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ARTICLES

Mysteries in Ca^{2+} Signaling During Osteoclast Differentiation

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Nuclear factor of activated T cells c1 (NFATc1) activation by Ca^{2+} signaling is an essential part of the signaling axis in osteoclast differentiation. Although the molecular signaling cascade downstream of receptor activator of nuclear factor-κB ligand (RANKL) has been extensively studied, the origin of the intracellular Ca^{2+} increase and the dynamics required for efficient NFATc1 activation have been unclear. Using mice lacking inositol 1,4,5-triphosphate (IP_3) receptor (IP_3R) type 2 (IP_3R2) and IP_3R3, a study from last year by Kuroda and coauthors found that RANKL-induced Ca^{2+} oscillation and osteoclast differentiation were impaired *in vitro*, but that there was no defect in osteoclastogenesis *in vivo* (1). To understand this discrepancy, the authors focused on the role of osteoblasts, which have the ability to induce osteoclastogenesis in $IP_3R2/3$-deficient cells. Surprisingly, Ca^{2+} oscillation was not detected in the coculture system, but NFATc1 induction and osteoclastogenesis were observed. Their findings suggest the existence of an as yet unidentified (possibly Ca^{2+} oscillation-independent) regulatory mechanism for NFATc1 activation mediated by osteoblasts, and may open up new directions for research on Ca^{2+} signaling during osteoclastogenesis.

RANKL Signaling During Osteoclastogenesis

Osteoclasts are cells of monocyte-macrophage origin that decalcify and degrade the bone matrix. The differentiation of osteoclasts is regulated primarily by three signaling pathways that are activated by RANKL, macrophage colony-stimulating factor (M-CSF), and immunoreceptor tyrosine-based activation motif (ITAM) (2). Whereas M-CSF promotes the proliferation and survival of bone marrow monocyte/macrophage precursor cells (BMMs) (3), RANKL activates the differentiation process by inducing the master transcription factor for osteoclastogenesis, NFATc1.

The robust induction of NFATc1 is dependent on Ca^{2+} signaling, which is mediated by the activation of ITAM in adaptor molecules such as DNAX-activating protein 12 (DAP12) and Fc receptor common γ subunit (FcRγ) (4;5). DAP12 and FcRγ associate with costimulatory receptors of the immunoglobulin superfamily, including osteoclast-associated receptor (OSCAR), triggering receptor expressed in myeloid cells-2 (TREM-2), signal-regulatory protein β1 (SIRPβ1), and paired immunoglobulin-like receptor-A (PIR-A) (4). Although the ligands of the immunoglobulin-like receptors remain to be elucidated, it has been shown that osteoblasts supply putative ligands for costimulatory receptors associated with FcRγ, suggesting an important role of osteoblasts in osteoclast costimulation (4).

The phosphorylation of ITAM results in the recruitment of the nonreceptor tyrosine kinase Syk. Meanwhile, RANKL stimulates Tec tyrosine kinases to form a complex with Syk, which activates phospholipase Cγ (PLCγ) (4-7). It is well-established that PLCγ generates IP_3 , which evokes Ca^{2+} release from the endoplasmic reticulum (ER) through IP_3Rs, with subsequent Ca^{2+} entry from the extracellular milieu taking place through plasma membrane channels (8;9).

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Although this mechanism has not been confirmed in osteoclasts, Ca^{2+} oscillation induced by RANKL has been thought to be important for the efficient activation of NFATc1 via the Ca^{2+}-dependent phosphatase calcineurin (4;10). Consistent with this, in most previous reports, osteoclastogenesis was impaired when Ca^{2+} oscillation was not observed (4;7;11;12). As osteoclastogenesis is also impaired in PLCγ2-deficient mice, it is expected that IP_3-mediated Ca^{2+} signaling plays an important role in osteoclastogenesis (Fig. 1).

