Journal of Cellular Biochemistry 97:45–55 (2006)

Calmodulin Is a Critical Regulator of Osteoclastic Differentiation, Function, and Survival

Eric C. Seales,[1] Keith J. Micoli,[1] and Jay M. McDonald[1,2]*

[1]Department of Pathology, University of Alabama at Birmingham, 619, 19th Street South, West Pavilion 220, Birmingham, Alabama 35233-7331
[2]Veterans Administration Medical Center, Birmingham, Alabama

Abstract Increased osteoclastic resorption and subsequent bone loss are common features of many debilitating diseases including osteoporosis, bone metastases, Paget's disease, and rheumatoid arthritis. While rapid progress has been made in elucidating the signaling pathways directing osteoclast differentiation and function, a comprehensive picture is far from complete. Here, we explore the role of the Ca^{2+}-activated regulator calmodulin in osteoclastic differentiation, functional bone resorption, and apoptosis. During active bone resorption, calmodulin expression is increased, and calmodulin concentrates at the ruffled border, the organelle utilized for acid transport and bone dissolution. Pharmacologic inhibitors of calmodulin, several of which are already used clinically as anti-cancer and anti-psychotic agents, inhibit osteoclastic acid transport, suggesting their potential as bone-sparing drugs. Recent studies also implicate calmodulin in osteoclast apoptosis through a mechanism involving its direct interaction with the death receptor Fas. During osteoclastogenesis, RANKL-induction stimulates a rise in intracellular Ca^{2+}, which in turn activates calmodulin and its downstream effectors. In particular, the Ca^{2+}/calmodulin-dependent phosphatase calcineurin and its targets, the NFAT family of transcription factors, have been posited as the master regulators of osteoclastogenesis. However, recent in vivo and in vitro studies demonstrate that another Ca^{2+}/calmodulin-regulated effector protein, CaMKII, is also involved. $CaMKII^{+/-}$ mutant mice have reduced osteoclast numbers, and CaMKII antagonists inhibit osteoclastogenesis in vitro. Furthermore, CaMKII is known to activate AP-1 transcription factors, which are also required for RANKL-induced osteoclast gene transcription, and recent findings suggest that CaMKII can down-regulate gp130, a cytokine receptor involved in bone remodeling and implicated in numerous osteo-articular diseases. J. Cell. Biochem. 97: 45–55, 2006. © 2005 Wiley-Liss, Inc.

Key words: calmodulin; osteoclast; osteoclastogenesis; calcium; calcineurin; calmodulin-dependent protein kinase II (CaMKII); acid transport

OSTEOBLASTS, OSTEOCLASTS, AND BONE TURNOVER

Bone is a dynamically-regulated organ system responsible for maintaining structural/mechanical integrity, and it also serves as the main reservoir of calcium in the body. Maintenance of stable bone mass and microarchitecture (homeostasis) relies on a constant, balanced process of turnover mediated by the specialized cells of the bone microenvironment, osteoblasts and osteoclasts. Osteoblasts, terminally-differentiated cells originating from local bone mesenchymal stem cell precursors, are primarily responsible for the production of organic bone matrix and its subsequent mineralization [Aubin, 1998]. Osteoclasts, large multinucleated cells derived from the hematopoietic, monocyte–macrophage lineage, perform the opposing function of bone resorption [Teitelbaum, 2000]. Resorption is carried out by a specialized osteoclast organelle, the ruffled membrane, which uses a vacuolar H^+-ATPase (V-ATPase) to pump bone-dissolving H^+ into a sealed extracellular compartment between the osteoclast and bone surface, a process which ultimately releases free calcium (Ca^{2+}) into the extracellular compartment [Blair et al., 1989]. While

*Correspondence to: Jay M. McDonald, Department of Pathology, University of Alabama at Birmingham, 619, 19th Street South, West Pavilion 220, Birmingham, AL 35233-7331. E-mail: mcdonald@path.uab.edu

Received 24 August 2005; Accepted 26 August 2005

DOI 10.1002/jcb.20659

© 2005 Wiley-Liss, Inc.

healthy bone maintains a net balance between the opposing processes of osteoclastic resorption and osteoblastic production, bone loss occurring in diseases such as osteoporosis, arthritis, and tumor metastases to bone is commonly due to increased osteoclast numbers and/or resorptive activity. Accordingly, bisphosphonates, which impede bone resorption, are currently the main pharmacologic tool for prevention of bone loss. While the hormone estrogen is capable of blocking osteoclastogenesis, estrogen's adverse tumorigenic and cardiovascular effects ultimately limit its clinical utility. Therefore, elucidation of the mechanisms controlling osteoclast proliferation, differentiation, bone resorptive activity, and survival is necessary in order to identify new targets for pharmacotherapeutic intervention.

RANKL, M-CSF, AND Ca^{2+} IN OSTEOCLAST DIFFERENTIATION AND FUNCTION

For many years, analysis of mechanisms directing osteoclastogenesis and bone resorption was hampered by the necessity of creating osteoclasts through co-culture of osteoclast precursors with bone stromal cells and osteoblasts [Zhang et al., 2003; Takayanagi, 2005]. Recently, two hematopoietic factors provided by this co-culture system, receptor activator of nuclear factor-κB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF), have been identified as the minimal essential drivers of osteoclastogenesis [Boyle et al., 2003]. Supplementation of culture media with RANKL and M-CSF is sufficient to generate osteoclasts in vitro in the absence of osteoblasts or bone stromal cells. However, other factors present in serum from cell culture media, such as IL-1, IFN-γ, TGF-β, and TNF-α, also regulate osteoclastogenesis, and many groups including our own have found that the reliability of in vitro osteoclastogenesis depends on the batch of serum used, strongly suggesting that presently unidentified serum factors are also necessary to modulate RANKL/M-CSF-directed osteoclastogenesis [Zhang et al., 2005].

