

# Role of zinc and zinc-modulated ion channels, ORAI1 and HCN in osteoclasts

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## Abstract

Zinc is a trace element in the mammalian body, and increasing evidence is suggesting that it plays a critical role in bone development and in the differentiation of bone cells such as osteoblasts, osteoclasts, and chondrocytes. In vivo and in vitro studies have shown that zinc affects osteoclast differentiation. Zinc-sensitive ion channels have been reported. Zinc-related ion channel is ORAI1, which is a store-operated  $\text{Ca}^{2+}$  entry channel subunit, and zinc inhibits the activity of this channel. ORAI1 channels play a significant role in regulating osteoclastic  $\text{Ca}^{2+}$  oscillations during osteoclast differentiation. Knockdown of ORAI1 inhibited osteoclast differentiation. Zinc also inhibited osteoclastogenesis however, its inhibition was reduced by ORAI1 knockdown. Interestingly, zinc can change the osteoclastic membrane potential. Based on this, hyperpolarization-activated cyclic nucleotide-modulated (HCN) channels were investigated and were found to be highly expressed in osteoclasts. High concentrations of zinc chloride increase  $I_h$  current which is generated by HCN channels, suggesting a complicated relationship between HCN channels and zinc. Zinc plays various roles in bone physiology through zinc-modulated ion channels. Signaling of these ion channels would be promising targets for treating skeletal diseases.

## Introduction

Bone is the main storage medium for calcium ( $\text{Ca}^{2+}$ ) and zinc [1]. Although  $\text{Ca}^{2+}$ -related signaling in bone biology is well studied [2-4], the biology of intracellular zinc signaling and related molecules remain unclear. Zinc is an essential trace element in physiological metabolic processes, including bone remodeling [5,6]. Numerous studies have shown that zinc deficiency leads to bone loss and increased risk of fractures [7-11]. Recently, some reports have demonstrated that zinc transporters plays critical roles in bone and cartilage development [5] and the pathology of osteoarthritis [12].

The mechanism of osteoclast differentiation and the related intracellular signaling has been abundantly studied [3,4,13,14]. The receptor activator of nuclear factor- $\kappa\text{B}$  (RANK)/RANK ligand (RANKL) signaling pathway induces changes in intracellular  $\text{Ca}^{2+}$  levels ( $[\text{Ca}^{2+}]_i$ ); these changes lead to  $\text{Ca}^{2+}$ -calcineurin-dependent dephosphorylation and activation of nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), which translocates to the nucleus from the cytosol [4]. Addition of zinc to the culture medium inhibits osteoclast differentiation by decreasing  $[\text{Ca}^{2+}]_i$  after NFATc1 translocation [15]. Furthermore, zinc-related molecules such as ion channels and transporters also play important roles in osteoclast differentiation [14-17]. A recent study suggested that the endoplasmic reticulum-localized L-type voltage-gated  $\text{Ca}^{2+}$  channel, which is activated by membrane depolarization, is involved in intracellular zinc signaling [18]. In addition, our previous study showed that zinc influences osteoclastic membrane potential and hyperpolarization-activated cyclic nucleotide-modulated channels (HCN) [14].

This study focuses on the roles of zinc on osteoclasts, as well as the roles of zinc-modulated ion channels in osteoclast differentiation. Recent advances in the understanding of intracellular zinc signaling, including zinc-related ion channels, ORAI1 and HCNs in osteoclasts, are presented. Because zinc exerts inhibitory effects on the activity of

various ion channels, the relationships between zinc and ion channels in bone physiology are described.

## Materials and methods

### Cell culture

RAW 264.7 mouse osteoclast precursor-like cells (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM (GE Healthcare Japan, Tokyo, Japan) supplemented with 10% FBS and 1% penicillin and streptomycin. All cells were maintained at 37°C and 5%  $\text{CO}_2$  in a humidified atmosphere. All chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) unless noted otherwise. For osteoclast differentiation, RAW 264.7 cells were seeded at  $2.0 \times 10^5$  cells/ $\text{cm}^2$  with the addition of soluble RANKL (sRANKL, 50 ng/mL; Oriental Yeast, Tokyo, Japan). TRAP staining was performed as previously described [3,14]. Orail-siRNA and control-siRNA were purchased from Thermo Fisher Scientific K.K. (Yokohama, Japan). The siRNA was transfected into RAW cells using RNAi-MAX (Thermo Fisher Scientific K.K.) according to manufacturer protocols. TRAP-positive cells with more than three nuclei were defined as multinucleated osteoclasts.