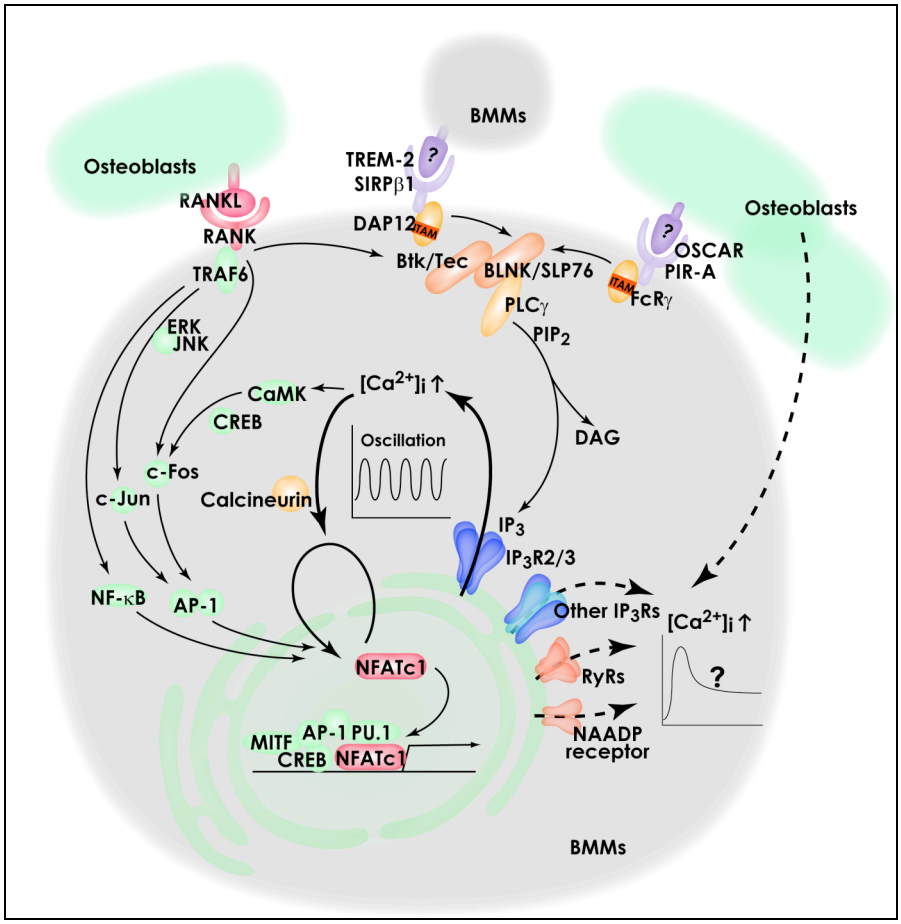

Fig. 1. Ca^{2+} signaling in osteoclast differentiation. Osteoclastogenesis is supported by osteoblasts or bone stromal cells, which provide RANKL, M-CSF and putative ligands for costimulatory receptors. RANKL binding to RANK results in the recruitment of TRAF6, which activates NF-κB and MAPKs (ERK and JNK), whereas AP-1 (containing c-Fos) is also activated by RANK stimulation. NFATc1 induction is dependent on the transcription factors AP-1 and NF-κB. Costimulatory signals for RANK activate non-receptor tyrosine kinase Syk through ITAM, which phosphorylates BLNK/SLP-76. Btk and Tec, which are activated by RANK, bind to BLNK/SLP-76 and phosphorylate PLCγ. PLCγ produces IP_3, which evokes Ca^{2+} release from the ER through IP_3R2 and IP_3R3, and subsequently generates Ca^{2+} oscillation. NFATc1 translocates into the nucleus after dephosphorylation by calcineurin, which is activated by Ca^{2+} signaling, and binds to its own promoter, resulting in the autoamplification of NFATc1 expression. NFATc1 regulates osteoclastogenesis together with other transcription factors, such as AP-1, PU.1, MITF and CREB so as to induce various osteoclast-specific genes, including *TRAP*, *cathepsin K*, *calcitonin receptor* and *OSCAR*. Osteoblasts may provide compensatory signals for the loss of $IP_3R2/3$, as indicated by the dotted arrows. One possibility is that IP_3Rs other than $IP_3R2/3$ may function in response to IP_3 production through costimulatory signaling mediated by FcRγ-associated immunoreceptors. Another possibility is that RyRs or NAADP receptors may be upregulated through an unknown mechanism mediated by osteoblasts, and these receptors may function as alternative channels to $IP_3R2/3$.