M-CSF binding to the c-fms receptor is required for the survival and proliferation of preosteoclasts, while RANKL binding to RANK is required for differentiation (Fig. 1). Activated RANK recruits TNF receptor-associated factors (TRAF) 1, 2, 3, 5, and 6 to its cytoplasmic tail. TRAFs in turn activate downstream signaling

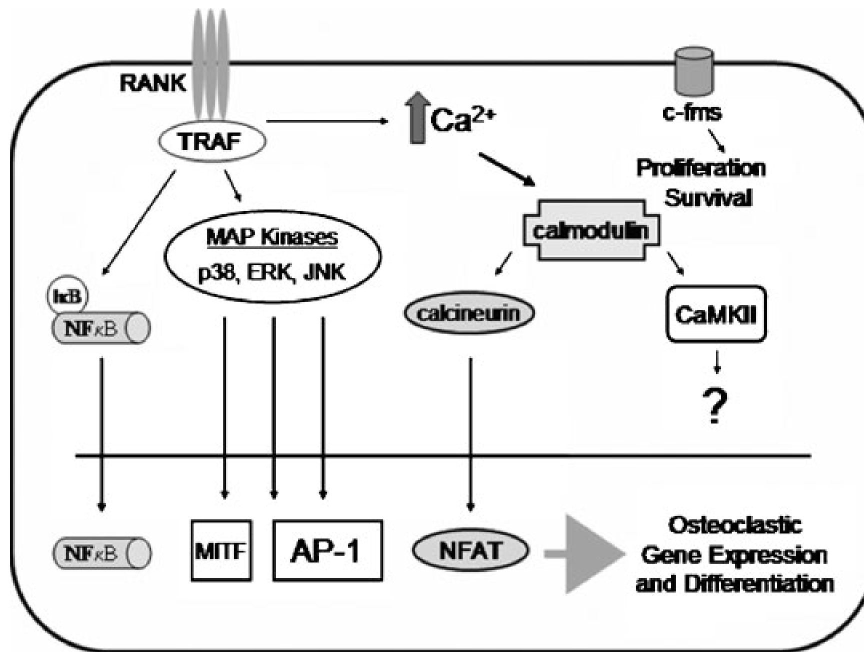

Fig. 1. Signaling cascades in osteoclastogenesis. Activation of c-fms promotes proliferation and survival of osteoclast precursors. Activation of RANK, a requirement for osteoclast differentiation, leads to TRAF association and downstream activation of NFκB and the MAPK family members: p38, ERK, and JNK. RANK activation also leads to a rise in intracellular Ca^{2+}, which, upon binding to the Ca^{2+}-transducer calmodulin, activates calcineurin and CaMKII. NFκB, MAPK-activated transcription factors including MITF and AP-1, and the calcineurin-activated NFATs then coordinately regulate osteoclast-specific gene transcription.

cascades critical for osteoclast differentiation, resorptive function, and survival. Targets of TRAFs include the nuclear factor-κB (NFκB) transcription factor, all three members of the MAPK family, ERK, JNK, and p38, as well as the serine/threonine kinase, Akt. p38 induces the transcription factor MITF; downstream effectors of ERKs and JNKs include the AP-1 transcription factors, a family of heterodimers formed between Fos family members (c-Fos, Fra-1, Fra-2, and FosB) and Jun family members (c-Jun, JunB, and JunD). In conjunction with other transcription factors, AP-1 factors regulate expression of osteoclast marker genes such as cathepsin K (CTSK), calcitonin receptor (CTR), tartrate-resistant acid phosphatase (TRAP), and αv and β3 integrin subunits [Boyle et al., 2003; Lee and Kim, 2003; Teitelbaum, 2004]. Another family of transcription factors, nuclear factor of activated T cells (NFAT) 1 and 2 have recently been implicated in osteoclastogenesis, with NFAT2 described as a "master regulator" of osteoclastogenesis due both to its cooperative interaction with AP-1 and NFκB in osteoclastic gene transcription and its proposed auto-amplification, a strategy known to be utilized for lineage commitment in T cells, cardiac, and skeletal muscle cells [Hogan et al., 2003; Takayanagi, 2005].

Involvement of NFATs directly implicates Ca^{2+} signaling in osteoclastogenesis since NFAT activation and subsequent nuclear translocation is directed by the Ca^{2+}/calmodulin-dependent serine/threonine phosphatase calcineurin [Hogan et al., 2003]. RANKL activation of RANK induces a rise in cytosolic and nuclear Ca^{2+} [Myers et al., 1999]. RANK activation-induced intracellular Ca^{2+} oscillations in pre-osteoclasts mediate osteoclastic differentiation [Takayanagi et al., 2002], and pharmacologic manipulation of intracellular Ca^{2+} levels affects osteoclastic AP-1 and NFκB signaling, differentiation, and apoptosis in the context of RANK induction [Yip et al., 2005]. Presumably, RANKL-stimulated elevation of intracellular Ca^{2+} exerts its downstream effects on osteoclast differentiation and function first by activating Ca^{2+}-binding proteins. In osteoclasts, evidence from our lab points strongly to the multifunctional Ca^{2+}-sensor calmodulin as a regulator of osteoclastic differentiation, function, and survival [Radding et al., 1994; Williams et al., 1996, 1997, 2000, 2003a,b; Manion et al., 2000; Zhang et al., 2003, 2005].

CALMODULIN: A TRANSDUCER OF Ca^{2+} SIGNALING IN OSTEOCLASTS

Metabolically, bone serves as the body's Ca^{2+} reservoir (with Ca^{2+} and phosphates bound in the form of hydroxyapatite crystals) and buffers free Ca^{2+} to a level of 10^{-3} M in extracellular fluids [Chin and Means, 2000]. This high extracellular Ca^{2+} level contrasts dramatically with levels in the intracellular compartment, where homeostatic mechanisms aggressively maintain cytosolic Ca^{2+} at roughly 10^{-7}–10^{-6} M. The tremendous potential energy maintained by this 10,000-fold concentration gradient across the plasma membrane makes Ca^{2+} ideally suited for its role as a an intracellular second messenger. Through control of both receptor-mediated Ca^{2+} entry and subsequent intracellular sequestration, the timing, amplitude, frequency, and sub-cellular localization of Ca^{2+} can be tightly regulated, allowing a simple ion to activate diverse signaling cascades, direct cell behavior, and regulate proliferation, differentiation, and even apoptosis in a wide range of cell types [Chin and Means, 2000; Soderling and Stull, 2001].

