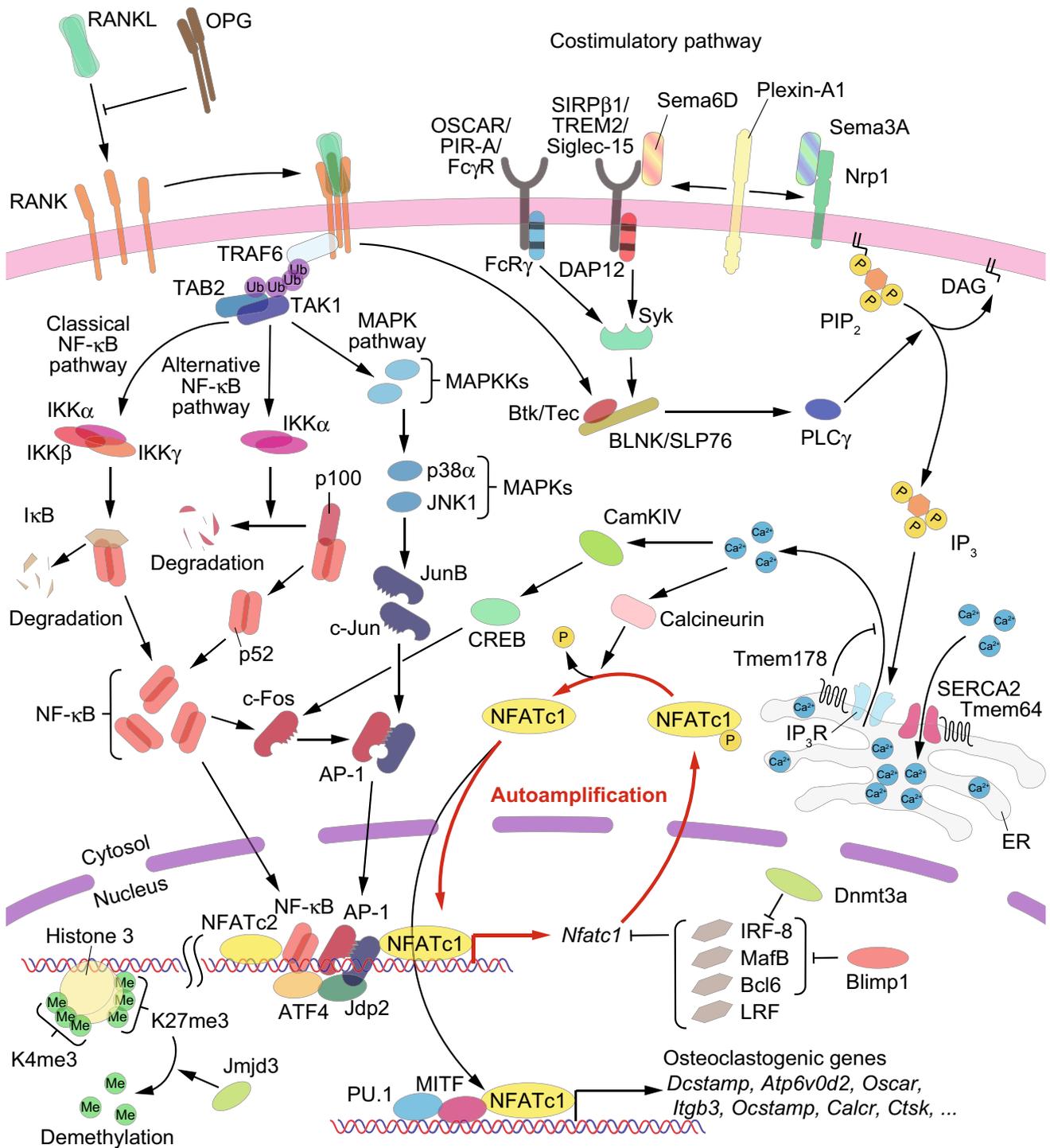
progenitor cells with a truly reliable strategy are absolutely required. Reports have shown that *Tnfrsf11a*$^{-/-}$ osteoclast progenitor cells are able to differentiate into osteoclasts via TNF-α stimulation under conditions in which progenitors are deficient in the transcription factor RBP-J or co-stimulated by TGF-β (Kim et al. 2005; Zhao et al. 2012). It has been also shown that a local injection of TNF-α induced osteoclastogenesis in the calvariae of *Tnfrsf11a*$^{-/-}$ mice, but the resulting osteoclasts were not functional (Li et al. 2000; Zhao et al. 2012). These together suggest that TNF-α as well as other cytokines may be able to induce osteoclastogenesis under certain conditions, but cannot completely substitute for RANKL.

