Review

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Effects and mechanisms of microenvironmental acidosis on osteoclast biology

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SUMMARY Due to continuous bone remodeling, the bone tissue is dynamic and constantly being updated. Bone remodeling is precisely regulated by the balance between osteoblast-induced bone formation and osteoclast-induced bone resorption. As a giant multinucleated cell, formation and activities of osteoclasts are regulated by macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor-kappaB ligand (RANKL), and by pathological destabilization of the extracellular microenvironment. Microenvironmental acidosis, as the prime candidate, is a driving force of multiple biological activities of osteoclast precursor and osteoclasts. The mechanisms involved in these processes, especially acid-sensitive receptors/channels, are of great precision and complicated. Recently, remarkable progress has been achieved in the field of acid-sensitive mechanisms of osteoclasts. It is important to elucidate the relationship between microenvironmental acidosis and excessive osteoclasts activity, which will help in understanding the pathophysiology of diseases that are associated with excess bone resorption. This review summarizes physiological consequences and in particular, potential mechanisms of osteoclast precursor or osteoclasts in the context of acidosis microenvironments.

Keywords acidosis, microenvironment, osteoclasts, physiological consequences, acid-sensitive receptors/pathways

1. Introduction

The normal functioning of cells depends on proper maintenance of acid-base balance in the extracellular microenvironment (*1*). The pH of arterial blood ranges between 7.36 and 7.44, whereas the pH of venous blood is approximately 7.6 (*2*). In the human body, several intracellular and extracellular buffers help to keep the pH within this narrow range. In the blood, the HCO_3^-/CO_2 buffer system, plasma proteins, and histidine residues of hemoglobin provide buffering activity for H^+ and HCO_3^- (*2,3*). It is worth noting that the interstitial fluid lacks pH buffers, and for this reason, the pH of this fluid is determined by multiple complex factors (*3*). In this case, pH of the microenvironment greatly depends on the type of tissue, metabolic activity of different cells, and the status of blood supply in the local environment. For the musculoskeletal system, the pH of bone tissue microenvironment is affected by the following factors, such as tumors (*3,4*), inflammation (*5*), infection (*6*), wound healing (*7*) or fracture (*8*).

Generally, microenvironmental acidosis has a negative effect on the musculoskeletal system of the human body. Once the acid-base equilibrium is broken, the function of pH-dependent enzymes and membrane transporters in cells is impaired leading to bone malfunction and metabolic dysfunction (*9*). For osteoblasts, extracellular acidosis reduces the activity of alkaline phosphatase (ALP), decreasing formation of extracellular matrix, thereby inhibiting most of the biological functions of osteoblasts, decreasing trabecular bone formation, and reducing bone density (*10*). For bone marrow mesenchymal stem cells (BMSCs), although short-term acidic stimulation enhances the stem cell phenotype, cell proliferation and viability, it reduces the migration ability of BMSCs (*11*). More importantly, acidosis impairs the osteogenic differentiation of BMSCs (*12*).

However, osteoclasts are an exception. Osteoclasts, as non-proliferative polykaryons that differentiate from monocyte precursors, are responsible for bone remodeling and maintenance of the dynamic calcium homeostasis. Osteoclasts have the ability

to sense and respond to acidosis in the extracellular microenvironment, and osteoclasts require proton stimulation for differentiation, bone resorption activities and survival (*13*). Bone resorption activities of osteoclasts can be maximized and cause bone mineral loss when microenvironmental pH is 6.9, and a weak alkaline condition inhibits osteoclastogenesis (*14-16*). In addition, bone resorption functions of osteoclasts depend on excretion of H^+ to the sealing zone through vacuolar H^+-ATPase (V-ATPase), which helps dissolve the bone matrix. In turn, excess protons enter the cytoplasm from the sealing zone and are eventually discharged out of the cell through vesicles, leading to rhythmic pH oscillations and gradual intracellular acidification during the resorption process (*13,17,18*). Taken together, osteoclast biology is closely associated with protons (Table 1).

A better understanding of the functions and molecular mechanisms of acid-sensitive receptors/channels of osteoclasts under microenvironmental acidosis will help in establishing whether and how they can be used as drug targets for patients with bone metabolism disorders that are characterized by bone loss. Therefore, we summarize and explain the physiological consequences and underlying mechanisms of microenvironmental acidosis on osteoclasts.

2. Effects of microenvironmental acidosis on osteoclast biology

2.1. Differentiation

Osteoclasts are giant multinucleate cells that are derived from hematopoietic precursor cells of the monocyte/macrophage lineage (*19*). During this process, osteoclast differentiation is promoted by macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor-kappaB ligand (RANKL) secreted by osteoblasts. RANKL binds its receptor, RANK, located on osteoclast precursor plasma membrane, and further, recruits TNF receptor associated factor 6 (TRAF6). TRAF6 activates downstream signaling pathways of nuclear factor-kappaB (NF-κB) and mitogen-activated protein kinases (MAPKs). The nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), a master regulator, is induced by NF-κB, MAPKs signaling cascades, calcium signal and autoamplification loop, which is indispensable for osteoclastogenesis (*13,19*). Extracellular protons, as a result of acidosis, play a significant role in fine-tuning osteoclastogenesis (*20-25*). Actually, extracellular acidosis has been shown to significantly elevate intranuclear NFATc1 levels in rat and rabbit osteoclast precursors through MAPKs (*20*) and Ca^{2+}/calcineurin pathways (*21-23*). Its effect of promoting osteoclastogenesis is comparable to that of RANKL (*21*). Kohtaro Kato *et al.* reported that one of the main action points for acidosis is in the final stages of osteoclastogenesis, especially during the 4-7 days of osteoclast precursor cultures. Moreover, protons can directly promote osteoclast differentiation, independently of bicarbonate ions (*24,25*).

Alkaline drugs or materials can counteract the negative effects induced by protons, such as K citrate or borosilicate glasses, which provide a new direction for clinical treatment of diseases with redundant osteoclastogenesis (*15,16*).

K citrate is commonly used to increase urine pH, thereby inhibiting solute precipitation and kidney stone formation. However, K citrate may also be beneficial in preventing the progression of bone loss. The potassium channel subfamily is an inhibitor of proton-induced osteoclastogenesis, and extracellular K^+ inhibits osteoclastogenesis in a dose-dependent manner. In addition, citrate has calcium-binding abilities, and it competitively binds intracellular Ca^{2+}, suppressing proton-induced NFATc1 signal transduction. Therefore, K citrate can counteract acidosis-induced negative effects, and can even overcome alendronate-associated drug resistance in a long-term acidic microenvironment (*15*). To rebuild bone regeneration balance, Wenlong Liu *et al.* established a local weakly alkaline microenvironment that was generated by biodegradation of borosilicate glasses, which further modulated osteoclast differentiation. Actually, the higher the pH, the lower the differentiation activity of osteoclasts. At a pH of 7.8, a threshold value for regulating osteoclast differentiation, this alkaline material almost completely shut down osteoclastogenesis (*16*).

2.2. Migration, adhesion and fusion

A prerequisite for bone resorption involves a series of complex events that osteoclast precursors must undergo, including attraction/migration, recognition, adhesion, membrane fusion, and finally, formation of giant multinucleated cells (*13*). These events are mainly attributed to DC-STAMP, osteopontin (OPN), Atp6v0d2 and CD47 among others, and defects in these genes inevitably lead to the generation of inactive osteoclasts (*26*). DC-STAMP, a membrane-bound receptor, with no definite ligand, is the main regulator of pre-osteoclast fusion (*26*). Some studies have reported that acidosis does not foster osteoclast precursor fusion, and that mRNA expression levels of DC-STAMP are rarely susceptible to pH changes (*24*). However, more reports showed that extracellular acidosis enhances the fusion of pre-osteoclasts and the largest surface area of mature osteoclasts (*27*), with redundant expression levels of DC-STAMP (*15,28*).