Since most Ca^{2+}-responsive proteins do not directly bind free Ca^{2+}, a cellular response to the intracellular Ca^{2+} signal first requires binding to an intermediary protein, which then in turn transduces the signal into downstream effects. The most important of these Ca^{2+} transducers is calmodulin, a highly conserved 17 kDa protein found ubiquitously throughout animals, plants, fungi, and protozoa. Structurally, calmodulin belongs to a family of proteins utilizing an "EF-hand" motif for Ca^{2+} binding. Calmodulin contains four Ca^{2+}-binding EF-hands arranged in pairs at both its globular N and C termini, with a long α-helix linking the two. Calmodulin's K_d for Ca^{2+} is in the 10^{-6}–10^{-7} M range, making it well-suited to respond to transitory intracellular Ca^{2+} spikes resulting from receptor-mediated stimulation events. In contrast, proteins with much higher Ca^{2+} affinity ($K_d < 10^{-7}$ M) are not as readily able to disengage from Ca^{2+} following a decline in intracellular Ca^{2+}; conversely, proteins with much lower affinity for Ca^{2+} ($K_d > 10^{-6}$ M) are not sensitive to the physiologic range of Ca^{2+} spikes and are more suited to the role of Ca^{2+} buffering (e.g., calsequestrin and calnexin). In response to transient increases in intracellular Ca^{2+}, Ca^{2+} binding by the four EF-hands of calmodulin

leads to a dramatic conformational shift, creating an open form of calmodulin characterized by solvent-exposed hydrophobic pockets within the EF-hands distinct from the Ca^{2+}-binding sites. It is this Ca^{2+}-directed conformational shift which primes calmodulin for activation of its downstream effector proteins [Chin and Means, 2000; Means, 2000; Soderling and Stull, 2001].

CALMODULIN IN OSTEOCLAST ACID TRANSPORT AND BONE RESORPTION

Osteoclastic bone resorption requires both H^+ secretion from a V-ATPase found at the ruffled membrane and coordinated delivery of acid proteases (such as CTSK) into the resorption pit, leading to degradation of bone matrix and release of massive quantities of free Ca^{2+} (Fig. 2). Exposure of osteoclasts to high extracellular Ca^{2+} likely exerts pressure on Ca^{2+} buffering mechanisms and modulates the function of Ca^{2+}-directed proteins such as calmodulin. This hypothesis is consistent with the discovery of a calmodulin-sensitive Ca^{2+}-ATPase in the osteoclast plasma membrane [Bekker and Gay, 1990], a finding which encouraged us to explore calmodulin-mediated regulation of acid secretion.

An early study showed that mature avian osteoclasts increase calmodulin expression twofold to threefold following bone attachment compared to plastic-attached cells, and calmodulin was enriched in areas of bone attachment and the ruffled membrane [Radding et al., 1994]. These observations suggested a functional role for calmodulin in bone resorption. In osteoclasts treated with the calmodulin antagonist trifluoroperazine (TFP), bone resorption was dose-dependently inhibited, an effect not due to TFP-mediated changes in osteoclastic bone attachment. Using acid transport assays with purified avian osteoclast ruffled membranes, the calmodulin inhibitor calmidazolium blocked vesicle acidification, an effect reversed by addition of calmodulin to the assay [Radding et al., 1994]. Collectively, these results showed that calmodulin concentrates at the ruffled border and regulates acid secretion.

Interestingly, both tamoxifen (TMX), a compound with both estrogen agonistic and antagonistic capacity, and its active metabolite

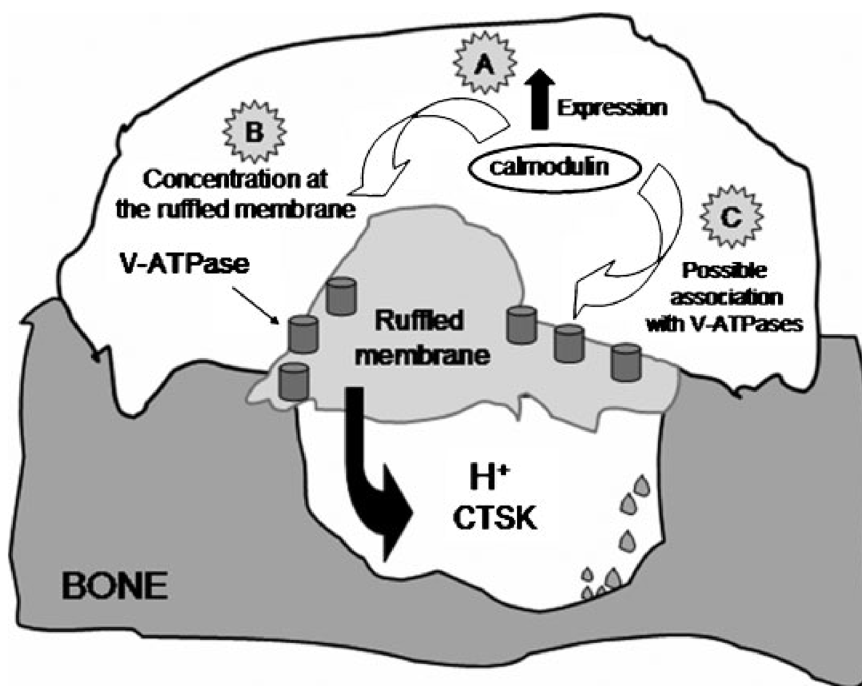

Fig. 2. Calmodulin and bone resorption. Following the attachment of mature osteoclasts to bone, calmodulin protein levels are upregulated twofold to threefold (**A**), and calmodulin is concentrated in areas of bone attachment, particularly the acid-secreting ruffled membrane (**B**). While the mechanisms by which calmodulin regulates acid secretion are not known, a likely scenario involves direct interaction with a subunit of V-ATPase (**C**).

4-hydroxytamoxifen, also inhibit resorption in bone-attached osteoclasts [Williams et al., 1996], a finding consistent with the seemingly paradoxical bone-sparing capacity of TMX [Turner et al., 1988]. Since TMX is a calmodulin antagonist with potency similar to TFP, TMX-induced inhibition of bone resorption could be mediated either through calmodulin or estrogen receptor signaling. However, neither estrogen receptor agonists (17-β-estradiol and diethylstilbestrol) nor the estrogen receptor antagonist ICI 182780 affect bone resorption at estrogen receptor-saturating concentrations, indicating that estrogen signaling pathways do not regulate osteoclastic bone resorption. ICI 182,780 does inhibit resorption at concentrations 1,000-fold higher than its K_d value for the estrogen receptor; however, at these concentrations, ICI 182780 was shown to inhibit calmodulin activity [Williams et al., 1996]. These findings showed TMX-induced inhibition of resorption is mediated through calmodulin, not estrogen receptor signaling.