◀**Fig. 3** Signal cascade in the process of osteoclastogenesis. RANKL and its receptor RANK transduce a signal via the adaptor molecule TRAF6. TRAF6 recruits TAB2 and TAK1, which in turn activates the NF-κB pathway and MAPK pathway. NF-κB induces c-Fos expression via IKKs. Activation of MAPK pathway results in the activation of the Jun proteins. The c-Fos and Jun proteins associate to form the complex AP-1. The expression of the master regulator of osteoclastogenesis, NFATc1, is driven by AP-1, NF-κB, ATF4, Jdp2, NFATc2 and NFATc1 itself. NFATc1 expression is also enhanced by the demethylation of the NFATc1 promoter by Jmjd3. The activation of NFATc1 is regulated by a co-stimulatory signal pathway. FcRγ, DAP12 and their associating molecules activate Syk, which forms a complex with Btk/Tec and BLNK/SLP76. This complex further activates PLCγ, resulting in calcium signaling. The influx and efflux of Ca^{2+} is regulated by Ca^{2+} channels, Tmem64 and Tmem178. The calcium signaling activates calcineurin, which dephosphorylates NFATc1, promoting its entry into the nucleus. Activated NFATc1 promotes its own expression making a autoamplification loop. The calcium signaling also induces c-Fos expression via CAMKIV and CREB. NFATc1, together with other factors including PU.1 and MITF, promotes the expression of osteoclastogenic genes. The expression of *Nfatc1* is negatively regulated by LRF, Bcl6, MafB and IRF-8, some of which are inhibited by Blimp1 and Dnmt3a

Osteoclastogenesis-supporting cells: sources of RANKL in the skeletal system

As described above, osteoblasts were found to support osteoclastogenesis, which has become a dogma ever since (Takahashi et al. 1988; Udagawa et al. 1989). However, some reports have suggested that osteoblasts do not exert effects on osteoclasts as supposed because there is no decrease in the osteoclast number in mouse models in which the osteoblast number or function have declined (Corral et al. 1998; Ogata et al. 2007; Weinstein et al. 2002). These reports have led to a rethinking of the dogma.

RANKL is expressed by several types of cells in bone (osteoblasts, osteocytes, immune cells, etc.), but the expression is higher in bone tissue (osteoblasts and osteocytes) than other cells (Nakashima et al. 2011). Purified osteocytes were shown to more vigorously express RANKL and support osteoclastogenesis than osteoblasts, suggesting that osteocytes have a crucial role in bone remodeling (Nakashima et al. 2011). To investigate this, several lines of genetically modified mice, in which *Tnfsf11* was deleted in specific cell types in the bone, were examined (Nakashima et al. 2011; Xiong et al. 2011, 2015). Analyses of neonatal or young mice in their growth period revealed that hypertrophic chondrocytes in the growth plate and osteoblasts, which are targeted by *Prrx1-Cre, Sp7-Cre, BGLAP-Cre, Dmp1-Cre* or *Col10a1-Cre*, but not osteocytes, serve as the source of RANKL in bone metabolism (Nakashima et al. 2011; Xiong et al. 2011). In older mice, on the other hand, osteoblasts do not contribute to RANKL expression; instead, osteocytes do. The long bones and spine become osteopetrotic with impaired osteoclastic bone resorption in *Tnfsf11*[f/f]*Dmp1-Cre* mice, *Tnfsf11*[f/Δ]*Dmp1-Cre* mice and *Tnfsf11*[f/f]*Sost-Cre* mice,

but not in *Tnfsf11*[f/f]*Sp7-Cre* mice (Nakashima et al. 2011; Xiong et al. 2011, 2015). Thus, it is indicated that osteocytes contribute to osteoclastogenesis in the adult, not in the developmental phase of the life course.

Osteocytes regulate bone remodeling more than only in the steady state. They have also been shown to be responsible for unloading-induced bone resorption and alveolar bone remodeling in orthodontic tooth movement (Shoji-Matsunaga et al. 2017; Xiong et al. 2011), indicating that osteocytes are involved in mechanical stress-induced bone remodeling. This seems reasonable because osteocytes are recognized to be mechanosensitive cells in bone. The osteocytes embedded in the bone lacunae are connected to other lacunae by bone canaliculi, within which osteocytes extend their dendrites. The bone lacunae and canaliculi are filled with tissue fluid and the osteocytes are anchored in the spaces by adhesive molecules. Upon mechanical loading, there occurs not only a structural deformation of bone matrix, but also a streaming of tissue fluid in the lacuno-canalicular system (Bonewald 2014). These stimuli are thought to be sensed by several different cellular components, such as the primary cilia, integrins, cytoskeletal proteins and ion channels, as a result inducing mechano-responsive gene activation (Nguyen and Jacobs 2013; Takano-Yamamoto 2014). However, the precise mechanism underlying the osteocyte response is largely unknown. Further studies are required to properly understand mechanical stress-induced bone remodeling.

RANKL signaling in osteoclast differentiation

The indispensability of RANKL in osteoclastogenesis has prompted scientists and clinicians to study the RANKL signaling pathway in terms of elucidating the whole picture of osteoclastogenesis and establishing the molecular basis for therapeutic strategies in bone disease. The RANKL signaling pathway is summarized in Fig. 3.

Adaptor molecules for RANK

RANK adopts a trimetric conformation upon binding to RANKL. The intracellular domain of the RANK trimer is thought to lack signaling domains, but instead, for signal transduction, recruits adaptor molecules: TNF receptor-associated factors (TRAFs), Grb-2-associated binder (Gab) 2 and four and a half LIM domain (FHL) 2 (Bai et al. 2005; Lamothe et al. 2007; Naito et al. 1999; Wada et al. 2005). Among TRAF1/2/3/5/6, which bind to RANK, TRAF6 is essential for osteoclastogenesis, as shown by the analyses of two lines of *Traf6*^{−/−} mice, which exhibit severe osteopetrosis (Lomaga et al. 1999; Naito et al. 1999). The distinctive role of TRAF6 may be due to the lowest C-terminal homology among the TRAF family molecules (Lomaga et al. 1999).