In addition to DC-STAMP, OPN, a matrix protein containing the Arg-Gly-Asp motif, and integrin avβ3, a subunit of the cell-surface receptor superfamily, coordinate to mediate the adhesion and migration of osteoclast precursors and osteoclasts (*26*). In fact, protons promote the expression of OPN, which in turn increases

its interaction with integrins avβ3, thereby inducing the activation of proline-rich tyrsosine kinase 2 (Pyk2) and Src protein-tyrosine kinase (Src) signals and the production of actin rings, which stimulate the migration and adhesion of osteoclast precursors (*20,22,29,30*).

Gap junction, a unique plasma membrane structure, is composed of two connexon hemichannels, connected to neighboring cells, and mediates the exchange of ions and small molecules (*31*). Osteoclast fusion is a multifactorial process that may involve gap junction communication (GJC) in addition to the above mentioned cytokines (*31*). To characterize the role of GJC, Elina Kylmäoja *et al.* showed that, at neutral pH (7.4), AAP10, a GJC agonist, was shown to promote the expression levels of connexin and maintained gap junctions in an open state, which led to the fusion of osteoclast precursors (*32*). Acidosis (pH 6.5) caused the gap junctions to close, offsetting the effects of AAP10, eventually inhibiting GJC-mediated fusion of osteoclast precursors (*32*). However, this will not affect osteoclastogenesis, which means that although GJC is an inhibitor of osteoclast fusion under acidic conditions, this inhibiting effect is insufficient to interfere with the promoter effect induced by cytokines mentioned above (*32*).

2.3. Bone resorption

After undergoing migration, adhesion and fusion of osteoclast precursors, giant multinucleated osteoclasts are involved in bone resorption and in deterioration of the skeletal microarchitecture (*13,33*). To improve efficiency of bone resorption, the bone-facing plasma membrane is transformed into a ruffled border, a specific late endosome-like domain, and releases H^+, Cl^-, cathepsin K and matrix metalloproteinase 9 (MMP-9) into the sealed area further acidifying the resorption lacuna and dissolving the bone matrix (*13,33*). This acidification is highly efficient and can reduce the pH to 3 within a few minutes of the resorption lacuna when osteoclasts exert bone resorption activity (*34*). Finally, products of bone resorption, including degraded collagen fragments, calcium, phosphate and extra protons, are released from the sealing zone to the extracellular matrix of the osteoclasts through transcytosis pathways (*18*).

Extracellular acidosis increases the size and number of bone resorption pits mediated by osteoclasts, and can even lead to calvarial bone perforations (*35*). In fact, the resorption area was shown to increase 14-fold as pH changed from pH 7.4 to 6.8, accompanied by a 3-fold increase in cathepsin K and V-ATPase activities (*27,36,37*). This response is highly sensitive, a decrease of 0.1 units in pH is enough to cause a two-fold trabecular bone loss (*38*). Interestingly, the relationship between the ability of bone resorption and pH is not linear, but curved. Bone resorption capacity reaches its peak when the extracellular pH drops to 6.8. Alkaline conditions and the peracid environment inhibits resorption activity (*27*). This effect is not desensitized in long-term cultures and bone resorption of osteoclasts continues (*27*).

Osteoclasts resorption activities are subject to paracrine regulation, including RANKL, OPN, Prostaglandin E2 (PGE2) and interleukin 6 (IL-6), which originate from surrounding stromal cells, including osteoblasts and its precursor (*39,40*). PGE2 redundantly expresses induction by osteoblasts under metabolic acidosis, which further stimulates bone resorption by osteoclasts (*39,40*). Cyclooxygenase-2 (COX-2), a cyclooxygenase that converts arachidonic acid into active prostaglandin metabolites, promotes PGE2 secretion. To further characterize paracrine effects on bone resorption, a series of studies were performed and it was found that acidosis specifically up-regulated $[Ca^{2+}]i$ levels, which induced the signal cascade from COX-2 to PEG2 in osteoblasts (*41*). PGE2 directly fosters RANKL expression in a paracrine manner, and RANKL binds the RANK receptor on plasma membranes of osteoclasts, which significantly increases bone resorption and net calcium efflux from the bone (*42-44*). This implies that not only do protons act on osteoclasts themselves, they also act on osteoblasts to promote the activities of osteoclasts in a paracrine manner.

NBCn1, an electrically neutral sodium bicarbonate cotransporter, is present in the ruffled border membrane of osteoclasts. NBCn1 removes bicarbonate from the sealing zone, and excretes bicarbonate into the upper space of osteoclasts through an electroneutral chloride-bicarbonate exchanger (*45*). This process contributes to maintenance of the acidic environment in the resorption cavity, therefore, inhibiting the activities of NBCn1 may provide a new method to inhibit the excessive bone resorption that osteoclasts suffer from acidosis (*45*).

2.4. Apoptosis

Osteoclasts are short-lived terminally differentiated cells. However, the acidic microenvironment can extend the lifespan of osteoclasts (*29*). Their survival rate was shown to have doubled at pH 7.0 when compared to pH 7.6 (*46*). To further define the mechanism of this phenomenon, Alexey Pereverzev *et al.* (*46*) found that these effects are due to Ca^{2+}/protein kinase C (PKC)/extracellular regulated protein kinases (ERK) signaling, rather than Ca^{2+}/calcineurin/NFAT signaling. Notably, when protons induce elevation of $[Ca^{2+}]i$ in osteoclasts, intracellular calcium signals stimulate the activation of PKC and ERK, two anti-apoptotic signals involved in multiple cell types, which promote the lifespan of osteoclasts (*47*).

3. The mechanism of microenvironmental acidosis on osteoclasts

Perception, response or adaptation of osteoclasts to extracellular acidosis is regulated by various proton-