TMX is also a protein kinase C (PKC) inhibitor [Horgan et al., 1986], and the PKC inhibitor bis-indolylmaleimide (bIM) can concentration-dependently inhibit bone resorption and V-ATPase-mediated acid transport activity. While this does implicate PKC in regulation of osteoclastic acid transport, half-maximal inhibitory concentrations of TMX and bIM are not additive, suggesting different mechanisms of action. Furthermore, calmodulin addition reverses the TMX-mediated, but not the bIM-mediated, inhibition, indicating once again that TMX inhibits resorption through calmodulin, not PKC [Williams et al., 2003b]. Interestingly, phorbol esters, which induce short-term activation of PKC but long-term downregulation of PKC protein levels, stimulate bone resorption [Williams et al., 2000]; however, this increased phorbol ester-mediated resorption occurred several days after the downregulated expression of all PKC isoforms. Phorbol ester-mediated increases in bone resorption were temporally correlated with an increase in calmodulin expression, and TMX and TFP inhibited the PMA-induced increases in both bone resorption and calmodulin expression [Williams et al., 2000], strongly suggesting that phorbol esters promote bone resorption through regulation of calmodulin rather than PKC.

Collectively, we have established calmodulin as a regulator of osteoclast acid transport. However, it is not known whether calmodulin regulates this process through direct interactions with proteins involved in acid transport or through activation of a downstream Ca^{2+}/calmodulin-directed effector. Cyclosporine A (CsA)-mediated inhibition of the Ca^{2+}/CaM-dependent phosphatase, calcineurin, exerts a negligible effect on osteoclastic bone resorption. Furthermore, isolated ruffled membranes treated directly with CsA or isolated from CsA-treated osteoclasts showed no inhibition of acid transport activity, demonstrating that calcineurin does not act downstream of calmodulin in regulating acid transport at the ruffled membrane [Williams et al., 2003a]. A more plausible scenario is direct interaction of calmodulin with the ruffled membrane V-ATPase. Indeed, a subunit of the yeast V-ATPase binds calmodulin and is functionally responsive to Ca^{2+}/calmodulin activation [Burgoyne and Clague, 2003].

CALMODULIN, Fas, AND APOPTOSIS

The use of calmodulin antagonists to prevent the function of mature osteoclasts is one way to preserve bone mass; however, recent reports also support the hypothesis that calmodulin plays a role in osteoclast apoptosis. Enhancing osteoclast apoptosis is another potential therapeutic mechanism to prevent bone loss. Bisphosphonates prevent apoptosis in osteoblasts and promote apoptosis in osteoclasts, thereby preserving bone mass [Plotkin et al., 1999]. Conversely, agents such as glucocorticoids induce apoptosis in osteoblasts but prevent apoptosis in osteoclasts, and thus cause loss of bone mass by simultaneously preventing new bone formation and enhancing bone resorption [Weinstein et al., 2002].

Bisphosphonates have been reported to induce osteoclast apoptosis in part through a Fas expression-dependent mechanism involving activation of caspase 3, although apoptosis could not fully account for the anti-resorptive effects of the bisphosphonates [Wang et al., 2000; Benford et al., 2001]. Fas is a transmembrane TNFR superfamily receptor that is expressed in a variety of cell types, and mediates cell death by induction of caspase activity. Recent reports show that Fas-mediated apoptosis may play a direct role in controlling bone homeostasis, as aged mice defective in Fas signaling (*lpr* and *gld*) have decreased bone mass [Wu et al., 2003, 2005a]. Expression of Fas has

been shown in both osteoblasts and osteoclasts, and defective Fas-mediated apoptosis has been implicated in the pathogenesis of rheumatoid arthritis [Mountz et al., 1994; Kawakami et al., 1997; Wu et al., 2005a]. Induction of the Fas pathway in osteoclasts is an attractive target for reducing osteoclast numbers and thus, preventing bone resorption. In vitro studies have shown that high levels of RANKL increase Fas expression during osteoclast differentiation but suppress Fas expression and sensitivity to Fas-mediated apoptosis in mature osteoclasts [Wu et al., 2005b].

Calmodulin has been shown to bind directly to Fas in osteoclasts and to play a role in Fas-mediated apoptosis, and calmodulin antagonists have been shown to induce apoptosis in several cell types [Wu et al., 2005a]. Calmodulin antagonists including TMX and TFP induce osteoclast apoptosis in a manner independent of Fas receptor activation. In fact, in Lpr^{-cg} mice, which lack binding of calmodulin to Fas, calmodulin antagonists induced greater levels of osteoclast apoptosis compared to wild-type controls. These studies suggest that calmodulin antagonists may also prevent or reverse bone loss by inducing osteoclast apoptosis.

CALMODULIN IN OSTEOCLASTOGENESIS AND RANKL SIGNALING

While calmodulin regulates acid transport in mature osteoclasts, its potential role in osteoclastogenesis has only recently been explored. During RANKL induction of human peripheral blood mononuclear cells, calmodulin gene expression is upregulated, suggesting a functional role for calmodulin in osteoclastogenesis [Day et al., 2004]. Recently, we showed that pharmacologic calmodulin antagonists (TFP, W7, and TMX) dose-dependently inhibited RANKL-induced osteoclastogenesis in both primary mouse bone marrow macrophages and in the RAW264.7 cell line as measured by reduced TRAP activity and numbers of multinucleated osteoclasts formed. Inhibition was not due to apoptosis since co-treatment with both TFP and a broad caspase inhibitor under identical conditions had no effect. Intriguingly, inhibitory effects on osteoclastogenesis occurred only during the last 24 h of RANKL stimulation, with 72 h calmodulin inhibitor treatment followed by withdrawal for the last 24 h exerting no effect [Zhang et al., 2003]. This 24 h time window from day 3 to 4 is when pre-osteoclasts begin fusing into multinucleated cells. How calmodulin might modulate fusion during late osteoclastogenesis is not known; however, calmodulin has been implicated in numerous cellular functions requiring membrane fusion including synaptic exocytosis and homotypic fusion of early endosomes [Burgoyne and Clague, 2003]. Both of these processes require activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), suggesting a role for CaMKII in osteoclastogenesis.

Since activation of RANK is critical for osteoclastogenesis, calmodulin-directed regulation of RANKL-induced signaling cascades was examined. TFP-induced calmodulin inhibition selectively downregulated RANKL-induced phosphorylation of both JNK and IκBα, the inhibitory subunit of the NFκB complex. In contrast, TFP did not block RANKL-induced phosphorylation of p38 [Zhang et al., 2003]. These findings implicate calmodulin in regulation of osteoclastic gene expression through both the NFκB and AP-1 transcription factor families.