In Vivo and *In Vitro* Analysis of $IP_3R2/3$-Deficient Mice

To address the physiological role of IP_3-mediated signals in the osteoclast lineage, Kuroda and colleagues (1) investigated the bone phenotype of $IP_3R2/3$-deficient mice, because they had found IP_3R2 and IP_3R3 to be the major IP_3Rs in osteoclasts among the 3 known types of IP_3Rs. However, bone mass and osteoclast number were normal in these mice, suggesting that IP_3-mediated Ca^{2+} signaling is dispensable for osteoclastogenesis, or that $IP_3R2/3$ are dispensable for transmitting IP_3-mediated Ca^{2+} signals. $IP_3R2/3$ are reportedly essential for transmitting IP_3-mediated Ca^{2+} signaling under certain conditions (13), but there is little information regarding the osteoclast lineage.

The authors then focused on the observation that Ca^{2+} oscillation during *in vitro* osteoclastogenesis was impaired in $IP_3R2/3$-deficient osteoclasts 48 hours after RANKL stimulation (1). They concluded that the combined deficiency of IP_3R2 and IP_3R3 results in impaired osteoclastogenesis *in vitro* due to a lack of Ca^{2+} oscillation and suggested that IP_3R2 and IP_3R3 are essential for Ca^{2+} oscillation in osteoclasts. However, it was also observed that impairment of osteoclast differentiation from $IP_3R2/3$-deficient BMMs was partially rescued by coculturing with osteoblasts. They proposed that the result in this coculture condition was consistent with the *in vivo* observation that osteoclastogenesis was normal in $IP_3R2/3$-deficient mice (1).

Surprisingly, they could not detect Ca^{2+} oscillation in $IP_3R2/3$-deficient multinuclear osteoclasts, although NFATc1 induction and osteoclast differentiation were observed. Furthermore, FK506 did not affect osteoclastogenesis in $IP_3R2/3$-deficient BMMs in coculture with osteoblasts (Table 1). Thus, the authors proposed the interesting hypotheses that 1) Ca^{2+} oscillation is not required for NFATc1 activation and osteoclast formation; 2) calcineurin is not essential for NFATc1 activation and osteoclast formation; and 3) oscillation-independent calcineurin-independent osteoclastogenesis is supported by osteoblasts.

Is NFATc1 Activation Dependent on Ca^{2+} Oscillation?

NFAT is activated by a low, but sustained Ca^{2+} plateau level (14), and Ca^{2+} oscillation is thought to be beneficial in keeping NFATc1 in the nucleus and ensuring the long-lasting transcriptional activation of NFATc1 that is required for terminal differentiation into osteoclasts (10). However, the genetic evidence for the Ca^{2+} signaling pattern required for osteoclastogenesis is lacking. The rare example is RGS10-deficient mice, which exhibit high bone mass and impaired osteoclastogenesis (12). RGS10 is important for RANKL-induced Ca^{2+} oscillation, suggesting the importance of Ca^{2+} oscillation in osteoclastogenesis. However, it is difficult to rule out the possibility that disruption of RGS10 affects other types of Ca^{2+} signaling.

Usually Ca^{2+} oscillation becomes detectable approximately 24 hours after RANKL stimulation, when NFATc1 induction becomes evident (4;7;10;11). Therefore, it is likely that Ca^{2+} signaling patterns other than oscillation are important for NFATc1 activation in the earlier phase. It is unclear up until which stage osteoclastogenesis is dependent on oscillation (15). Thus, it remains to be determined which type of Ca^{2+} signal is absolutely necessary for osteoclastogenesis in each differentiation stage. In this study (1), Ca^{2+} oscillation was evaluated at a limited number of time points, and it can be difficult to detect Ca^{2+} signaling in mixed cell culture systems like cocultures of osteoblasts and osteoclast precursor cells. It will first be necessary to determine the Ca^{2+} signaling pattern required for osteoclastogenesis at multiple stages and then to analyze the differences in Ca^{2+} signaling events in knockout mice, which together will provide the requisite fundamental information on the role of the distinct Ca^{2+} signaling pattern.