Table 1. Effects of acidosis on osteoclasts biology

Author/year (*Ref.*)	pH value	Exposure time	Osteoclast source	Acidosis-induced actions	The effects of acidosis on osteoclast biology
T R Arnett 1986 (*36*)	6.8, 7.0, 7.2, 7.4	2h, 24h	long bones of rat pups	None	increase bone resorption
P Goldhaber 1987 (*95*)	6.94, 7.15, 7.28	7 d	neonatal mouse calvaria	increase net cell-mediated calcium release	increase bone resorption
A Teti 1989 (*30*)	6.5, 7.05, 7.4, 7.6	90 min	medullary bone of laying hens	increase formation of podosomes of osteoclasts	increase adhesion
T R Arnett 1994 (*94*)	6.76, 7.07, 7.20, 7.30	24h	long bones of rat pups	None	increase bone resorption
T R Arnett 1996 (*38*)	6.6, 6.8, 7.0, 7.2, 7.4	26h	long bones of littermate rat	None	increase bone resorption
T Nordström 1997 (*37*)	6.5, 7.0, 7.5	24, 48h	long bones of new zealand white rabbit	upregulation of vacuolar type H^+ ATPase activity	increase bone resorption
D M Biskobing 2000 (*95*)	6.5, 6.75, 7.4	4h	primary marrow cells of mice	upregulation of carbonic anhydrase II and calcitonin receptor genes	increase osteoclastogenesis and bone resorption
N S Krieger 2000 (*39*)	6.8, 7.1, 7.4	24, 48,51 h	calvariae of neonatal mouse	upregulation of PGE2 synthesis, increase net calcium efflux	increase bone resorption
S Meghji 2001 (*35*)	6.9, 7.0, 7.1, 7.2, 7.3,7.4	72h	calvariae of mice	increase net calcium efflux	increase bone resorption and even lead to bone perforations
D A Bushinsky 2001 (*40*)	7.1, 7.5	24, 48,51 h	calvariae of neonatal mice	upregulation PGE2 levels, increase net calcium efflux	increase bone resorption
Kevin K Frick 2003 (*43*)	7.1, 7.5	24h, 48h	calvariae of mice	increase net calcium efflux	increase bone resorption
Svetlana V Komarova 2005 (*21*)	7.0, 7.6	15, 45, 75, 90 min	RAW 264.7 mouse monocytic cell line	activation of Ca^{2+}/calcineurin/NFAT pathway	increase osteoclastogenesis and bone resorption
Kevin K Frick 2005 (*44*)	7.1, 7.4	24h, 48h	calvariae of mice	increase net calcium efflux	increase bone resorption
Jin-Man Kim 2007 (*20*)	7.0, 7.5, 8.0	1, 2, 3, 4 d	RAW 264.7 mouse monocytic cell line and bone marrow monocytes	activation of MAPK pathway, upregulation of osteopontin protein	increase osteoclastogenesis and migration
Nancy S Krieger 2007 (*41*)	7.1, 7.4	24h, 48h, 51h	calvariae of COX-2 wildtype($^{+/+}$), heterozygous(+/-) and homozygous knockout ($^{-/-}$) littermates	upregulation of COX-2 mRNA and protein, and net calcium efflux	increase bone resorption
Mariusz Muzylak 2007 (*27*)	6.92, 7.15, 7.25	7, 14 d	peripheral blood of cat	upregulation of the expression level of trap, cathepsin K and proton pump enzymes	increase osteoclastogenesis, fusion and bone resorption
Kaori Iwai 2007 (*96*)	7.0, 7.4	75min	bone marrow monocytes of C57BL/6J mouse and RAW 264.7 cell line	activation of OGR1/NFAT pathway	increase osteoclastogenesis
Alexey Pereverzev 2008 (*46*)	6.8, 7.0, 7.2, 7.4, 7.6	18h	long bones of neonatal Wistar rats and RAW 264.7 mouse monocytic cell line	activation of OGR1/Ca^{2+}/PKC signaling	increase osteoclasts survival and suppress osteoclast apoptosis
Riikka Riihonen 2010 (*45*)	6.0,7.2	none	CD14 positive cells from human peripheral blood	upregulation of NBCn1 protein expression	increase bone resorption
Nancy S Kriege 2011 (*42*)	7.1,7.4	24h, 48h	calvariae of neonatal CD-1 mouse	activation of OGR1/Ca^{2+}/COX2, PGE2/ RANKL signaling of osteoblast	increase bone resorption
Kohtaro Kato 2011 (*24*)	6.8, 7.0, 7.2, 7.4	pH 7.4 for 3 d and then pH 6.8 for 21 h	spleen mononuclear cells and bone marrow cells of male mice	upregulation of TRPV4 activity	increase osteoclastogenesis and fusion, especially in the last phase
Heejin Ahn 2012 (*29*)	7.0, 7.5	24h, 40h	bone marrow-derived macrophages of C57BL6 male mice	None	increase osteoclast adhesion, migration, bone resorption activity and survival
Kohtaro Kato 2013 (*25*)	6.8, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.2	1, 2, 3, 7, 14 d	bone marrow cells of male ddY mice	upregulation of TRPV1, 4 mRNA	increase osteoclastogenesis
Xia Li 2013 (*22*)	6.0, 6.5, 7.0, 7.5	18 h	bone marrow-derived macrophages of rats	activation of [Ca^{2+}]i/NFATc1 signaling mediated by ASIC1a	increase osteoclastogenesis
Carlotta Reni 2016 (*23*)	7.2, 7.4	6d	bone marrow cells of mice	activation of TRPV1 ion channel	increase osteoclastogenesis

PGE2, Prostaglandin E2. NFAT, Nuclear Factors of activated T. MAPK, pathway mitogen-activated protein kinase pathway. COX-2, Cyclooxygenase-2. OGR1, ovarian cancer G protein coupled receptor 1. RANKL, Receptor Activator for Nuclear Factor-κ B Ligand. TRPV, transient receptor potential vanilloid. NFATc1, Nuclear factor of activated T-cells cytoplasmic. ASIC1a, acid-sensing ion channels 1a. Pyk2, proline-rich tyrsosine kinase 2. Src, Src protein-tyrosine kinase. RANK, receptor activator of NF-KB ligand. MMP-9, matrix metalloprotein-9. Tracp, tartrate resistant acid phosphatase. min, minute. h, hour. d, day.

Table 1. Effects of acidosis on osteoclasts biology (continued)

Author/year (Ref.)	pH value	Exposure time	Osteoclast source	Acidosis-induced actions	The effects of acidosis on osteoclast biology
X Li 2017 (*82*)	6.0,7.4	6, 12, 18 h	bone marrow cells of rat femurs	upregulation the expression of αvβ3 integrin and phosphorylation of Src and Pyk2 mediated by ASIC1a	increases osteoclast migration and adhesion
Donatella Granchi 2017 (*97*)	6.9,7.4	2, 4, 7, 14d	RAW 264.7 cell line and peripheral blood mononuclear cells of health volunteers	upregulation of the expression level of RANK, CD44, DC-STAMP, Cathepsin K and MMP-9 mRNA	increase osteoclastogenesis, fusion and bone resorption activity
Elina Kylmäoja 2018 (*32*)	6.5, 7.4	14d	bone marrow mononuclear cells and peripheral blood mononuclear cells of human	None	increase osteoclastogenesis, fusion and bone resorption activity
Wenlong Liu 2019 (*16*)	7.21-7.32,7.43-7.50,7.59-7.64,7.70-7.78,7.9-8.04	5d	RAW 264.7 cell line	None	increase osteoclastogenesis and bone resorption activity
Pedro Henrique Imenez Silva 2020 (*98*)	7.05, 7.4	none	bone marrow of both $OGR1^{-/-}$ and $OGR1^{+/+}$ mice	None	increase bone resorption
Nancy S Krieger 2021 (*28*)	7.1,7.4	45min, 1d, 2d, 4d	bone marrow or spleen cell of both $OGR1^{-/-}$ and $OGR1^{+/+}$ mice	upregulation of the expression level of Cathepsin K, MMP-9, Tracp, Dc-stamp, NFATc1 and RANK RNA mediated by OGR1	increase osteoclastogenesis and bone resorption activity

PGE2, Prostaglandin E2. NFAT, Nuclear Factors of activated T. MAPK, pathway mitogen-activated protein kinase pathway. COX-2, Cyclooxygenase-2. OGR1, ovarian cancer G protein coupled receptor 1. RANKL, Receptor Activator for Nuclear Factor-κ B Ligand. TRPV, transient receptor potential vanilloid. NFATc1, Nuclear factor of activated T-cells cytoplasmic. ASIC1a, acid-sensing ion channels 1a. Pyk2, proline-rich tyrsosine kinase 2. Src, Src protein-tyrosine kinase. RANK, receptor activator of NF-KB ligand. MMP-9, matrix metalloprotein-9. Tracp, tartrate resistant acid phosphatase. min, minute. h, hour. d, day.

sensitive receptor/channels, which fall into three overall categories, including G-protein-coupled receptor (GPCR), transient receptor potential vanilloid (TRPV) and acid-sensing ion channel (ASIC) (Table 2).

3.1. GPCR

Currently, four subfamilies of GPCRs are known, which are ovarian cancer G protein coupled receptor 1 (OGR1), G protein coupled receptor 4 (GPR4), T cell death associated gene 8 (TDAG8), and G2 accumulation protein (G2A) (*48*). Compared to TRPV and ASIC, OGR1 is sensitive to weak acids, approximately at pH 6-8. For the musculoskeletal system, OGR1 signaling was initially reported in the plasma membrane of osteoblasts (*48*). $[Ca^{2+}]$i and inositol phosphate concentrations have been directly associated with the degree of OGR1 and subsequently Gq activation in osteoblasts (*49*). $[Ca^{2+}]$i is a fundamental second messenger, which can lead to a series of signaling cascades, one of which is COX2/PEG2 in osteoblasts, which stimulate bone resorption of osteoclasts and calcium release from bones *via* the paracrine system (*50*).