Though TFP and other calmodulin antagonists can also inhibit PKC [Brown et al., 2000], we found that treatment with the PKC inhibitor bIM, even at concentrations 10-fold higher than its IC_{50}, failed to inhibit osteoclastogenesis, strongly suggesting TFP and other calmodulin inhibitors block osteoclastogenesis through antagonizing calmodulin and not PKC. This hypothesis is supported by the observation that calmodulin overexpression protects RAW264.7 cells against the inhibitory effects of TFP. Since the calmodulin antagonist TMX could potentially affect osteoclastogenesis through interaction with estrogen receptors, the effects of the pure estrogen antagonist ICI 182780 was examined, both at concentrations known to antagonize estrogen receptors and at a much higher concentration known to inhibit calmodulin. ICI 182780 did not block osteoclastogenesis in the lower concentration range; in contrast, at a calmodulin-inhibitory concentration, osteoclastogenesis was inhibited significantly, demonstrating that TMX inhibits osteoclastogenesis through calmodulin antagonism and not through its affects on estrogen signaling. Interestingly, TFP was able to reduce osteoclast numbers and restore trabecular bone volume in ovariectomized mice just as effectively as estrogen [Zhang et al., 2003]. In these

studies, the effective serum concentration of TFP (an antipsychotic agent sold under the brand name Stelazine) averaged 93 nM. This is comparable to the therapeutic range in humans (8–83 nM), making TFP, or its analogs, a potentially useful agent for prevention of bone loss.

CALMODULIN EFFECTOR PROTEINS IMPLICATED IN OSTEOCLASTOGENESIS

Over 50 calmodulin-binding proteins have been identified including kinases and phosphatases, nitric-oxide synthase (NOS), numerous receptors, ion channels, G-coupled proteins, and transcription factors [Berridge et al., 2000; Hoeflich and Ikura, 2002]. Of particular interest are two Ca^{2+}/calmodulin-activated proteins experimentally implicated in osteoclastic differentiation and/or function: the serine/threonine phosphatase calcineurin and the multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII).

Calcineurin and NFAT2

NFAT transcription factors, which regulate a large numbers of genes and direct differentiation in numerous cell types, are distinct in their regulation by the Ca^{2+}/calmodulin-dependent serine/threonine phosphatase calcineurin. Structurally, calcineurin is a heterodimer consisting of a catalytic subunit (calcineurin A) bound to a Ca^{2+}-binding regulatory subunit (calcineurin B); despite calcineurin B's Ca^{2+}-binding potential, Ca^{2+}-mediated activation requires Ca^{2+}/calmodulin binding to the regulatory domain of calcineurin A. Upon Ca^{2+}/calmodulin stimulation, calcineurin dephosphorylates cytoplasmic NFATs, allowing their subsequent nuclear translocation and transcriptional activity [Klee et al., 1998; Hogan et al., 2003].

Recently, activation of calcineurin and, in particular, NFAT2 (a.k.a. NFATc1) has been proposed as a necessary step in osteoclastogenesis. In both murine bone marrow cultures and RAW264.7 cells, the calcineurin inhibitor CsA dose-dependently blocked RANKL-induced formation of $TRAP^{+}$ multinucleated osteoclasts [Ishida et al., 2002; Takayanagi et al., 2002]. The essential requirement for NFAT2 was demonstrated both by the complete inability of $NFAT2^{-/-}$ embryonic stem cells to undergo osteoclastic differentiation [Takayanagi et al., 2002], and by inhibition of RANKL-induced osteoclast formation in RAW264.7 cells treated with an NFAT2 antisense construct [Ishida et al., 2002].

Sustained upregulation of NFAT2 is a crucial aspect of lineage commitment in numerous cell types, and this same pattern appears to hold true in osteoclastogenesis. However, whether sustained NFAT2 upregulation involves auto-amplification (as it does in T cells) is still controversial. In the early stages of RANKL induction, NFAT2 expression is upregulated, most likely in response to transcriptional induction by NFAT1, NFκB, and the AP-1 family members, c-Fos and c-Jun [Hogan et al., 2003; Teitelbaum, 2004]. The presumption has been that, following early induction of NFAT2, NFAT2 can auto-amplify itself through binding to NFAT consensus sites within its own promoter. This sustained auto-amplification thus "locks" the pre-osteoclast into a terminal differentiation pathway. Supporting this hypothesis, the calcineurin inhibitor FK506 was shown to block NFAT2 amplification in bone marrow pre-osteoclasts, and NFAT2 overexpression induced expression of endogenous NFAT2 [Takayanagi et al., 2002]. However, other studies cast doubt on the NFAT auto-amplification hypothesis. For instance, CsA-mediated calcineurin inhibition failed to block RANKL-induced upregulation of NFAT2 expression in RAW264.7 cells despite CsA's ability to dose-dependently inhibit nuclear transport of NFAT2 [Ishida et al., 2002]. Furthermore, in human osteoclasts, RANKL-induced upregulation of NFAT2 was not blocked by co-treatment with high doses of CsA (1 μg/ml) [Day et al., 2005]. Clearly, further studies will be required to confirm or disprove the existence of an NFAT2 autoregulatory loop in osteoclastogenesis.

Unfortunately, elucidation of the functional role of calcineurin/NFAT signaling in osteoclastogenesis has been hampered in most studies by a heavy reliance on the calcineurin inhibitors, CsA and FK506. CsA and FK506 first require binding to cyclophilins and FK506 binding proteins (collectively known as immunophilins) before they can inhibit calcineurin. In most cell types, immunophilin concentrations are unknown, and only certain immunophilins can form active calcineurin-inhibiting complexes with CsA or FK506 [Kahl and Means, 2003], Thus, the effective concentrations of CsA or FK506 needed for calcineurin inhibition are not established and vary widely from one cell

type to another. Furthermore, recent studies using CsA or FK506 to inhibit osteoclastogenesis did not directly measure calcineurin activity [Ishida et al., 2002; Takayanagi et al., 2002], raising the possibility that inhibitory effects were mediated through other signaling pathways besides calcineurin such as p38 and SAP/JNK, which are known to be downregulated by CsA [Su et al., 1994; Matsuda et al., 1998] CsA and FK506 also block cell proliferation [Kahl and Means, 2003], and a recent study showed that CsA and FK506 induce apoptosis in mouse bone marrow cultures [Igarashi et al., 2004]. In this study, apoptosis was induced with an FK506 concentration of 0.01 μM (8 ng/ml); therefore, apoptosis rather than calcineurin/NFAT2 inhibition, may have been the true cause for the complete inhibition of $TRAP^+$ multinucleated cell formation observed by Takayanagi at 100 ng/ml (0.125 μM) FK506 [Takayanagi et al., 2002].