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Table 1. $IP_3R2/3$ deficiency and osteoclastogenesis, from Kuroda *et al*. (1).

	RANKL-induced osteoclasto-genesis without osteoblasts	Coculture with osteoblasts	*In vivo*
Oscillation	× (48 hrs after RANKL stimulation)	× (In multinuclear osteoclasts)	ND
NFATc1 induction	×	○	ND
Osteoclast differentiation	×	○ (50% of WT cells)	○
Effect of 1 μM FK506	ND	Not affected	ND

×= abrogated, ○= normal, ND = not determined

Is NFATc1 Activation Dependent on Calcineurin?

It is widely accepted that calcineurin is a central regulator of NFAT and that immunosuppressants such as cyclosporine A and FK506 inhibit the activation of calcineurin, thereby blocking the nuclear import and transcriptional activation of NFAT. However, the authors demonstrated that FK506 only partially inhibited osteoblast-mediated osteoclastogenesis and did not inhibit osteoblast-mediated osteoclastogenesis in $IP_3R2/3$-deficient cells, suggesting that NFATc1 activation is not completely dependent on calcineurin. Recent reports have suggested calcineurin-independent NFAT activation mechanisms (16), but it is important to be careful about reaching conclusions based only on inhibitor or overexpression experiments. Since calcineurin-deficient mice are available, it will be important to analyze NFATc1 activation and osteoclastogenesis in these mice.

What Compensates for the Loss of $IP_3R2/3$?

Since there is no obvious bone phenotype in $IP_3R2/3$-deficient mice, certain compensatory mechanism(s) are obviously at work in these animals. The authors (1) propose that osteoblasts mediate this mechanism in a Ca^{2+} oscillation-independent manner, but is this compensatory mechanism Ca^{2+}-independent? Considering the increased bone mass in PLCγ2-deficient mice, it is highly likely that PLCγ-mediated IP_3 production and subsequent activation of ER Ca^{2+} release play a substantial role. Therefore, it is plausible that IP_3-mediated Ca^{2+} signaling would have to be achieved by the compensatory mechanism. One possibility is that IP_3Rs other than $IP_3R2/3$ (possibly IP_3R1) may be functional.

In non-excitable cells, IP_3Rs function as major Ca^{2+} channels that release Ca^{2+} from the ER, but it is also reported that Ca^{2+} release from the ER is mediated in an IP_3-independent manner via ryanodine receptors (RyRs) and/or nicotinic acid adenine dinucleotide phosphate (NAADP) receptors (17;18). Another possibility is that IP_3-independent Ca^{2+} release (possibly mediated by RyRs, or NAADP receptors, etc.) is compensatorily upregulated by an as yet unknown mechanism (Fig. 1).

The remaining issue is why these complex compensatory mechanisms are activated only *in vivo* or in the presence of osteoblasts. It is of crucial importance to identify the compensatory mechanism, which will provide molecular insight into bone remodeling based on the functional coupling between osteoblasts and osteoclasts.

New Directions for Research into Osteoclastogenesis

It is often the case that genetically-modified mice exhibit no obvious phenotype, despite a defect observed in suggestive *in vitro* experiments. The study from Kuroda *et al*. (1) provided an interesting example in which osteoblast-mediated signals are

compensatory for the loss of certain types of Ca^{2+} signaling. These findings have shed light on an unexpected mechanism underlying osteoblast-mediated NFATc1 activation, which will lead to new directions in research into NFATc1 regulation and Ca^{2+} signaling.

Conflict of Interest: None reported.

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References

1. Kuroda Y, Hisatsune C, Nakamura T, Matsuo K, Mikoshiba K. Osteoblasts induce Ca^{2+} oscillation-independent NFATc1 activation during osteoclastogenesis. *Proc Natl Acad Sci U S A*. 2008 Jun 24;105(25):8643-8.