TRAF6 contains an amino-terminal ring finger domain, which has ubiquitin-ligase activity for ubiquitinating TRAF6 itself (Lamothe et al. 2007). This ubiquitination does not serve as a signal for protein degradation, but as a scaffold for TGF-β-activated kinase (TAK) 1-binding protein (TAB) 2 and TAK1(Mizukami et al. 2002). A defect in the kinase activity of TAK1 in osteoclasts has been shown to lead to an impairment in osteoclastic bone resorption (Lamothe et al. 2013; Sumiya et al. 2015). In osteoclastogenesis, TAK1 activates inhibitory κB (IκB) kinases (IKKs) and mitogen-activated protein kinases (MAPKs) for further signaling.

NF-κB signaling in osteoclastogenesis

NF-κB is one of the key transcription factors. It is composed of dimeric transcription molecules: Rel proteins (RelA, RelB and c-Rel), NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100). This transcription factor is inactive in the cytosol in the steady state and translocates to the nucleus upon activation, which is dependent on the classical or the alternative pathways (Ghosh and Karin 2002). In the classical pathway, the IKK complex that consists of IKKα, IKKβ and IKKγ (also known as NF-κB essential modulator: NEMO) phosphorylates IκBs, the inactivators of NF-κB. The phosphorylation of the IκB molecules leads to their ubiquitination and subsequent degradation, freeing NF-κB. In the alternative pathway, the homodimer of IKKα cleaves p100 to generate p52. Activated NF-κB dimers induce c-Fos and nuclear factor of activated T cell c1 (NFATc1) expression (Yamashita et al. 2007). (The functions of c-Fos and NFATc1 are described below.) NF-κB has been shown to be essential for osteoclastogenesis, as $Nfkb1^{-/-}Nfkb2^{-/-}$ mice (mice deficient in both p50 and p52) develop osteopetrosis (Franzoso et al. 1997). Likewise, it is reported that $Ikbkb^{f/f}Mx1$-Cre mice, which are deficient in IKKβ in osteoclast progenitors, display an osteopetrotic phenotype, indicating the significance of IKK–NF-κB signaling in osteoclastogenesis (Ruocco et al. 2005).

MAPK signaling in osteoclastogenesis

TAK1 serves as a MAPK kinase kinase (MAPKKK) to phosphorylate the MAPK kinases (MAPKKs) that further phosphorylate the MAPKs, including p38 and c-Jun N-terminal kinase (JNK). Among the MAPKKs, MKK3 was shown to be involved in p38 phosphorylation during osteoclastogenesis (Boyle et al. 2014). The bones of $Map2k3^{-/-}$ mice, which are deficient in MKK3, are osteopetrotic with a tendency towards reduced osteoclasts (Boyle et al. 2014). p38 consists of four subtypes: p38α, p38β, p38γ and p38δ. p38α is most abundantly expressed in osteoclasts and the knockdown of this signaling molecule results in impaired osteoclastogenesis (Bohm et al. 2009). Analyses of younger aged

mice in which p38 is deleted specifically in monocytes and macrophages ($Mapk14^{f/f}LysM$-Cre mice) revealed increased bone mass along with a decrease in osteoclastic bone resorption (Cong et al. 2017).

JNK1 is a MAPK activated by RANKL. JNK1-deficient bone marrow cells ($Mapk8^{-/-}$ BMMs) fail to differentiate into osteoclasts, suggesting its involvement in osteoclastogenesis (David et al. 2002). In these cells, activation of a Jun protein, c-Jun was impaired. MKK7 is a MAPKK that activates JNK1. JNK1 phosphorylation is inhibited by the overexpression of a dominant negative form of MKK7, suggesting a possible role for MKK7 in osteoclastogenesis (Yamamoto et al. 2002). Another member of the Jun proteins, JunB, also has a role in osteoclastogenesis, as $Junb^{f/f}LysM$-Cre mice exhibit an osteopetrotic bone phenotype (Kenner et al. 2004). There are several other MAPKs and related kinases the functions of which in osteoclastogenesis remain unclear. Therefore, studies using genetically modified mice are required for the clarification of the functions of these molecules (Okamoto et al. 2017).

NFATc1, the master transcription factor in osteoclastogenesis

NF-κB and MAPK signals are not RANKL-specific pathways but RANKL almost exclusively induce osteoclastogenesis, which indicated the existence of osteoclastogenesis-specific transcription factors. In search for such factors, NFATc1 was discovered (Takayanagi et al. 2002). Deficiency in $Nfatc1$ results in the complete loss of osteoclastic bone resorption, indicating its essentiality in osteoclastogenesis (Aliprantis et al. 2008; Asagiri et al. 2005; Takayanagi et al. 2002).

Downstream of NF-κB and MAPK signaling, c-Fos and Jun proteins are induced as described above. These proteins dimerize to form activator protein-1 (AP-1). NF-κB, AP-1, NFATc2, ATF4 and Jdp2 are recruited to the promoter region of the $Nfatc1$ gene after RANKL stimulation, together inducing NFATc1 (Asagiri et al. 2005; Cao et al. 2010; Maruyama et al. 2012; Matsuo et al. 2004). The expression of this transcription factor is greatly upregulated after RANKL stimulation by autoamplification machinery (Asagiri et al. 2005; Takayanagi et al. 2002). NFATc1 expression is epigenetically regulated as well. Histone modification profiling in the $Nfatc1$ promoter region showed an obvious change during osteoclastogenesis, from bivalent H3K4me3/H3K27me3 to monovalent H3K4me3 (Yasui et al. 2011). A histone demethylase, Jmjd3, has been shown to be upregulated and recruited near the $Nfatc1$ promoter region after RANKL stimulation and enhance osteoclastogenesis (Yasui et al. 2011).