### Gene expression assay

Gene expression assay was performed as previously described [3,14]. Total RNA was extracted using a RNeasy kit (Qiagen K. K., Tokyo, Japan) according to the manufacturer's protocol. First-strand cDNA was synthesized from total RNA using a high-capacity cDNA

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**Received:** October 22, 2019; **Accepted:** November 08, 2019; **Published:** November 12, 2019

reverse transcription kit (Thermo Fisher Scientific). The amount of total RNA used in each reaction of synthesis was 1 µg. Quantitative real-time PCR (qRT-PCR) was performed using a StepOne real-time PCR system (Thermo Fisher Scientific), SYBR Green (Fast SYBR Green master mix, Thermo Fisher Scientific), and specific forward and reverse primers. Transcript levels were normalized relative to those of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

## Electrophysiology

Electrophysiological recordings were made from RAW-derived osteoclasts cultured for 3–5 days after addition of RANKL as previously described [14,19]. For whole cell recordings, the standard external solution contained 145 mM NaCl, 5 mM KCl, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 10 mM glucose, 10 mM HEPES (pH 7.3), or 10 mM 2-morpholinoethanesulfonic acid (MES) (pH 5.5), and 0.1% bovine serum albumin. To investigate the effects of high concentration of zinc, 1mM  $\text{ZnCl}_2$  was added to the bath. The pipette solutions contained 130 mM K-gluconate, 20 mM KCl, 3 mM  $\text{MgCl}_2$ , 1 mM EGTA, 1 mM ATP, 0.5 mM GTP, and 10 mM HEPES (pH 7.3). The pH of the bath and pipette solutions was adjusted by adding NaOH and KOH. Osmolality was maintained between 280 and 300 mOsm.

The borosilicate glass pipettes had a resistance of 5–8 MΩ. The reference electrode was an Ag-AgCl wire connected to the bath solution through a Ringer agar bridge. The zero current potential before formation of the gigaseal was taken as 0 mV. Current signals were recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA), digitized at 1–2 kHz with a Digidata 1200 analog-digital converter (Axon Instruments), and analyzed using pCLAMP software (Axon Instruments). Voltage steps (from -30 to -150 mV) were applied at holding potential of -30 mV every 10–30 s.

## Results and discussion

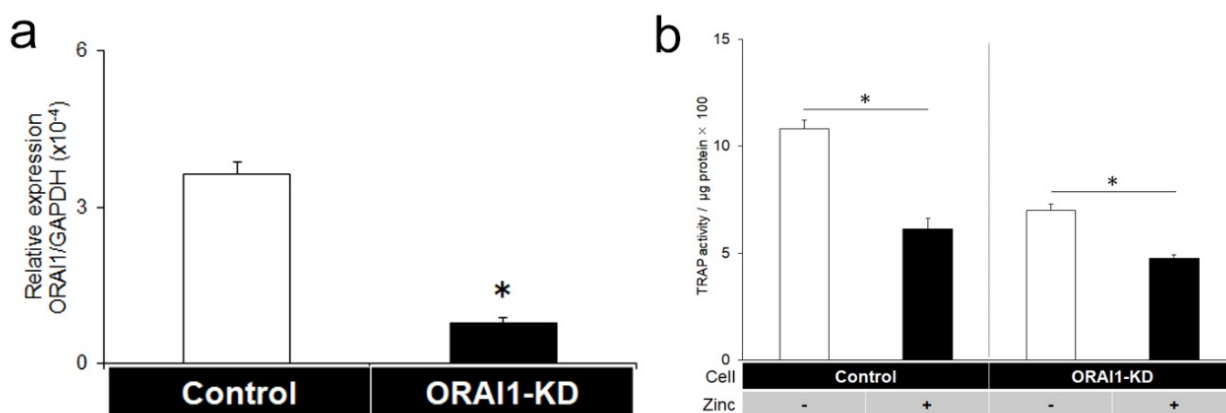
### Zinc and ORAI1 in osteoclasts

Ion channels that are selective for zinc have not been identified yet. However, zinc modulates the activities of various ion channels [20–22]. One of these channels, the voltage-gated  $\text{H}^+$  channel ( $\text{H}^+$  channel), is highly expressed in osteoclasts and contribute to regulation of the intracellular pH [19,23]. Zinc inhibits proton currents generated from  $\text{H}^+$  channels suggesting that zinc affects osteoclastic intracellular pH.