A Role for CaMKII in Osteoclastogenesis

CaMKII is a complex Ca^{2+}/calmodulin-activated serine/threonine kinase best known for its regulation of neural and cardiac development and function. CaMKII is encoded by four genes (α, β, γ, and δ), which can be modified into numerous subunit isoforms by alternate splicing. The most distinctive quality of CaMKII is its holo-enzymatic structure, consisting of stacked hexameric rings of associated subunits with their catalytic domains oriented away from center like the spokes of a wheel. Calmodulin binding to the regulatory domain of a CaMKII subunit allows for immediate auto-phosphorylation of the regulatory domain of the adjacent subunit in the holo-enzyme. This auto-phosphorylation imparts two important qualities to CaMKII: 1) the affinity for bound Ca^{2+}/calmodulin after Ca^{2+} dissociation increases 1,000-fold (calmodulin-trapping) and 2) CaMKII retains partial enzymatic activity in the absence of calmodulin [Soderling and Stull, 2001; Zhang et al., 2005]. Thus, CaMKII's response can be finely tuned to the duration, amplitude, and spatio-temporal quality of Ca^{2+} signals, and CaMKII can direct cellular functions long after Ca^{2+} signal dissipation (molecular memory).

While little attention has been paid to CaMKII in the bone biology field, we have recently established that CaMKII is involved in differentiation of both osteoblasts and osteoclasts [Zayzafoon et al., 2005; Zhang et al., 2005]. CaMKIIα mutant mice (CaMKIIα$^{+/-}$) have a reduced $TRAP^+$ osteoclast staining area in tibial and femoral bone sections; furthermore, the CaMKII inhibitor KN93 dose-dependently inhibited TRAP activity in RANKL-stimulated mouse bone marrow precursors from normal mice [Zhang et al., 2005], demonstrating that, despite the confounding influence of osteoblasts in the CaMKIIα mutant mice, CaMKIIα plays a direct role within the osteoclast.

One likely mechanism for CaMKII-mediated regulation of osteoclastogenesis is through activation of AP-1 transcription factors, which direct expression of numerous osteoclast-specific genes (Fig. 3). In murine osteoblastic MC3T3 cells, CaMKIIα induces *c-fos* gene expression and subsequent AP-1 activation [Zayzafoon et al., 2005], and the induction of CD44 expression in human promonocytic cells by TNFα, a cytokine closely-related to RANKL, is mediated by CaMKII-regulated activation of AP-1 [Mishra et al., 2005].

Another potential target for CaMKII regulation during osteoclastogenesis is the gp130 receptor family. The glycoprotein receptor gp130, in conjunction with one of several different membrane-associated binding partners, can bind the cytokines CT-1, LIF, OsM, IL-6, and IL-11. This leads to activation of Janus kinases (JAKs), which phosphorylate gp130, followed by gp130 dimerization and activation of the STAT and/or SHP2/ras/MAPK pathways. LIF, IL-11, 1L-6, CT-1, and OsM have been reported to affect both osteoclastic and osteoblastic differentiation, and these cytokines are implicated in osteo-articular pathologies such as Paget's disease, bone metastases, and rheumatoid arthritis. Mutant gp130$^{-/-}$ mice are osteopenic and have increased osteoclast numbers. Furthermore, RANKL-induced osteoclastogenesis of ex vivo bone marrow precursors is dramatically increased in mice expressing a mutated gp130 which cannot activate the SHP2/ras/MAPK pathway [Sims et al., 2004], suggesting a suppressive role for the gp130 receptor in osteoclastogenesis. [Heymann and Rousselle, 2000; Sims et al., 2004].

A recent report determined that LIF-induced phosphorylation of Ser-782 in the gp130 tail was mediated by CaMKII [Gibson et al., 2005]. Ser-782 is adjacent to a di-leucine motif which regulates internalization and downregulation of the gp130 receptor, and transient expression of a non-phosphorylatable mutant gp130

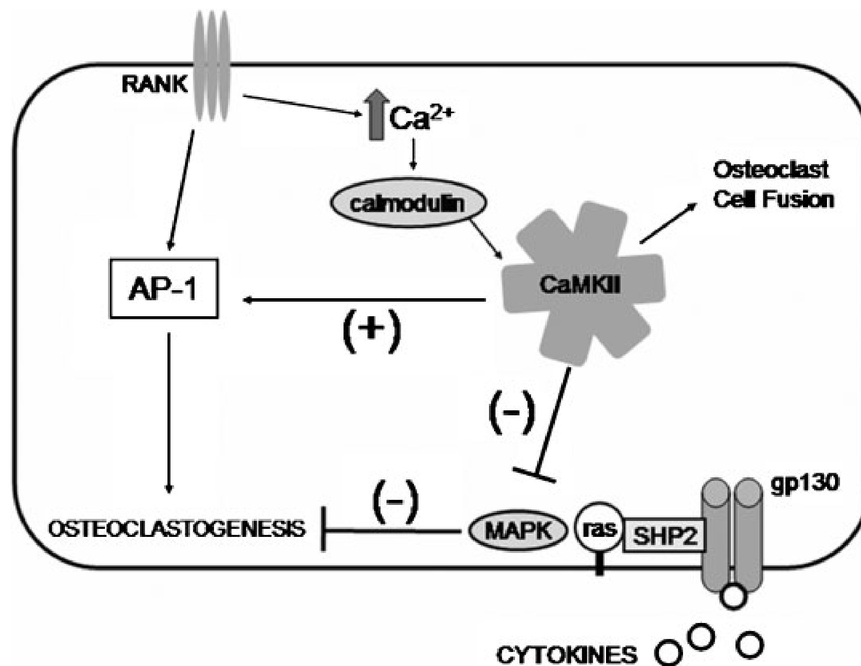

Fig. 3. Proposed actions of CaMKII in osteoclastogenesis. RANK-induced increases in intracellular Ca^{2+} can activate calmodulin and its downstream effector CaMKII. Possible roles for CaMKII in osteoclastogenesis include: (**A**) regulation of cell–cell fusion, (**B**) activation of the AP-1 transcription factor family, and (**C**) blockade of down-regulatory gp130 signaling cascades.

($^{S}782^{A}$) leads to increased gp130 cell surface expression and activation potential [Gibson et al., 2000], suggesting that CaMKII may promote osteoclastogenesis through downregulation of an inhibitory gp130 receptor cascade (Fig. 3).