Similarly, OGR1 causes calcium mobilization in osteoclasts (Figure 1). On the one hand, $[Ca^{2+}]$i promotes osteoclastogenesis (*51*), especially in the early stages (*52*), and bone resorption through the $[Ca^{2+}]$i -calcineurin- NFAT signal (*21*). On the other hand, it inhibits osteoclasts apoptosis through $[Ca^{2+}]$i/PKC/ERK1/2 signaling (Figure 1) (*46*). To further investigate the functions of OGR1, Nancy S. Krieger *et al.* (*53*) established mice with a genetic null mutation in OGR1, and found that both trabecular bone and cortical bone volume increased in $OGR1^{-/-}$ mice when compared to wild-type mice. *In vitro*, the number of $OGR1^{-/-}$ mice-derived osteoblasts increased, and expressions of alkaline phosphatase, type I collagen, osterix, runx2, and RANKL were up-regulated. However, interestingly, the number of tartrate-resistant acid phosphatase (TRAP) stained-positive osteoclasts derived from $OGR1^{-/-}$ mice similarly increased, which is contrary to evidence from OGR1 studies currently. There are two probable explanations for this controversial phenomenon. One, the function of osteoblasts is enhanced so much that the function of osteoclasts that should be suppressed is promoted through paracrine signals in OGR1 global knockout mice. However, this explanation is less likely, because the activity of osteoblasts should also decrease after OGR1 is globally knocked out, but this possibility cannot be fully excluded. Two, there are other proton-sensing channels that play a compensatory role for osteoclasts, such as GPR4 and TRPV4 among others, although OGR1 is inhibited. To explain this discrepancy, this team specifically deleted OGR1 in osteoclasts, and found that $OGR1^{-/-}$ mice bone densities increased (*54*), which is comparable to the previous study (*53*). However, *in vitro*, $OGR1^{-/-}$ mice-derived osteoclast

Table 2. The acid-sensitive mechanism of osteoclasts

Author/year (Ref.)	Receptor/Channels	Cell type	Downstream	The effects of acidosis on osteoclasts biology
Svetlana V Komarova 2005 (*21*)	OGR1	osteoclasts derived from RAW 264.7 cells	PLC- Ca^{2+}-calcineurin- NFATc1	increase osteoclastogenesis, bone resorption
Meiheng Yang 2006 (*52*)	OGR1	osteoclasts derived from long bones osteopetrotic rats	none	increase osteoclastogenesis at early stages
Kaori Iwai 2007 (*96*)	OGR1	osteoclasts derived from RAW264.7 and bone marrow cells	$[Ca^{2+}]i$ - NFATc1	increase osteoclastogenesis
Alexey Pereverzev 2008 (*46*)	OGR1	osteoclasts derived from long bones of neonatal Wistar rats	$[Ca^{2+}]i$- PKC- ERK1/2	increase survival
Hideaki Tomura 2008 (*50*)	OGR1	osteoblasts derived from human osteoblastic cell line	Gq/11-PLC-Ca^{2+}	increase osteoclastogenesis
Kevin K Frick 2008 (*49*)	OGR1	primary bone cells derived from CD-1 mouse calvariae	none	increase $[Ca^{2+}]i$
Hui Li 2009 (*51*)	OGR1	osteoclasts derived from $OGR1^{+/+}$ or $OGR1^{-/-}$ mice	none	increase osteoclastogenesis
Nancy S Krieger 2016 (*53*)	OGR1	osteoclast derived from $OGR1^{+/+}$ or $OGR1^{-/-}$ mice	none	increase osteoclastogenesis, bone resorption
Nancy S Krieger 2021 (*54*)	OGR1	osteoclasts derived from osteoclast-specific deletion of OGR1 mice	none	increase osteoclastogenesis, bone resorption
Asuka Okito 2015 (*55*)	GPR4	osteoblasts derived from mouse bone marrow cells	cAMP/PKA	increase osteoclastogenesis
	TDAG8	osteoclasts derived from mouse bone marrow cells	cAMP	
Hisako Hikiji 2014 (*56*)	TDAG8	osteoclasts derived from bone marrow cells	none	decrease osteoclastogenesis, bone and calcium resorption
Bram C J van der Eerden 2005 (*57*)	TRPV5	osteoclasts derived from $TRPV5^{+/+}$ or $TRPV5^{-/-}$ mice	none	decrease osteoclastogenesis and the number of nuclei per osteoclasts, increase bone resorption
Ritsuko Masuyama 2008 (*77*)	TRPV4	osteoclasts derived from WT and $Trpv4^{-/-}$ mice	$[Ca^{2+}]i$-NFATc1	increase osteoclasts terminal differentiation, bone resorption and survival
Tom Nijenhuis 2008 (*60*)	TRPV5	osteoclasts derived from $TRPV5^{+/+}$ or $TRPV5^{-/-}$ mice	none	increase the number of resorption pits
Rossi F 2009 (*74*)	TRPV1	osteoclasts derived from healthy subjects	$[Ca^{2+}]i$	increase osteoclastogenesis
Aymen I Idris 2010 (*67*)	TRPV1	osteoclasts derived from long bones of mice	IκB and ERK1/2	increase osteoclastogenesis, bone resorption and decrease apoptosis
Estelle Chamoux 2010 (*62*)	TRPV5	osteoclasts derived from human cord blood	$[Ca^{2+}]i$ - RANKL	decrease bone resorption
Francesca Rossi 2011 (*68*)	TRPV1	osteoclasts derived from peripheral blood of menopausal women and healthy subjects	$[Ca^{2+}]i$	increase the number of nuclei per osteoclast and osteoclasts activity of healthy subjects
Kohtaro Kato 2011 (*24*)	TRPV4	osteoclasts derived from male mice	Ca^{2+}-calcineurin-myosin IIa	increase osteoclastogenesis, fusion and migration of osteoclasts
Peng Yan 2010 (*61*)	TRPV5	osteoclasts derived from bone marrow cells of the tibiae and femurs of SD rats	$[Ca^{2+}]i$	increase bone resorption
E Verron 2012 (*99*)	TRPV5	osteoclasts derived from RAW 264.7 cell line	none	None
Ritsuko Masuyama 2012 (*78*)	TRPV4	osteoclasts derived from Trpv4 null mice	$[Ca^{2+}]i$ -calmodulin	increase osteoclastogenesis, migration and bone resorption
Kainat Khan 2012 (*75*)	TRPV1	osteoclasts derived from bone marrow cells of Balb/cByJ mice	$[Ca^{2+}]i$	increase osteoclastogenesis
B C J van der Eerden 2013 (*79*)	TRPV4	osteoclasts derived from $TRPV4^{+/+}$ or $TRPV4^{-/-}$ mice	none	increase osteoclastogenesis and bone resorption for male mice instead of female mice
Fangjing Chen 2014 (*65*)	TRPV6	osteoclasts derived from $TRPV6^{+/+}$ or $TRPV6^{-/-}$ mice	none	decrease osteoclastogenesis and bone resorption
Fangjing Chen 2014 (63)	TRPV5	osteoclasts derived from SHAM and ovariectomy operation mice	none	decrease osteoclastogenesis and bone resorption
F Rossi 2014 (*69*)	TRPV1	osteoclasts derived from $TRPV1^{+/+}$ or $TRPV1^{-/-}$ mice	none	increase osteoclastogenesis and bone resorption
Francesca Rossi 2014 (*72*)	TRPV1	osteoclasts derived from β-thalassemia major patient	none	decreases osteoclastogenesis
Bram C J van der Eerden 2016 (*58*)	TRPV5	osteoclasts derived from $Trpv5^{+/+}$ and $Trpv5^{-/-}$ mice	none	increase osteoclastogenesis and bone resorption

OGR1, ovarian cancer G protein coupled receptor 1. PLC, phospholipase C. NFATc1, Nuclear factor of activated T-cells cytoplasmic 1. PKC, protein kinase C. ERK1/2, extracellular regulated protein kinases 1/2. COX-2, cyclooxygenase-2. PGE2, Prostaglandin E2. cAMP, cyclic adenosine monophosphate. PKA, protein kinase A. RANKL, receptor activator of nuclear factor-kappaB ligand. TGFβ-3, transforming growth factorβ-3. GPR4, G protein-coupled receptor 4. TDAG8, T cell deathassociated G protein 8. TRPV, transient receptor potential vanilloid. WT, Wild Type. IκB, inhibitor of NF-κB. TRAP, tartrate-resistant acid phosphatase. IGF, insulin like growth factor. ASIC1, acid sensing ion channel 1. PI3K, phosphatidylinositol 3-kinase. Pyk2, proline-rich tyrsosine kinase 2. Src, Src protein-tyrosine kinase.