Recently,  $\text{H}^+$  channels in RAW264.7-derived osteoclasts are reported to be activated by extracellular phosphates, and are suggested to support production of reactive oxygen species (ROS), an essential mediator in RANK-RANKL signaling cascade [24]. ROS products are decreased by inhibition of  $\text{H}^+$  channel, which might down-regulate osteoclastogenesis.

The other zinc-related ion channel is the ORAI1  $\text{Ca}^{2+}$  channel, which is a store-operated  $\text{Ca}^{2+}$  entry (SOCE) channel subunit (25). ORAI1  $\text{Ca}^{2+}$  channels play an important role in the maintenance of osteoclastic  $\text{Ca}^{2+}$  oscillations during osteoclast differentiation [16]. The knockdown of ORAI1 inhibits osteoclastogenesis and decreases nuclear translocation of NFATc1. It should be noted that zinc inhibits the activity of the SOCE channel and decreases  $\text{Ca}^{2+}$  entry into cells [26–28]. Based on these reports, we have investigated the relationships between zinc and ORAI1 during osteoclast differentiation in RAW 264.7 cells. ORAI1-siRNA transfection significantly reduced mRNA expression of ORAI1 compared with control-siRNA transfection (Figure 1a). Zinc inhibited tartrate-resistant acid phosphatase (TRAP) activity in controls; however, less reduction in TRAP activity was observed in ORAI1-knockdown cells (Figure 1b). This result indicates that zinc-induced inhibitory effects on osteoclastogenesis include, in part, the inhibition of ORAI1 activity.

Recently we found that zinc transiently hyperpolarizes osteoclastic cell membrane potential (14), although the mechanism of such changes to the membrane potential remains unclear. This finding implies that zinc regulates cell function via voltage-gated ion channels. Based on our findings, we focused on HCNs, which have important functions in excitable cells [29–32]. HCNs are non-selective cation channels that control the rhythmic activity of cardiac myocytes and the timing of neuron firing, and are known as “pacemaker channels” [32,33]. The physiological roles of HCNs have been investigated in the heart and brain because all four subunits (HCN1–4) have been cloned. One of these subunits, HCN1, localizes abundantly in the cerebellum, hippocampus, and cortex, and is considered to be involved in learning and memory [32,34]. HCNs exert their effects by generating specific hyperpolarization-activated currents (known as  $I_h$  or  $I_f$ ), which were recognized in the 1970s [35].  $I_h$  plays a significant role in determining the resting membrane potential in these excitable cells [33,36].



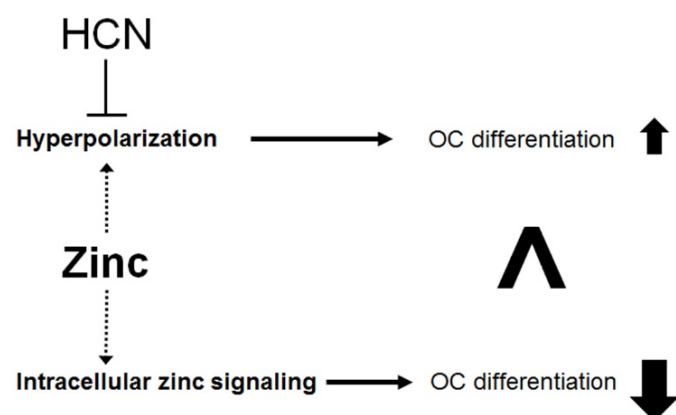
**Figure 1.** ORAI1 knockdown reduced the zinc-induced inhibitory effects on osteoclastogenesis in RAW264.7 cells. (a): ORAI1 knockdown by siRNA transfection, the mRNA expression of ORAI1 was reduced at 24 h after transfection of control-siRNA or ORAI1-siRNA (Mean  $\pm$  SD,  $n=5$ , \* $p<0.01$ ). (b): TRAP activities in the presence or absence of zinc chloride (100 µM) in control and ORAI1-knockdown (ORAI1-KD) cells 3 days after the addition of RANKL (50 ng/ml) ( $n=8$ ). \* $P<0.01$ . Zinc inhibited osteoclastogenesis (1<sup>st</sup> vs. 2<sup>nd</sup> column from the left); however, its inhibition was reduced by ORAI1 knockdown (3<sup>rd</sup> vs. 4<sup>th</sup> column)