The induced NFATc1 in turn drives a number of osteo-clastogenic genes including *Dcstamp* (dendritic cell-specific transmembrane protein: DC-STAMP), *Atp6v0d2* (v-type proton ATPase subunit d2), *Oscar, Itgb3* (integrin β3), *Ocstamp* (osteoclast stimulatory transmembrane protein: OC-STAMP), *Acp5* (tartrate-resistant acid phosphatase: TRAP), *Calcr* (calcitonin receptor) and *Ctsk*, together with other transcription factors such as PU.1 and microphthalmia transcription factor (MITF) (Kim et al. 2008; Matsumoto et al. 2004; Matsuo et al. 2004; Miyamoto et al. 2012).

Co-stimulatory signaling and calcium signaling in osteoclastogenesis

In T cells, NFATc1 has been known to localize in the cytosol in the steady state in a hyperphosphorylated, latent form that needs to be dephosphorylated by calcineurin for trans-location into the nucleus (Hirotani et al. 2004). Likewise, during osteoclastogenesis, nucleus-directed mobilization of NFATc1 was observed and the activation of NFATc1 was shown to be sufficient to induce osteoclastogenesis (Hirotani et al. 2004; Takayanagi et al. 2002).

Immunoglobulin-like receptors (IgLRs) are a group of proteins composed of an extracellular region, which has an immunoglobulin-like domain and an intracellular region, which associates with immunoreceptor tyrosine-based activation motif (ITAM)- or immunoreceptor tyrosine-based inhibitory motif (ITIM)-harboring adaptor proteins. IgLRs and their adaptors are expressed on osteoclasts as well as immune cells. Fc receptor common γ subunit (FcRγ) and DNAX-activating protein (DAP) 12 are ITAM-harboring adaptors expressed in osteoclasts that have been shown to have an essential role in osteoclastogenesis through NFATc1 induction (Koga et al. 2004). Paired immunoglobulin-like receptor-A (PIR-A), osteoclast-associated receptor (OSCAR) and Fcγ receptors (FcγRs) are reported to associate with FcRγ; whereas triggering receptor expressed in myeloid cells (TREM)-2, signal-regulatory protein (SIRP) β1, sialic acid-binding Ig-like lectin (Siglec)-15 and myeloid DAP12-associating lectin (MDL)-1 associate with DAP12 (Joyce-Shaikh et al. 2010; Kameda et al. 2013; Koga et al. 2004; Negishi-Koga et al. 2015).

The semaphorins have a variety of functions in organo-genesis, immune responses and tumorigenesis. They are divided into 8 subfamilies and most of them use plexins as their receptors. It was demonstrated that Semaphorin (Sema) 6D and its receptor plexin-A1 (PlxnA1), which associate with TREM-2–DAP12, promote osteoclastogenesis (Takegahara et al. 2006). Sema3A and its receptor neuropilin-1 (Nrp1) inhibit osteoclastogenesis by competing with Sema6D for Plxn1, thus protecting bone from excessive resorption (Hayashi et al. 2012).

Phosphorylation of the ITAM motif of FcRγ and DAP12 leads to the activation of spleen tyrosine kinase (Syk), which promotes the formation of the Bruton agammaglobulinemia tyrosine kinase (Btk)/Tec–B cell linker protein (BLNK)/SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) complex (Shinohara et al. 2008). This complex activates phospholipase Cγ (PLCγ), which mediates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) in the cellular membrane, mediating inositol 1,4,5-triphosphate (IP_3) release (Shinohara et al. 2008). IP_3 binds to its receptor IP_3R to induce the release of Ca^{2+} out of the endoplasmic reticulum (ER). Sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) 2 reuptakes the released Ca^{2+} into ER. Such influx and efflux of Ca^{2+} leads to a repetitive fluctuation of $[Ca^{2+}]$ in the cytosol, i.e. calcium oscillation (Yang et al. 2009). Transmembrane protein (Tmem) 64 was shown to associate with SERCA2 and to be essential for its function (Kim et al. 2013). Tmem178 is an NFATc1- and PLCγ2-dependent molecule and suppresses the excessive efflux of Ca^{2+} (Decker et al. 2015). Calcium oscillation activates calcineurin, which dephosphorylates NFATc1, enabling it to enter the nucleus, so as its expression becomes amplified. Calcium signaling is also reported to induce the c-Fos via Ca^{2+}/calmodulin-dependent kinase (CAMK) IV-cAMP response element-binding protein (CREB) pathway (Sato et al. 2006).