Table 2. The acid-sensitive mechanism of osteoclasts (continued)

Author/year (Ref.)	Receptor/Channels	Cell type	Downstream	The effects of acidosis on osteoclasts biology
Carlotta Reni 2016 (*23*)	TRPV1	osteoclasts derived from type 1 diabetic mice	none	increase osteoclastogenesis
Giulia Bellini 2017 (*70*)	TRPV1	osteoclasts derived from dperipheral blood mononuclear cells of healthy subjects	none	increase osteoclastogenesis and bone resorption
Lin-Hai He 2017 (*73*)	TRPV1	osteoclasts derived from Trpv1 $^{+/+}$ and Trpv1 $^{-/-}$ mice	none	increase osteoclastogenesis
Tengfei Song 2018 (*64*)	TRPV5	osteoclasts derived from RAW 264.7 cell line and bone marrow-derived macrophages	none	decreases bone resorption activity and promote osteoclast apoptosis
Boran Cao 2019 (*80*)	TRPV4	osteoclasts derived from RAW 264.7 cell line	[Ca^{2+}]i-calcineurin-NFATc1	increase osteoclastogenesis and autophagy of osteoclasts
Shu Yan 2019 (*76*)	TRPV1	osteoclasts derived from bone marrow of C57/BL6 mice	none	increase osteoclastogenesis
Haruki Nishimura 2020 (*100*)	TRPV1, 4	osteoclasts derived from TRPV1/TRPV4 double knockout mice and wild type mice	none	increase osteoclastogenesis
Jun Ma 2021 (*66*)	TRPV6	osteoclasts derived from wild type and Trpv6 $^{-/-}$ mice	IGF-PI3K-AKT	decrease osteoclastogenesis, and bone resorption
Xia Li 2013 (*22*)	ASIC1	osteoclasts derived from bone marrow-derived macrophages of rats	[Ca^{2+}]i-calcineurin-NFAT	increase osteoclastogenesis
Xia Li 2017 (*82*)	ASIC1	osteoclasts derived from bone marrow of rats	αvβ3 integrin-Pyk2-Src	increase migration and adhesion

OGR1, ovarian cancer G protein coupled receptor 1. PLC, phospholipase C. NFATc1, Nuclear factor of activated T-cells cytoplasmic 1. PKC, protein kinase C. ERK1/2, extracellular regulated protein kinases 1/2. COX-2, cyclooxygenase-2. PGE2, Prostaglandin E2. cAMP, cyclic adenosine monophosphate. PKA, protein kinase A. RANKL, receptor activator of nuclear factor-kappaB ligand. TGFβ-3, transforming growth factorβ-3. GPR4, G protein-coupled receptor 4. TDAG8, T cell deathassociated G protein 8. TRPV, transient receptor potential vanilloid. WT, Wild Type. IκB, inhibitor of NF-κB. TRAP, tartrate-resistant acid phosphatase. IGF, insulin like growth factor. ASIC1, acid sensing ion channel 1. PI3K, phosphatidylinositol 3-kinase. Pyk2, proline-rich tyrsosine kinase 2. Src, Src protein-tyrosine kinase.

Figure 1. The acid-sensitive mechanism of osteoclasts mediated by OGR1. OGR1 activated by protons promotes the release of calcium from the endoplasmic reticulum. The calcium signal activates calmodulin-dependent kinase, dephosphorylates NFATc1 and promotes NFATc1 entry into the nucleus, thereby promoting osteoclastogenesis. Moreover, elevated [Ca^{2+}]i promotes the survival of osteoclasts through PKC signaling. (OGR1: ovarian cancer G protein-coupled receptor 1; CaM: calmodulin; IP3: inositol 1,4,5-trisphosphate; IP3R: inositol 1,4,5-trisphosphate receptor; CaMK: calmodulin-dependent kinase; PKC: protein kinase C; ERK1/2: extracellular regulated protein kinases 1/2.)

precursors significantly inhibited differentiation and pit formation, as well as the expression of cathepsin, MMP-9, tartrate resistant acid phosphatase (TRACP), DC-STAMP, NFATc1 and RANKL. This implies that, in the absence of interference from osteoblasts, through OGR1, protons promote osteoclastogenesis and activities of osteoclasts (*54*).

In addition to OGR1, other GPCR subfamily members, such as GPR4 and TDAG8, exhibit proton sensing abilities in the musculoskeletal system. OGR4, and the subsequent cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signals promote the release of RANKL from osteoblasts, and osteoblasts exhibit a phenotype that promotes osteoclast mineralization under acidic conditions, thereby modulating the generation and activity of osteoclasts (*55*). In contrast, TDAG8 is the only known member of GPCRs that inhibits bone resorption activities. A lack of TDAG8 leads to a significant increase in osteoclast formation, osteoclastic calcium resorption, and prevents abnormal morphologic changes of osteoclasts (*56*). Characterization of the role of TDAG8 in inhibition of bone resorption may help in elucidating the equilibrium mechanism of bone remodeling in the acidic condition.

3.2. TRPV

The transient receptor potential (TRP) family consists of several subfamilies, one of which is TRPV. TRPV1/2, TRPV3, TRPV4, and TRPV5/6 are four members in TRPV. Moreover, TRPV1/2, TRPV4 and TRPV5/6 are expressed in osteoclasts.

3.2.1. TRPV5/6

At the amino acid level, TRPV5 and TRPV6 share a 75% homology. TRPV5/6 are regulated strictly by Ca^{2+} because they are the only high-Ca^{2+} selective channels in the TRPV family. TRPV5/6 is a universal gatekeeper for epithelial cell Ca^{2+} transport (*57*). It is well known that the Ca^{2+} signal is also essential for osteoclast formation and activities.

TRPV5 is the first acid-sensitive TRPV channel member discovered in osteoclasts. However, the effects of TRPV5 on bone resorption by osteoclasts have not been conclusively determined. TRPV5-deficient mice showed gross phenotypic dysregulation of Ca^{2+} homeostasis, severe hypercalciuria and excess bone loss in the musculoskeletal system (*57-59*). Interestingly, bone resorption was significantly inhibited in *in vitro* cell cultures from TRPV5-deficient mice *(57,58,60)*. Apparently, contradictory phenotypes of reduced bone resorption and excessive bone loss concurrently appearing in the same mice are inconsistent. This implies that, *in vivo*, TRPV5-deficient mice have bone resorption compensatory mechanisms. $1,25(OH)_2D_3$ and TRPV6, as potential candidates, may contribute to this mechanism (*57,58,60*). $1,25(OH)_2D_3$ is a powerful regulator that maintains Ca^{2+} homeostasis and compensates for the loss of Ca^{2+} in the absence of TRPV5, but at the expense of enhanced bone resorption (*57*). This means that bone loss should be attributed to $1,25(OH)_2D_3$, rather than TRPV5 deletion, *in vivo*. Especially, in long-term bred TRPV5-deficient mice, old TRPV5-deficient mice bone resorption function was found to be essentially the same as that of wild mice, which is attributed to over expression of $1,25(OH)_2D_3$ in old mice compared to young ones (*58*). In short, this evidence has confirmed that TRPV5 promotes bone resorption in wild type mice (*57,58,60,61*).