Zinc-induced hyperpolarization of osteoclastic cell membrane potential is reduced by ZD7288, which is an HCN inhibitor [14]. Both HCN1 and HCN4 are expressed in osteoclasts: the main subtype is HCN4, based on qRT-PCR analysis and Ih current recording from whole-cell patch clamps. To confirm the relationships between HCN4 and zinc, HCN4 was knocked down using siRNA. Zinc-induced inhibition of osteoclastogenesis was diminished. The investigation of the effects of membrane potentials on osteoclast differentiation was challenging because the non-invasive methods were required for regulating the membrane potential. To resolve this problem, we have generated the RAW cell line that expresses a light-driven pump (Arch). The Arch is activated by yellow-green light and hyperpolarizes via proton transport [37]. Using this light-controlled system, the hyperpolarization was shown to promote osteoclastogenesis. Therefore, zinc inhibits osteoclast differentiation through intracellular signaling cascade, while osteoclast differentiation is promoted via zinc-induced hyperpolarization. Under physiological conditions, zinc-induced promotion of osteoclastogenesis (via changes to the membrane potential) is suppressed by HCN function (Figure 2). These findings suggest that zinc affects the activity of voltage-gated ion channels via changes to the membrane potential.

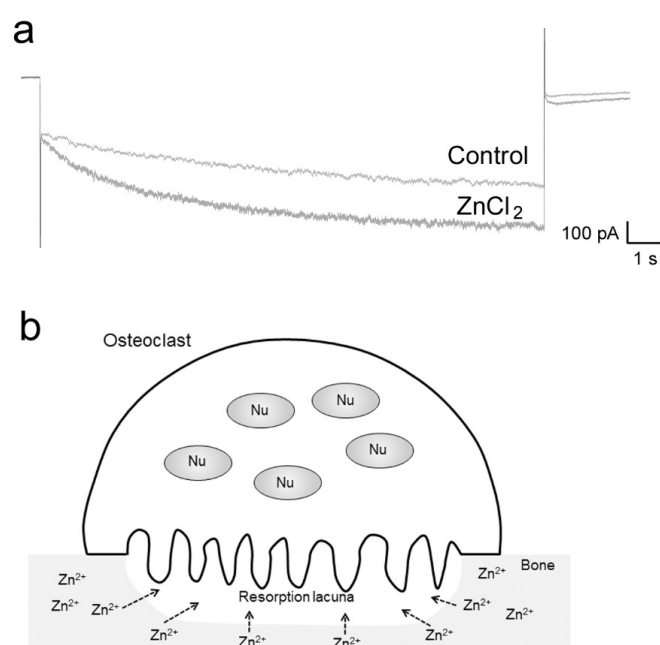
In addition to these results, we have found that high concentrations of zinc chloride increase Ih current (Figure 3a), suggesting a complicated relationship between HCN channels and zinc. This increase in Ih current implies the acceleration of HCN channel activity; thus, high concentrations of zinc itself would reduce hyperpolarization-induced osteoclastogenesis. Although the mechanism of the effects of high zinc concentrations on bone physiology is unclear, the resorption lacuna might be exposed to high concentrations of zinc (Figure 3b) during bone resorption. Effects of high concentrations of zinc on osteoclasts will be investigated in our subsequent experiments involving HCN channels.

## Conclusion

Zinc is abundant in bone; however, its physiological role in bone biology is unclear. Zinc directly affects osteoclastic cell functions via zinc transporters. Because zinc functions as an inhibitor of certain types of ion channels and causes changes to the membrane potential, understanding zinc-modulated ion channel functions would lead to novel findings in bone biology.



**Figure 2.** The proposed relationships between HCN and zinc in osteoclastogenesis



**Figure 3.** Effects of high concentrations of zinc on Ih currents (a: Representative whole-cell currents evoked by hyperpolarization pulses (-150 mV) applied at a holding potential of -30 mV, before (Control) and after addition of ZnCl<sub>2</sub> (100 μM). ZnCl<sub>2</sub> increased hyperpolarization-activated inward current in RAW-derived OC. b: The proposed physiological condition for high concentration of zinc. Nu: nucleus)

## Conflicts of interest

The authors declare no conflict of interest.

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