Mice deficient in the factors above exhibit impaired calcium oscillation and subsequent abnormal osteoclastic bone resorption: $Tyrobp^{-/-}$ mice (deficient in DAP12) display mild osteopetrosis and $Tyrobp^{-/-}$ cells have impaired calcium oscillation (Kaifu et al. 2003; Koga et al. 2004; Negishi-Koga et al. 2015); $Tyrobp^{-/-}Fcer1g^{-/-}$ mice (deficient in DAP12 and FcRγ) are osteopetrotic and $Tyrobp^{-/-}Fcer1g^{-/-}$ cells have a defect in calcium oscillation (Koga et al. 2004; Sato et al. 2006); $Fcgr3^{-/-}$ mice (deficient in FcγRIII) are osteopenic and the amplitude of calcium oscillation is higher in $Fcgr3^{-/-}$ cells (Negishi-Koga et al. 2015); $Tec^{-/-}Btk^{-/-}$ mice are osteopetrotic and calcium oscillation in $Tec^{-/-}Btk^{-/-}$ cells is attenuated (Shinohara et al. 2008); $Atp2a2^{+/-}$ mice (haploinsufficient in SERCA2) exhibited osteopetrosis and no calcium oscillation in $Atp2a2^{+/-}$ cells (Yang et al. 2009); $Tmem64^{-/-}$ mice are osteopetrotic and $Tmem64^{-/-}$ cells are defective in calcium oscillation (Kim et al. 2013); and $Tmem178^{-/-}$ mice are osteopenic, with a high level and slow efflux of Ca^{2+} in $Tmem178^{-/-}$cells (Decker et al. 2015).

Negative regulation of osteoclastogenesis

NFATc1 expression and activity are not only enhanced, but also suppressed by transcription factors so that osteoclastic bone resorption does not become excessive. Interferon regulatory factor (IRF)-8 is one such molecule. In $Irf8^{-/-}$ cells, the expression of *Nfatc1* after RANKL stimulation is high

and osteoclastic bone resorption is enhanced in $Irf8^{-/-}$ mice, indicating its suppressive effect on osteoclastogenesis (Zhao et al. 2009). The expression of $Irf8$ was shown to be high in osteoclast progenitor cells and to gradually decrease during osteoclastogenesis, which is mediated by DNA methyltransferase (Dnmt) 3A through methylation of the 3′-flanking region of the gene (Nishikawa et al. 2015; Zhao et al. 2009).

MafB is a bZIP motif-containing transcription factor that has been known to stimulate macrophage differentiation. $Mafb$ expression was reported to be downregulated by RANKL stimulation, which is under the control of p38 or JNK MAPKs. MafB was shown to suppress the expression of $Nfatc1$ and $Oscar$ by inhibiting c-Fos, MITF and NFATc1, leading to the suppression of osteoclastogenesis (Kim et al. 2007).

B cell lymphoma (Bcl) 6 is a transcription factor with a BTB/POZ domain in the N-terminus and a zinc finger domain in the C-terminus. Like $Irf8$ and $Mafb$, the expression of $Bcl6$ decreases during osteoclastogenesis. Bcl6 was revealed to suppress osteoclastogenesis as $Bcl6^{-/-}$ cells underwent enhanced osteoclastogenesis and $Bcl6^{-/-}$ mice developed an osteoporotic phenotype due to the increased osteoclastic bone resorption. The mechanism underlying this suppression is explained by the repression of the transcription of $Nfatc1, Dcstamp$ and $Ctsk$ (Miyauchi et al. 2010).

B lymphocyte-induced maturation protein (Blimp) 1 is a transcriptional repressor, the expression of which is upregulated by NFATc1 (Nishikawa et al. 2010). Osteoclast-specific Blimp1-deficient mice ($Prdm1^{f/\Delta}Ctsk^{Cre/+}$ mice) were reported to be osteopetrotic because of reduced osteoclastic bone resorption (Miyauchi et al. 2010; Nishikawa et al. 2010). Blimp1 was shown to suppress the expression of $Irf8, Mafb$ and $Bcl6$ by binding to their promoter regions (Miyauchi et al. 2010; Nishikawa et al. 2010).

Leukemia/lymphoma-related factor (LRF) is a transcriptional repressor belonging to the POZ/BTB and Krüppel (POK) family and is involved in various biological processes. $Zbtb7a$ (LRF) expression was shown to be very low before and highly elevated after RANKL stimulation (Kukita et al. 2011; Tsuji-Takechi et al. 2012). LRF is a unique transcription factor in that it regulates osteoclastogenesis in a stage-specific manner. Overexpression of LRF in osteoclast progenitors in the early phase results in suppression and $Zbtb7a^{f/\Delta}Mx1$-Cre mice have osteopenia, suggesting an inhibitory function in osteoclastogenesis. On the other hand, $Zbtb7a^{f/\Delta}Ctsk^{Cre/+}$ mice were osteopetrotic, indicating a promoting function (Tsuji-Takechi et al. 2012).

The studies discussed here have done much to uncover the mechanisms underlying osteoclastogenesis, in which RANKL/RANK signaling plays a central role. The discovery of new mechanisms and molecules reflects the technological advances that have occurred in biology, such as ones in epigenetics. Although key molecules for osteoclastogenesis have been elucidated, there still remain processes, in which the underlying mechanisms are not well understood. The continuing search for novel factors involved in osteoclastogenesis should lead to the establishment of therapeutic strategy for bone diseases.

Osteoclastic bone resorption

As osteoclastogenesis proceeds, osteoclast progenitors fuse so as to generate a large, multinucleated cell. Such enlargement of a cell enables it to widely seal the bone surface, increasing bone resorption efficiency. Inside the sealed zone, bone tissue is degraded by H^+, Cl^- and catalytic enzymes. Inorganic components of the bone are resorbed under the acidic environment established by HCl. Organic components are degraded by various enzymes including CtsK and MMP-9. This section reviews how osteoclasts become multinucleated and resorb bone matrix (summarized in Fig. 4).