CONCLUSIONS

This is an exciting time in the field of bone biology. Identification of RANKL and M-CSF as critical factors for osteoclastogenesis has opened the door to rapid and reliable methods for in vitro analysis of the osteoclast, and, ultimately, to a better understanding of the osteoclast's role in the bone loss associated with painful and debilitating diseases such as osteoporosis and rheumatoid arthritis. However, despite the rapid advancements made in recent years towards understanding the mechanisms directing osteoclastic differentiation and functional activity, comprehensive models to explain these processes are far from complete.

While Ca^{2+} signaling is critical for osteoclastic processes, there is currently very little understanding of how Ca^{2+}-directed signaling pathways work within osteoclasts. Not surprisingly, the primary intracellular calcium receptor protein, calmodulin, is involved in regulating all aspects of the osteoclast lifespan, including genesis, function and death. In recent years, the Ca^{2+}/calmodulin dependent phosphatase, calcineurin, and its downstream effectors, the NFAT family of transcription factors, have been characterized as master regulators of osteoclastogenesis. However, most experimental analyses of the calcineurin/NFAT system in osteoclasts have relied heavily on inhibitors such as CsA, which can downregulate other signaling pathways besides calcineurin/NFAT, including p38 and ERK. Thus, despite claims to the contrary, a complete picture of calcineurin/NFAT signaling in osteoclastogenesis has not been established.

Intriguingly, recent studies have demonstrated that another Ca^{2+}/calmodulin-dependent effector, CaMKII, plays a role in osteoclastogenesis. While the mechanisms of CaMKII-mediated signaling in osteoclasts have not been studied, CaMKII can activate c-Fos and AP-1 transcription factors, which can in turn drive NFAT2 expression. Therefore, it is possible that CaMKII and calcineurin play redundant and/or overlapping roles in Ca^{2+}/calmodulin signaling. Furthermore, CaMKII may act as a negative regulator of the gp130 cytokine receptor,

which is involved in osteoclast differentiation, bone remodeling, and numerous osteo-articular diseases.

REFERENCES

Aubin JE. 1998. Bone stem cells. J Cell Biochem Suppl 30–31:73–82.

Bekker PJ, Gay CV. 1990. Characterization of a Ca2(+)-ATPase in osteoclast plasma membrane. J Bone Miner Res 5:557–567.

Benford HL, McGowan NW, Helfrich MH, Nuttall ME, Rogers MJ. 2001. Visualization of bisphosphonate-induced caspase-3 activity in apoptotic osteoclasts in vitro. Bone 28:465–473.

Berridge MJ, Lipp P, Bootman MD. 2000. The versatility and universality of calcium signaling. Nat Rev Mol Cell Biol 1:11–21.

Blair HC, Teitelbaum SL, Ghiselli R, Gluck S. 1989. Osteoclastic bone resorption by a polarized vacuolar proton pump. Science 245:855–857.

Boyle WJ, Simonet WS, Lacey DL. 2003. Osteoclast differentiation and activation. Nature 423:337–342.

Brown OA, Sosa YE, Castro MG, Goya RG. 2000. Studies on the prolactin-releasing mechanism of histones H2A and H2B. Life Sci 66:2081–2089.

Burgoyne RD, Clague MJ. 2003. Calcium and calmodulin in membrane fusion. Biochim Biophys Acta 1641:137–143.

Chin D, Means AR. 2000. Calmodulin: A prototypical calcium sensor. Trends Cell Biol 10:322–328.

Day CJ, Kim MS, Stephens SR, Simcock WE, Aitken CJ, Nicholson GC, Morrison NA. 2004. Gene array identification of osteoclast genes: Differential inhibition of osteoclastogenesis by cyclosporin A and granulocyte macrophage colony stimulating factor. J Cell Biochem 91:303–315.

Day CJ, Kim MS, Lopez CM, Nicholson GC, Morrison NA. 2005. NFAT expression in human osteoclasts. J Cell Biochem 95:17–23.

Gibson RM, Schiemann WP, Prichard LB, Reno JM, Ericsson LH, Nathanson NM. 2000. Phosphorylation of human gp130 at Ser-782 adjacent to the Di-leucine internalization motif. Effects on expression and signaling. J Biol Chem 275:22574–22582.

Gibson RM, Laszlo GS, Nathanson NM. 2005. Calmodulin-dependent protein kinases phosphorylate gp130 at the serine-based dileucine internalization motif. Biochim Biophys Acta 1714:56–62.

Heymann D, Rousselle AV. 2000. gp130 cytokine family and bone cells. Cytokine 12:1455–1468.

Hoeflich KP, Ikura M. 2002. Calmodulin in action: Diversity in target recognition and activation mechanisms. Cell 108:739–742.

Hogan PG, Chen L, Nardone J, Rao A. 2003. Transcriptional regulation by calcium, calcineurin, and NFAT. Genes Dev 17:2205–2232.

Horgan K, Cooke E, Hallett MB, Mansel RE. 1986. Inhibition of protein kinase C mediated signal transduction by tamoxifen. Importance for antitumour activity. Biochem Pharmacol 35:4463–4465.

Igarashi K, Hirotani H, Woo JT, Stern PH. 2004. Cyclosporine A and FK506 induce osteoclast apoptosis in mouse bone marrow cell cultures. Bone 35:47–56.

Ishida N, Hayashi K, Hoshijima M, Ogawa T, Koga S, Miyatake Y, Kumegawa M, Kimura T, Takeya T. 2002. Large scale gene expression analysis of osteoclastogenesis in vitro and elucidation of NFAT2 as a key regulator. J Biol Chem 277:41147–41156.

Kahl CR, Means AR. 2003. Regulation of cell-cycle progression by calcium/calmodulin-dependent pathways. Endocr Rev 24:719–736.

Kawakami A, Eguchi K, Matsuoka N, Tsuboi M, Koji T, Urayama S, Fujiyama K, Kiriyama T, Nakashima T, Nakane PK, Nagataki S. 1997. Fas and Fas ligand interaction is necessary for human osteoblast apoptosis. J Bone Miner Res 12:1637–1646.

Klee CB, Ren H, Wang X. 1998. Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. J Biol Chem 273:13367–13370.

Lee ZH, Kim HH. 2003. Signal transduction by receptor activator of nuclear factor kappa B in osteoclasts. Biochem Biophys Res Commun 305:211–214.

Manion MK, Villain M, Pan ZG, McDonald JM, Blalock JE. 2000. Cellular uptake and in situ binding of a peptide agonist for calmodulin. Biochem Biophys Res Commun 277:462–469.