2. Asagiri M, Takayanagi H. The molecular understanding of osteoclast differentiation. *Bone*. 2007 Feb;40(2):251-64.

3. Ross FP, Teitelbaum SL. alphavbeta3 and macrophage colony-stimulating factor: partners in osteoclast biology. *Immunol Rev*. 2005 Dec;208:88-105.

4. Koga T, Inui M, Inoue K, Kim S, Suematsu A, Kobayashi E, Iwata T, Ohnishi H, Matozaki T, Kodama T, Taniguchi T, Takayanagi H, Takai T. Costimulatory signals mediated by the ITAM motif cooperate with RANKL for bone homeostasis. *Nature*. 2004 Apr 15;428(6984):758-63.

5. Mócsai A, Humphrey MB, Van Ziffle JA, Hu Y, Burghardt A, Spusta SC, Majumdar S, Lanier LL, Lowell CA, Nakamura MC. The immunomodulatory adapter proteins DAP12 and Fc receptor gamma-chain (FcRgamma) regulate development of functional osteoclasts through the Syk tyrosine kinase. *Proc Natl Acad Sci U S A*. 2004 Apr 20;101(16):6158-63.

6. Mao D, Epple H, Uthgenannt B, Novack DV, Faccio R. PLCgamma2 regulates osteoclastogenesis via its interaction with ITAM proteins and GAB2. *J Clin Invest*. 2006 Nov;116(11):2869-79.

7. Shinohara M, Koga T, Okamoto K, Sakaguchi S, Arai K, Yasuda H, Takai T, Kodama T, Morio T, Geha RS, Kitamura D, Kurosaki T, Ellmeier W, Takayanagi H. Tyrosine kinases Btk and Tec regulate osteoclast differentiation by linking RANK and ITAM signals. *Cell*. 2008 Mar 7;132(5):794-806.

8. Bootman MD, Berridge MJ. The elemental principles of calcium signaling. *Cell*. 1995 Dec 1;83(5):675-8.

9. Clapham DE. Calcium signaling. *Cell*. 2007 Dec 14;131(6):1047-58.

10. 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. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev Cell*. 2002 Dec;3(6):889-901.

11. Gohda J, Akiyama T, Koga T, Takayanagi H, Tanaka S, Inoue J. RANK-mediated amplification of TRAF6 signaling leads to NFATc1 induction during osteoclastogenesis. *EMBO J*. 2005 Feb 23;24(4):790-9.

12. Yang S, Li YP. RGS10-null mutation impairs osteoclast differentiation resulting from the loss of $[Ca^{2+}]i$ oscillation regulation. *Genes Dev*. 2007 Jul 15;21(14):1803-16.

13. Futatsugi A, Nakamura T, Yamada MK, Ebisui E, Nakamura K, Uchida K, Kitaguchi T, Takahashi-Iwanaga H, Noda T, Aruga J, Mikoshiba K. IP_3 receptor types 2 and 3 mediate exocrine secretion underlying energy metabolism. *Science*. 2005 Sep 30;309(5744):2232-4.

14. Dolmetsch RE, Lewis RS, Goodnow CC, Healy JI. Differential activation of transcription factors induced by Ca^{2+}

response amplitude and duration. *Nature*. 1997 Apr 24;386(6627):855-8.

15. Masuyama R, Vriens J, Voets T, Karashima Y, Owsianik G, Vennekens R, Lieben L, Torrekens S, Moermans K, Vanden Bosch A, Bouillon R, Nilius B, Carmeliet G. TRPV4-mediated calcium influx regulates terminal differentiation of osteoclasts. *Cell Metab*. 2008 Sep;8(3):257-65.

16. Aramburu J, Heitman J, Crabtree GR. Calcineurin: a central controller of signalling in eukaryotes. *EMBO Rep*. 2004 Apr;5(4):343-8.

17. Grafton G, Thwaite L. Calcium channels in lymphocytes. *Immunology*. 2001 Oct;104(2):119-26.

18. House SJ, Potier M, Bisaillon J, Singer HA, Trebak M. The non-excitable smooth muscle: calcium signaling and phenotypic switching during vascular disease. *Pflugers Arch*. 2008 Aug;456(5):769-85.