Cellular fusion in osteoclast formation

The fusion of osteoclast progenitors is thought to be crucial for the optimization of the resorption activity. Cellular fusion is achieved by the remodeling of phospholipids, components of the lipid bilayer of the cellular membrane. The phospholipids are divided into five groups: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and sphingomyelin (SM). PC and SM preferentially reside on the outer leaf of the lipid bilayer, whereas PE, PS and PI reside on the inner leaf (Zachowski 1993). Although it is known that the composition and distribution of the phospholipids can change during the course of cellular fusion, our knowledge on the phospholipid dynamics in the osteoclast fusion is currently inadequate (Irie et al. 2017).

One study recently showed that fusing osteoclasts highly express PE, especially on the surface of their filopodia, although PE is normally localized in the inner leaflet. Inhibition of the externalization of PE on osteoclast progenitors resulted in impaired cellular fusion, but the expression of osteoclastogenic genes was relatively preserved (Irie et al. 2017). It was shown that during osteoclast fusion, PE is synthesized and externalized by Acyl-CoA: lysophosphatidylethanolamine acyltransferase 2 (LPEAT2, encoded by $Lpeat2$) and two ABC transporters (ABCB4, encoded by $Abcb4$ and ABCG1, encoded by $Abcg1$), respectively (Irie et al. 2017).
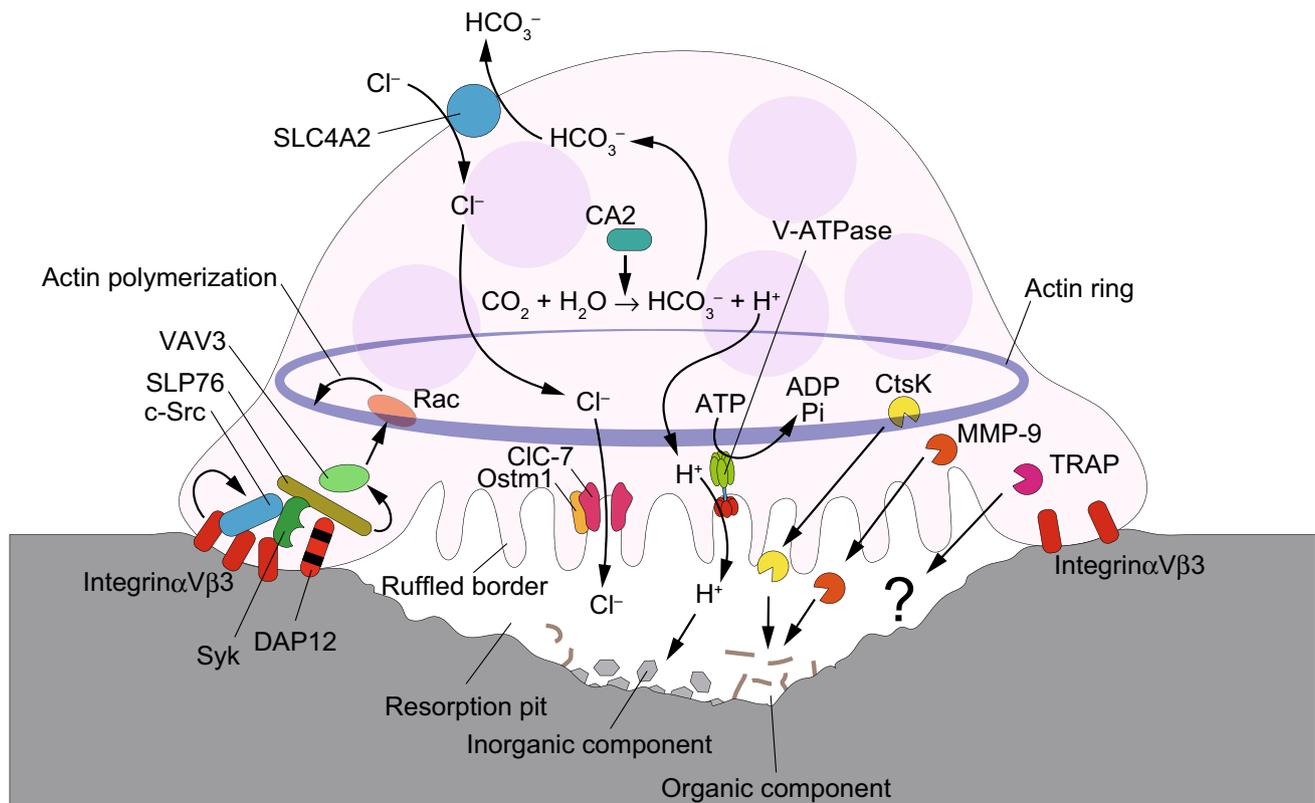
Fig. 4 Osteoclastic bone resorption. The integrins expressed on osteoclasts are responsible for the adherence of these cells to the bone surface. Inside osteoclasts, integrins activate c-Src, leading to the formation of a complex with Syk, DAP12, SLP76 and VAV3. This complex activates the Rho-GTPase Rac. Rac induces actin ring formation and isolates the resorption pit. Osteoclasts secrete H^+ and Cl^- into the isolated pit via ion channels. H^+ is produced by the dehydrogenation of a carbonic acid. Cl^- is imported into osteoclasts in exchange for HCO_3^-. Acidification of the resorption pit leads to the decalcification of the bone, as well as the activation of CtsK. CtsK and MMP-9 degrade the organic components, including collagens, whereas TRAP is likely a phenotypic marker