The above research also has another puzzling phenomenon (*57*). Although the bone resorption capacity of osteoclasts is weakened, the number of osteoclasts increases in the absence of TRPV5. Estelle Chamoux *et al.* (*62*) postulated that there are two explanations for this puzzling phenomenon. One is that the lack of TRPV5 promotes survival of osteoclast precursors, which are TRAP-positive cells, but they exhibit low resorption activities, while the other is that a lack of TRPV5 produces dysfunctional mature osteoclasts. These are closely related to the differentiation of osteoclasts. In order to reduce disruption of differentiation, differentiated osteoclasts were used to establish study models by Estelle Chamoux *et al.* (*62*), and Estelle Chamoux *et al.* reported that TRPV5 promotes stable Ca^{2+} influx at the ruffled border and significantly inhibits human osteoclast-mediated bone resorption, inconsistent with the above research results. Subsequent research further confirmed that, TRPV5, as a target of estrogen (E2), is able to suppress osteoclastogenesis, formation of F-actin ring and an increase in osteoclast apoptosis, which diminishes bone loss. This implies that TRPV5 is a potential option for inhibiting hyperabsorption (*63,64*).

Although the function of TRPV5 has not been conclusively determined, current evidence for TRPV6 is unified. TRPV6 is a negative regulator of osteoclast differentiation and activity. In fact, TRPV6 exhibits several similar characteristics to TRPV5. For example, TRPV6 is located on the ruffled border and is associated with calcium homeostasis, and mice lacking TRPV6 were shown to exhibit a bone loss phenotype and an apparent increase in the expression of $1,25(OH)_2D_3$ (*57*). However, unlike TRPV5, TRPV6 is capable of inhibiting osteoclast formation and bone resorption activities, and these moderating effects have nothing to do with RANKL-induced calcium oscillations, therefore, other signals are involved (*65*). Differentiations and activities of osteoclasts are susceptible to some non-Ca^{2+}-independent pathways, and insulin like growth factor (IGF) is one of the candidates. NVP-AEW540, an inhibitor of IGF-1R/InsR, inhibits the increase of osteoclastogenesis induced by TRPV6-deletion. Further exploration of downstream signal transduction of IGF revealed that ratios of p-P85/P85, p-phosphoinositide dependent kinase-1(PDK1)/PDK1 and p-AKT (also known as PKB, protein kinase B)/AKT were elevated in osteoclasts isolated from TRPV6-deficient mice. Apparently, Trpv6 may aid in reducing the ratio of phosphoprotein/total protein in the IGF- phosphatidylinositol 3-kinase (PI3K)-AKT signaling pathway and lead to unfavorable functions of osteoclasts (Figure 2) (*66*).

3.2.2. TRPV1/2

TRPV1, as a non-selective cation channel, is activated by its agonists capsaicin and resiniferatoxin (RTX) as well as heat (thermal threshold > 43°C) or microenvironmental acidosis (protons) (*67*). TRPV2 is sensitive to noxious heat (thermal threshold > 52°C) and mechanical stimulation, but not to protons. Therefore, we discuss the pathological mechanisms of osteoclast overactivation under the induction of TRPV1 in acidic environments.

TRPV1 is involved in the pathophysiological processes of certain bone metabolic diseases, such as menopause (*67-69*), type 1 diabetes (*23*), glucocorticoids (*70*), or disuse (*71*) induced osteoporosis, thalassemia (*72*), and non-union after fracture (*73*). This implies that TRPV1 exerts potential impacts on osteoclasts. $TRPV1^{-/-}$ mice were found to exhibit higher bone densities, bone volume/total volume, trabecular thickness and trabecular number (*73*), and prevented bone loss associated with over-differentiation of osteoclasts in ovariectomized mice (*67,72,74*). *In vitro*, $[Ca^{2+}]i$ concentrations were found to be significantly up-regulated in osteoclasts (*69,72-74*), and TRAP-positive cells, cathepsin K expressions were also found to be elevated after administration of TRPV1 agonists, such as RTX (*74-76*). However, it is worth

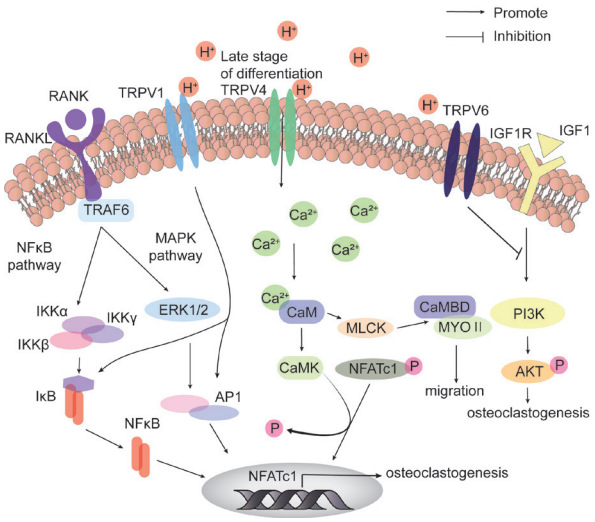

Figure 2. The acid-sensitive mechanism of osteoclasts mediated by TRPV1, 4 and 6. RANKL and its receptor RANK activates two classic pathways through TRAF6, namely the NF-κB pathway and the MAPK pathway. TRPV1 stimulated by protons respectively promotes the NF-κB pathway and the MAPK pathway, and ultimately promotes osteoclastogenesis. In the late stage of osteoclast differentiation, TRPV4 activated by protons induces an increase in the concentration of $[Ca^{2+}]i$. On the one hand, $[Ca^{2+}]i$ promotes the nuclear translocation of NFATc1 and osteoclastogenesis. On the other hand, $[Ca^{2+}]i$ and CaM forms a complex and promotes migration of osteoclast precursors under the mediation of MLCK. As a non-calcium-dependent pathway, the activation of IGF signal promotes osteoclastogenesis, however, the TRPV6 ion channel stimulated by protons inhibits the signal cascade of IGF, thereby reducing differentiation of osteoclasts. (RANKL: receptor activator of nuclear factor-kappaB ligand; RANK: receptor activator of nuclear factor-kappaB; TRAF6: TNF receptor associated factor 6; NF-κB: nuclear factor-kappa B; MAPK: mitogen-activated protein kinase; IKK: i kappaB kinase; IκB: inhibitory κB; ERK: extracellular regulated protein kinases; AP1: activator protein 1; TRPV: transient receptor potential vanilloid; CaM: calmodulin; CaMK: calmodulin-dependent kinase; MLCK: myosin light chain kinase; CaMBD: CaM-binding domain; MYO II: myosin II; IGF: insulin like growth factor; PI3K: phosphatidylinositol 3-kinase. NFATc1: nuclear factor of activated T-cells cytoplasmic 1.)

noting that some evidence proves that activated TRPV1 exerts adverse effects on the physiology of osteoclasts, which may be a consequence of desensitization of TRPV1 after long-term activation, such as in long term osteoporosis patients, or after administration of an excessive dose of agonists (*67-69,72*). Moreover, this does not imply that TRPV1 is absolutely insensitive to external stimulus after desensitization. Francesca Rossi *et al.* found that, under RTX stimulation, desensitized TRPV1 from osteoporosis patients could still promote the expression of NFκB, although it was not strong enough to affect osteoclast biology. On the contrary, with TRPV1 genetic ablation, desensitization or when subjected to its antagonists, such as capsazepine or 5′-iodo-resiniferatoxin (I-RTX), $[Ca^{2+}]i$ concentrations, peak and wave numbers of intracellular calcium oscillations were weakened (*73*), TRAP-positive cells as well as expression levels of NFATc1, cathepsin K were suppressed, RANKL-induced NFκB and MAPKs signaling were inhibited, fracture healing was impaired while caspase-3 induced apoptosis of osteoclasts was enhanced (*67-74*). Therefore, TRPV1 promotes osteoclast activities, and this effect is not compensated by osteoblasts (Figure 2) (*67*).