Matsuda S, Moriguchi T, Koyasu S, Nishida E. 1998. T lymphocyte activation signals for interleukin-2 production involve activation of MKK6–p38 and MKK7–SAPK/JNK signaling pathways sensitive to cyclosporin A. J Biol Chem 273:12378–12382.

Means AR. 2000. Regulatory cascades involving calmodulin-dependent protein kinases. Mol Endocrinol 14:4–13.

Mishra JP, Mishra S, Gee K, Kumar A. 2005. Differential involvement of calmodulin-dependent protein kinase II-activated AP-1 and c-Jun N-terminal kinase-activated EGR-1 signaling pathways in tumor necrosis factor-alpha and lipopolysaccharide-induced CD44 expression in human monocytic cells. J Biol Chem 280:26825–26837.

Mountz JD, Wu J, Cheng J, Zhou T. 1994. Autoimmune disease. A problem of defective apoptosis. Arthritis Rheum 37:1415–1420.

Myers DE, Collier FM, Minkin C, Wang H, Holloway WR, Malakellis M, Nicholson GC. 1999. Expression of functional RANK on mature rat and human osteoclasts. FEBS Lett 463:295–300.

Plotkin LI, Weinstein RS, Parfitt AM, Roberson PK, Manolagas SC, Bellido T. 1999. Prevention of osteocyte and osteoblast apoptosis by bisphosphonates and calcitonin. J Clin Invest 104:1363–1374.

Radding W, Williams JP, Hardy RW, McDonald JM, Whitaker CH, Turbat-Herrera EA, Blair HC. 1994. Calmodulin concentrated at the osteoclast ruffled border modulates acid secretion. J Cell Physiol 160: 17–28.

Sims NA, Jenkins BJ, Quinn JM, Nakamura A, Glatt M, Gillespie MT, Ernst M, Martin TJ. 2004. Glycoprotein 130 regulates bone turnover and bone size by distinct downstream signaling pathways. J Clin Invest 113:379–389.

Soderling TR, Stull JT. 2001. Structure and regulation of calcium/calmodulin-dependent protein kinases. Chem Rev 101:2341–2352.

Su B, Jacinto E, Hibi M, Kallunki T, Karin M, Ben-Neriah Y. 1994. JNK is involved in signal integration during costimulation of T lymphocytes. Cell 77:727–736.

Takayanagi H. 2005. Mechanistic insight into osteoclast differentiation in osteoimmunology. J Mol Med 83:170–179.

Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, Saiura A, Isobe M, Yokochi T, Inoue J, Wagner EF, Mak TW, Kodama T, Taniguchi T. 2002. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. Dev Cell 3:889–901.

Teitelbaum SL. 2000. Bone resorption by osteoclasts. Science 289:1504–1508.

Teitelbaum SL. 2004. RANKing c-Jun in osteoclast development. J Clin Invest 114:463–465.

Turner RT, Wakley GK, Hannon KS, Bell NH. 1988. Tamoxifen inhibits osteoclast-mediated resorption of trabecular bone in ovarian hormone-deficient rats. Endocrinology 122:1146–1150.

Wang XM, Yu SF, Yang ZP. 2000. Apoptosis of osteoclast-like cells induced by alendronate is related to Fas gene expression. Chin J Dent Res 3:26–32.

Weinstein RS, Chen JR, Powers CC, Stewart SA, Landes RD, Bellido T, Jilka RL, Parfitt AM, Manolagas SC. 2002. Promotion of osteoclast survival and antagonism of bisphosphonate-induced osteoclast apoptosis by glucocorticoids. J Clin Invest 109:1041–1048.

Williams JP, Blair HC, McKenna MA, Jordan SE, McDonald JM. 1996. Regulation of avian osteoclastic H^+-ATPase and bone resorption by tamoxifen and calmodulin antagonists. Effects independent of steroid receptors. J Biol Chem 271:12488–12495.

Williams JP, McDonald JM, McKenna MA, Jordan SE, Radding W, Blair HC. 1997. Differential effects of tamoxifen-like compounds on osteoclastic bone degradation, H(+)-ATPase activity, calmodulin-dependent cyclic nucleotide phosphodiesterase activity, and calmodulin binding. J Cell Biochem 66:358–369.

Williams JP, McKenna MA, Thames AM III, McDonald JM. 2000. Tamoxifen inhibits phorbol ester stimulated osteoclastic bone resorption: An effect mediated by calmodulin. Biochem Cell Biol 78:715–723.

Williams JP, McKenna MA, Thames AM, III, McDonald JM. 2003a. Effects of cyclosporine on osteoclast activity: Inhibition of calcineurin activity with minimal effects on bone resorption and acid transport activity. J Bone Miner Res 18:451–457.

Williams JP, Thames AM III, McKenna MA, McDonald JM. 2003b. Differential effects of calmodulin and protein kinase C antagonists on bone resorption and acid transport activity. Calcif Tissue Int 73:290–296.

Wu X, McKenna MA, Feng X, Nagy TR, McDonald JM. 2003. Osteoclast apoptosis: The role of Fas in vivo and in vitro. Endocrinology 144:5545–5555.

Wu X, Ahn EY, McKenna MA, Yeo H, McDonald JM. 2005a. Fas binding to calmodulin regulates apoptosis in osteoclasts. J Biol Chem 280:29964–29970.

Wu X, Pan G, McKenna MA, Zayzafoon M, Xiong WC, McDonald JM. 2005b. RANKL regulates Fas expression and Fas-mediated apoptosis in osteoclasts. J Bone Miner Res 20:107–116.

Yip KH, Zheng MH, Steer JH, Giardina TM, Han R, Lo SZ, Bakker AJ, Cassady AI, Joyce DA, Xu J. 2005. Thapsigargin modulates osteoclastogenesis through the regulation of RANKL-induced signaling pathways and reactive oxygen species production. J Bone Miner Res 20:1462–1471.

Zayzafoon M, Fulzele K, McDonald JM. 2005. Calmodulin and calmodulin-dependent kinase IIalpha regulate osteoblast differentiation by controlling c-fos expression. J Biol Chem 280:7049–7059.

Zhang L, Feng X, McDonald JM. 2003. The role of calmodulin in the regulation of osteoclastogenesis. Endocrinology 144:4536–4543.

Zhang L, McKenna MA, Said-Al-Naief N, Wu X, Feng X, McDonald JM. 2005. Osteoclastogenesis: The role of calcium and calmodulin. Crit Rev Eukaryot Gene Expr 15:1–13.