Certain osteoclast-specific molecules are also known to function in the fusion of osteoclasts. DC-STAMP, one of the osteoclastogenic genes, is essential for cellular fusion of the osteoclast progenitors. Its deficiency results in a failure in osteoclast multinucleation, giving rise to many TRAP$^+$ mononuclear cells both in vitro and in vivo (Kukita et al. 2004; Yagi et al. 2005). *Dcstamp*$^{-/-}$ TRAP$^+$ mononuclear cells normally express *Fos, Nfatc1* and *Ctsk*. These cells also form a ruffled border and actin ring normally. However, bone resorption activity was lower in the mutant cells (Yagi et al. 2005). These results indicate that DC-STAMP is necessary specifically for the fusion process during osteoclastogenesis and that cellular fusion is crucial for bone resorption.

OC-STAMP is another factor that regulates the fusion of osteoclast progenitors. *Ocstamp*$^{-/-}$ cells develop into TRAP$^+$ mononuclear cells by RANKL stimulation, similarly to *Dcstamp*$^{-/-}$ cells, with normal expression in osteoclastogenic genes, normal formation of the ruffled border and sealing zone, and reduced bone resorption activity (Miyamoto et al. 2012). Interestingly, *Ocstamp*$^{-/-}$ mice do not exhibit significant osteopetrosis, indicating the difference in the contribution of DC-STAMP and OC-STAMP to bone resorption (Miyamoto et al. 2012).

Atp6v0d2 is a subunit of the vacuolar H$^+$-ATPases (V-ATPases) and its expression has been detected in osteoclasts. There were fewer multinuclear osteoclasts differentiated from *Atp6v0d2*$^{-/-}$ cells by RANKL stimulation, but the number of TRAP$^+$ cells and the expression of osteoclastogenic genes were unaffected (Lee et al. 2006). Although TRAP$^+$ cells were shown to be normally formed in *Atp6v0d2*$^{-/-}$ mice, osteoclastic bone resorption decreased, suggesting a contribution of Atp6v0d2 to cellular fusion and subsequent bone resorption (Lee et al. 2006). Although these osteoclastogenic molecules are essential for osteoclast fusion, their functional role in phospholipid dynamics remains unclear.

Isolation of the specific site of bone resorption

Osteoclasts adhere to the bone surface via integrin αVβ3, which activates the non-receptor tyrosine kinase c-Src.

Activated c-Src then phosphorylates Syk to facilitate the formation of a complex with DAP12 and SLP-76, further activating VAV3 and the Rho-GTPase Rac, leading to actin polymerization (Faccio et al. 2005; Izawa et al. 2012; Zou et al. 2007). Polymerized actin fibers form an actin ring, which is observed as a clear zone under transmission electron microscopy (TEM). The bone surface targeted for resorption is sealed with this structure so that acids and catabolic enzymes do not leak, enabling a fine-tuned targeting of the bone resorption site.

Inside the sealed zone, H^+, Cl^-, CtsK and MMP-9 are secreted to degrade the inorganic components consisting of hydroxyapatite along with the organic components (mostly collagen). To increase the efficiency of bone resorption, the cellular membrane facing the zone becomes folded, making the ruffled border. It is reported that the osteoclasts of $Src^{-/-}$ mice lack the ruffled border, resulting in a deficiency of bone resorbing activity, suggesting its contribution to bone resorption (Boyce et al. 1992; Soriano et al. 1991). The formation of the ruffled border is closely linked to bone resorption activity because the osteoclasts of mice deficient in synaptotagmin VII, a molecule responsible for exocytosis of enzymes, are unable to form the structure (Zhao et al. 2008).

Ion channels and the degradation of the inorganic components of bone

Osteoclasts secrete H^+ via V-ATPases located on the ruffled border. They consist of V_1 and V_0 domains, which carry out ATP hydrolysis and H^+ translocation, respectively (Qin et al. 2012). A deficiency in the subunits of these domains is reported to lead to osteopetrosis. Mice deficient in the a3 subunit of the V_0 domain (encoded by the *Tcirg1* gene) exhibit an osteopetrotic phenotype, although they do have osteoclasts in the bone (Li et al. 1999; Scimeca et al. 2000). As mentioned in section II-C, mutations in *TCIRG1* lead to ARO. A deficiency in the d2 subunit V_0 domain (*Atp6v0d2$^{-/-}$* mice) resulted in osteopetrosis. However, this seems to be due to a deficiency in cellular fusion than bone resorption activity (see section V-A) (Lee et al. 2006).

ClC-7 and its subunit Ostm1 neutralize the positive charge of the resorption pit by translocating Cl^-. *Clcn7$^{-/-}$* mice develop a severe osteopetrosis that is consistent with human patients (see section II-C) (Kornak et al. 2001). These mice have TRAP$^+$ osteoclasts but they have few ruffled border and no bone resorption activity (Kornak et al. 2001). Ostm1 was shown to constitute a complex with ClC-7 and is suggested to stabilize it (Lange et al. 2006). Grey-lethal mice (*gl/gl* mice), which are deficient in *Ostm1*, as well as human patient with mutations in the orthologous gene exhibit osteopetrosis (see section II-C) (Chalhoub et al. 2003). These

findings, taken together, indicate the key role for the ion channel in bone resorption.