Interestingly, in addition to TRPV1 functions, a clear relationship between the endovanilloid and endocannabinoid system has been determined, which may relate to the mechanisms involved in osteoclasts overactivity. Cannabinoids (CB) exert their physiological activities through CB1 and CB2 cannabinoid receptors, which are co-localized with TRPV1 in osteoclast plasma membranes, and they share some endogenous agonists, such as anandamide (AEA), and some endogenous antagonists, such as URB597. When osteoclasts are exposed to the TRPV1 agonist for 48 h, CB1 and CB2 were found to be significantly up-regulated, and the CB2 gene transcription level was increased 10-fold. Vice versa, the CB1 receptor antagonist tends to enhance the expression levels of TRPV1 (*68*). Therefore, it is considerably meaningful to study the cross-talk of these receptors or channels for bone microenvironmental acidosis and metabolic disorders. Indeed, TRPV1 and CB1 play a synergistic role in the promotion of osteoclast activity, while CB2 inhibits osteoclast activities (*68-70,72,74*). Therefore, when TRPV1 is agonized and CB2 is antagonized, TRAP-positive osteoclasts and expression levels of cathepsin K are significantly increased, compared to pure agonism or antagonism (*68-70,72,74*). This implies that drugs that are characterized by inhibition of TRPV1 and promotion of CB2 may aid in the treatment of diseases that are characterized by excess osteoclast activity. However, the cross-talk between endovanilloid/endocannabinoid systems is modulated by multifactorial items, and PKCβII is one of them. Glucocorticoids, a common inducer of osteoporosis, increase in TRPV1 expression and decreases in CB2 expression in osteoclasts, however, these outcomes can be counteracted by inhibition of PCKβII. The combination of TRPV1 and PCKβII was shown to induce PCKβII activation, the activated PCKβII further phosphorylates TRPV1 and reinforces osteoclast reactivity. Therefore, PCKβII is a positive regulator of the crosstalk between the endovanilloid/endocannabinoid system (*70*).

Sirtuin 1 (SIRT1), a nicotinamide adenine dinucleotide dependent lysine deacetylase, can inhibit bone resorption. TRPV1 is a key component in SIRT1 inhibition of bone resorption (*76*). When SIRT1 is silenced, TRPV1 and its ligands are up-regulated and osteoclastogenesis is enhanced. Therefore, SIRT1 inhibits osteoclast activities by weakening TRPV1 channel activities, in other words, TRPV1-associated activation of osteoclasts is attributed to suppression of SIRT1 (*76*).

3.2.3. TRPV4

TRPV4, a non-selective Ca^{2+} channel, is stimulated

by mechanical stress, protons, heat, and its agonists, such as 4a-PDD (*77*). Unlike TRPV5, which is located on the apical side of osteoclasts, it is involved in Ca^{2+} transport during bone resorption, TRPV4, located on the basolateral membrane, is responsible for Ca^{2+} uptake during osteoclast differentiation (*24,77*). Maintaining $[Ca^{2+}]i$ concentration is an important determinant of osteoclastogenesis, and subsequently bone resorption. Sources of $[Ca^{2+}]i$ are classified into two categories, including intracellular organelles, such as endoplasmic reticulum, while the second involves the influx of $[Ca^{2+}]o$ through the Ca^{2+} channel in the plasma membrane. In the early stages of differentiation, spikes in calcium oscillation depend on the supply of intracellular organelles rather than TRPV4, however, in the latter stages, the persistent influx of Ca^{2+} induced by TRPV4 can stabilize the high concentrations of $[Ca^{2+}]i$ and induce osteoclastogenesis *via* $[Ca^{2+}]i$-NFATc1 signaling (*77,78*). Ca^{2+} binds the Calmodulin (CAM) binding domain to form a Ca^{2+}/CAM complex when $[Ca^{2+}]o$ enters pre-osteoclasts under the induction of TRPV4. As a result, the Ca^{2+}/CAM complex stimulates the expression of calmodulin kinase and further potentiates dephosphorylation and nuclear translocation of NFATc1. In addition, the Ca^{2+}/CAM complex also exerts its effects on phosphorylation of CAM-dependent myosin light chain intermediates by the myosin light chain kinase (MLCK), which initiates cytoskeletal contraction and migration of pre-osteoclasts or osteoclasts (Figure 2) (*78*). Consistent with *in vitro* experiments, $TRPV4^{-/-}$ mice exhibited a phenotype of increased bone mass, decreased osteoclastogenesis and bone resorption, however, decreased stress resistance on long bones. This outcome may partially be attributed to compensatory mechanisms of osteocytes, although it has not been confirmed (*79*).

Interestingly, *in vivo* TRPV4 studies, male animals, instead of females, are the most common models. Regarding whether gender affects the function of TRPV4, B C J van der Eerden *et al.* reported that TRPV4 predisposes males to disorders related to bone metabolic perturbation and increases the risk of fractures (*79*). Notably, male mice lacking TRPV4 showed a decrease in the number of osteoclasts and bone resorption, but, interestingly, these were not observed in female mice. In line with this would be that adult men are more at risk of fractures associated with TRPV4 compared to women. Taken together, TRPV4-induced osteoclast activity is associated with a distinct sexual dimorphism, and TRPV4 can be used as a predictor of male-specific bone mass and bone strength (*79*).

Autophagy, as a control system for maintaining cell homeostasis, significantly facilitates the production of ruffled borders, secretion of protons and degradation of the bone matrix of osteoclasts. Moreover, autophagy is involved in TRPV4-induced osteoclastogenesis (*80*). The autophagy-related protein, LC3, is redundantly expressed, and the LC3-I/LC3-II ratio is elevated when TRPV4 is overexpressed. Mechanistically, Ca^{2+} attributed to TRPV4 activation is involved in calcineurin-NFATc1 signaling, and thereby, contributes to the yield of autophagy-related proteins, which modulate osteoclastogenesis (*80*).

3.3. ASIC

ASICs are ligand-gated cation channels that are responsible for perception and response to extracellular protons by osteoclasts when pH is lower than 7.0 (*81*). ASICs contain four subunits, ASIC 1-4. The expression level of ASIC2 is the highest, followed by ASIC1, and ASIC3, while ASIC4 is rarely expressed in osteoclasts (*81*). During osteoclast differentiation, expression levels of ASIC2 are significantly suppressed, and its expression in mononuclear osteoclast precursors is 4-fold that of mature multinuclear osteoclasts.

Bone resorption of osteoclasts involves multiple steps, including differentiation of mononuclear osteoclast precursors, migration, adhesion to the bone surface, and finally, resorption of the bone matrix. ASIC1a are involved in all processes, exhibit acid sensitivity, and regulate the activities of osteoclasts in cases of acidosis (*22,82*). To further define the potential mechanisms, as expected, ASIC1a enhances the concentrations of $[Ca^{2+}]i$ in osteoclasts, leading to transcriptional as well as nuclear translocation of NFATc1 and differentiation of osteoclast precursors in acidic conditions (Figure 3) (*22*). Besides differentiation, activation of ASIC1a potentiates the expression of αvβ3 integrins, and elevates phosphorylation levels and protein interactions of Pyk2 and Src, which enhances the formation of actin rings that are required for migration and adhesion of pre-

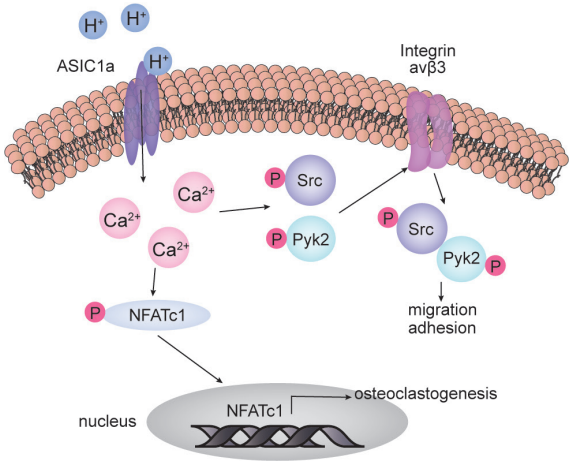

Figure 3. The acid-sensitive mechanism of osteoclasts mediated by ASICs. The acid-activated ASICs1a not only increases the concentration of $[Ca^{2+}]i$, promotes osteoclastogenesis by increasing the nuclear translocation of NFATc1, but also enhances expression of αvβ3 integrin, and its signal cascade is essential for migration and adhesion of osteoclast precursors. (ASICs1a: acid-sensing ion channels 1a; NFATc1: nuclear factor of activated T-cells cytoplasmic 1; Src: Src protein-tyrosine kinase; Pyk2: proline-rich tyrsosine kinase 2.)

osteoclasts or osteoclasts (Figure 3) (*82*).