CA2 catalyzes a reaction that gives rise to HCO_3^- and H^+ out of carbonic acid. The H^+ produced by this reaction is secreted by V-ATPase into the resorption pit. HCO_3^- is exported by anion exchangers, including SLC4A2, which in turn import Cl^- into the cytosol. Mice deficient in CA2 or SLC4A2 were shown to be osteopetrotic (Margolis et al. 2008; Wu et al. 2008). Mutations in *CA2* cause ARO, as discussed in section II-C (Sly et al. 1983). Taken together, these findings have indicated that CA2 functions as a supplier of ions for the other functional molecules described here.

Enzymes that degrade the organic components of the bone

Acidification of the resorption site is necessary not only for the dissolution of bone minerals, but also for activating certain enzymes. The cathepsins belong to the class of the cysteine proteinases, which can efficiently degrade the most abundant extracellular matrix (ECM) protein in the bone, type I collagen. CtsK was originally identified as a possible cysteine proteinase from a rabbit osteoclast cDNA library and later found in human bone tissues (Inaoka et al. 1995; Tezuka et al. 1994). The expression of *Ctsk* is the highest among the known cathepsins in osteoclasts (Ishibashi et al. 2001). Mutations in *CTSK* have been reported in human patients of an autosomal recessive osteochondrodysplasia, pycnodysostosis (Gelb et al. 1996). Hematopoietic cell-or monocyte/macrophage lineage-specific deletion of *Ctsk* in mice results in an osteopetrotic phenotype. These mice have an increased number of osteoclasts, but their bone resorption activity is low. In addition, these osteoclasts produced a higher level of sphingosine-1-phosphate (S1P), which increases osteoblastic bone formation (Lotinun et al. 2013).

The MMPs are a group of enzymes that can degrade ECM proteins. MMP-9 is expressed predominantly in osteoclasts and thought to be involved in bone resorption. *Mmp9$^{-/-}$* mice were shown to have a deficiency in cartilage resorption during development that was compensated in the later stage, suggesting that MMP-9 plays a role in development (Vu et al. 1998). TRAP begins to be expressed in the osteoclast progenitors just before fusion. Because the functions of TRAP in osteoclasts have not been elucidated well, TRAP is typically thought of more as a phenotypic marker than as a functional molecule.

Imaging of bone resorption

Accumulating evidence has led to a much improved understanding of the functions of osteoclasts. In vitro experiments have enabled a detailed investigation of the mechanisms by which osteoclasts develop and function. However, it has long

been known that the osteoclasts generated in the culture dish do not appear as how they are in the body, raising the concern that the cultured osteoclast phenotype does not reflect the actual osteoclasts in the body. Furthermore, the kinetics of these cells cannot be analyzed by in vitro experiments. To address this issue, live imaging techniques have been developed.

The use of animals, soft tissues of which are transparent, such as medaka and zebra fish has provided a simple and effective solution. Using *acp5-gfp* transgenic or *mmp9-rfp* transgenic medaka fish, osteoclasts were labeled and the gene expression levels were quantified with the fluorescence (Chatani et al. 2015, 2016). Quantification of *mmp9* expression by live imaging suggested that the unloading by spaceflight enhances bone resorption (Chatani et al. 2016). With the use of molecular probes that have the capacity to detect pH variance (described below), more detailed analyses of bone resorption would be achieved.

Intravital imaging is a technique in which spatiotemporal information on tissues, cells and molecules is obtained by detecting fluorescence using two-photon excitation microscopy (TPEM). Bone is an advantageous tissue in this process in that motion artifacts are negligible compared to those that occur in the lung and the heart. However, hydroxyapatite in the bone matrix scatters the excitation light and the light cannot penetrate as deep as soft tissues, making it difficult to observe deep inside. Efforts have been made and now TPEM is applicable to the observation of bone marrow cells for several hours in calvarial bone, the cortical bone of which is thin enough for the analysis (Ishii et al. 2009). The mobilization of osteoclast progenitors and bone resorption by osteoclasts have been successfully visualized using the intravital imaging of the bone marrow (Ishii et al. 2009, 2010; Maeda et al. 2016).

For the purpose of accurate observation, fluorescent proteins or molecular probes with high photostability, intense brightness and a high accessibility toward the targets are required. A molecular probe that is able to make pH variation in the resorption pit visible has been developed (Maeda et al. 2016). The information acquired by such techniques are processed with analytical procedures for the objective and quantitative assessment of osteoclast function. The development of microscopy techniques, design of molecular probes and performance of mathematical analysis require the cooperation of experts from a variety of research fields. With the help of such experts, the elucidation of osteoclast functions will further proceed.

Conclusion

Thus, with the series of studies discussed here, knowledge of the mechanisms underlying osteoclastogenesis has been advanced quite remarkably over approximately the last 20 years. In these studies, comprehensive analyses of transcriptomes/proteomes and genetically modified mice have been employed as powerful tools in the identification of a novel factor related to osteoclasts and in proving its significance in vivo. The accumulated findings might give the impression that most of the crucial processes of osteoclastogenesis have been uncovered, thus discouraging further investigation. However, with the help of novel technologies such as intravital live imaging and mathematical analysis, another edge of the wedge has been delivered and begun to provide further novel insights into osteoclast biology. Findings from such analyses would lead to the development of novel therapeutic strategies for variety of bone diseases. Therefore, from both the scientific and clinical points of view, there remains a room for further studies on the osteoclast and RANK/RANKL biology.

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