4. The effects of microenvironmental acidosis on bone metastases

Bone metastasis is a common and serious complication in patients with multiple myeloma, breast cancer, lung cancer, prostate cancer and kidney cancer (*83*). Once tumor cells develop in bone tissue, they disrupt the balance between osteoblasts and osteoclasts, leading to osteogenic or osteolytic lesions. Osteolytic bone metastasis results in higher morbidity compared with osteogenic bone metastasis (*83*). When osteoclast-mediated bone resorption dominates, it leads to excessive remodeling of local bone and lytic lesions.

Microenvironmental acidosis is an important factor that promotes osteolytic bone metastasis (*84*). Protons in the osteolytic bone metastasis microenvironment come from two categories of sources. The first source is glycolysis in tumor cells. In fact, proliferating tumor cells exhibit a high degree of glycolysis, which produces a large amount of protons or lactic acid in the extracellular matrix, and this is known as the Warburg effect (*85*). The second source of protons is excessive bone resorption by osteoclasts (*13,17,18*).

The V-ATPase is considered to be the primary pH regulator of bone metastasis because it is exists both in tumor cells and osteoclasts (*13,17,18*). In addition to V-ATPase, other ion/proton pumps contribute to bone metastases, such as the Na^+/H^+ exchanger, monocarboxylate transporters, and carbonic anhydrase 9 (*84*). These ion/proton pumps collectively lead to acidosis in the microenvironment of osteogenic bone metastasis.

Extracellular acidification of bone metastasis generally has three consequences. The first consequence is that it enhances invasion and aggressiveness of tumor cells (*86*). The second outcome is the promotion of differentiation and activity of osteoclasts. As mentioned above, osteoclasts are sensitive to protons, hence extracellular acidification stimulates various proton sensing pathways/receptors of osteoclasts leading to excessive bone remodeling (*27,36-40*). The third consequence is cancerous bone pain (*87*).

Of note, once tumor cells settle in the bone tissue, they are stimulated by protons thereby affecting the functions of osteoclasts through parathyroid hormone related protein (PTHrP), interleukin (IL)-11, and Jagged 1 (*88*). PTHrP and IL-11 enhance the production of RANKL, which stimulates the formation and activation of osteoclasts. On the other hand, Jagged 1 promotes the fusion of osteoclast precursors by directly binding to monocytes (*88*). Moreover, being a multinucleated giant cell with complex functions, osteoclasts consume a lot of energy during bone resorption (*33*). The acidified bone metastasis microenvironment improves mitochondrial function, thereby promoting the survival of osteoclasts and maintaining bone resorption (*89*).

Bone pain is a common symptom in patients with osteolytic bone metastases. Protons can directly stimulate acid-sensitive ion channels (such as TRPV1 and ASIC3) expressed on bone sensory neurons, thereby triggering pain-causing signals (*87*). Studies have indicated that suppressing acid-sensitive ion channels using specific ASIC3 antagonist APETx2 and specific TRPV1 inhibitor JNJ-17203212 can inhibit cancer-induced bone pain (*90,91*).

Taken together, the extracellular microenvironment of osteolytic bone metastasis is protonated by glycolytic tumor cells and excessive activity of osteoclasts (*13,17,18,85*). Extracellular acidosis of osteolytic bone metastasis can, not only enhance the invasion and aggressiveness of tumors, but also evoke osteolysis, and cancer-induced bone pain (*27,36-40,86,87*).

5. Conclusion

Bone remodeling is precisely regulated by the dynamic balance between bone formation induced by osteoblasts and bone resorption induced by osteoclasts, which occurs in the bone microenvironment (*13,17,25,73*). Virtually, it will inevitably elicit perturbation of the musculoskeletal system homeostasis and emergence of osteogenic or osteolytic disorders when this equilibrium is broken (*13,17,73*). Osteoclasts are multinucleated giant cells that are activated by M-CSF and RANKL, which play a pivotal role in diseases that are characterized by bone loss, such as osteoporosis, multiple myeloma and Paget's disease among others (*13,14,19*). The activity of osteoclasts is also influenced by the pathological state of the extracellular microenvironment, such as hypoxia, inflammation, mechanical stress and particularly, acidosis (*14*).

Protons are closely associated with osteoclast formation and functions. When osteoclasts are directed to move to resorption sites, H^+, Cl^-, and certain acid proteases are pumped into the sealing zones, leading to regional dissolution of the bone matrix (*13,33*). H^+ are obtained from three sources, the first is that they are byproducts of high mitochondrial metabolism in osteoclasts. In fact, in the process of bone resorption, osteoclasts require mitochondrial hyperactivation to maintain their high metabolic activities (*33*). The second is carbonic acid produced by carbonyl anhydrase II (CA II), which tends to split into protons and bicarbonate (*92,95*). Finally, they are obtained from extracellular acidosis. In *in vitro* simulation of osteoclasts, [pH]i was shown to drop to 6.8 as a consequence of [pH]o droping to 6.5, implying that osteoclasts are the local responding units for protons (*38*).

Numerous acid-sensitive channel subunits provide the basis for the response to pH of osteoclasts. Most of them share the feature with specificity increases $[Ca^{2+}]$i of osteoclast induced by protons. Ca^{2+} is a

common second messenger that induces various cellular biological functions, especially in osteoclasts. The [Ca^{2+}]i and the subsequent signaling cascade is essential for osteoclastogenesis (*46,80*). Although calcium oscillations are initially regulated by organelles that store Ca^{2+}, at late stages of osteoclast differentiation, elevated concentrations of [Ca^{2+}]i rely on the acid-sensitive channel, such as TRPV4 (*77*). Furthermore, for acid-sensitive Ca^{2+} channels, their functions correspond with membrane positioning. For example, high quantities of degradation products, including broken collagen, Ca^{2+} and phosphate are generated in the sealing zone during bone resorption (*13,33*), TRPV5/6, located on the apical side, is responsible for mediating Ca^{2+} translocation into the cytoplasm, thereby promoting the pumping out of Ca^{2+} (*57*). In contrast, TRPV4, located on the basolateral membrane, is involved in Ca^{2+} uptake in the late stages of osteoclast differentiation (*77*), furthermore, TRPV1, located on the ruffled border membrane, is involved in calcium mobilization along with the endocannabinoid system (*73,74*), and promotes the formation of osteoclasts. In addition to osteoclasts themselves, the paracrine elements of osteoblasts are also involved in activation of osteoclasts in acidic microenvironments, among them, PEG2 and RANKL are involved in facilitation of osteoblasts to osteoclasts (*39-42*).

Acid-sensitive channels promote multiple pivotal physiological activities of osteoclasts, however, evidence suggests that some inhibit, at least in part, osteoclast activities, such as TDAG8 and TRPV6 (*56,65*). Therefore, the acidosis microenvironment dynamically and bidirectionally regulates the activity of osteoclasts instead of invariably and unidirectionally, however, the members of inhibitory receptors and especially its mechanism are incomplete. In addition, genetic ablation of certain acid-sensitive receptors/pathways leads to contradictory bone phenotypes (*57,67,79*), implying that the relationship between the acidosis microenvironment and bone remodeling is multifactorial and complex *in vivo*. Although 1,25 $(OH)_2D_3$ or parathyroid hormone (PTH) are involved in compensation after certain acid-sensitive pathways are suppressed as far as we know (*57,60*), it is still incompletely understood to a large extent, which requires additional new insights into this issue.

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