The Vitamin D Analogue ED71 but Not 1,25(OH)\textsubscript{2}D\textsubscript{3} Targets HIF1\textalpha Protein in Osteoclasts

Yuiko Sato\textsuperscript{1,2}, Yoshiteru Miyauchi\textsuperscript{1,9}, Shigeyuki Yoshida\textsuperscript{3,9}, Mayu Morita\textsuperscript{3}, Tami Kobayashi\textsuperscript{1,4}, Hiroya Kanagawa\textsuperscript{1}, Eri Katsuyama\textsuperscript{1}, Atsuhiro Fujie\textsuperscript{1}, Wu Hao\textsuperscript{1}, Toshimi Tando\textsuperscript{1}, Ryuichi Watanabe\textsuperscript{1}, Kana Miymoto\textsuperscript{1}, HIDEO MORIOKA\textsuperscript{1}, Morio Matsumoto\textsuperscript{1}, Yoshiaki Toyama\textsuperscript{1}, Takeshi Miyamoto\textsuperscript{1,4*}

\textsuperscript{1}Department of Orthopedic Surgery, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan, \textsuperscript{2}Department of Musculoskeletal Reconstruction and Regeneration Surgery, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan, \textsuperscript{3}Department of Dentistry and Oral Surgery, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan, \textsuperscript{4}Department of Integrated Bone Metabolism and Immunology, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan

Abstract

Although both an active form of the vitamin D metabolite, 1,25(OH)\textsubscript{2}D\textsubscript{3}, and the vitamin D analogue, ED71, have been used to treat osteoporosis, anti-bone resorbing activity is reportedly seen only in 1,25(OH)\textsubscript{2}D\textsubscript{3}-treated patients. In addition, how ED71 inhibits osteoclast activity in patients has not been fully characterized. Recently, HIF1\textalpha expression in osteoclasts was demonstrated to be required for development of post-menopausal osteoporosis. Here we show that ED71 but not 1,25(OH)\textsubscript{2}D\textsubscript{3} suppress HIF1\textalpha protein expression in osteoclasts in vitro. We found that 1,25(OH)\textsubscript{2}D\textsubscript{3} or ED71 function in osteoclasts requires the vitamin D receptor (VDR). ED71 was significantly less effective in inhibiting M-CSF and RANKL-stimulated osteoclastogenesis than was 1,25(OH)\textsubscript{2}D\textsubscript{3} in vitro. Downregulation of c-Fos protein and induction of Ifn\beta mRNA in osteoclasts, both of which reportedly block osteoclastogenesis induced by 1,25(OH)\textsubscript{2}D\textsubscript{3} in vitro, were both significantly higher following treatment with 1,25(OH)\textsubscript{2}D\textsubscript{3} than with ED71. Thus, suppression of HIF1\textalpha protein activity in osteoclasts in vitro, which is more efficiently achieved by ED71 rather than by 1,25(OH)\textsubscript{2}D\textsubscript{3}, could be a reliable read-out in either developing or screening reagents targeting osteoporosis.

Introduction

A cause for concern in developed countries is the increasing number of osteoporosis patients and individuals suffering fragility fractures due to osteoporosis [1]. Estrogen-deficiency due to menopause is a risk factor for both [2]. Vitamin D insufficiency is also reportedly observed in osteoporosis patients with fragility fractures and considered a cause of osteoporotic fractures [3]. Indeed, vitamin D is known to play a crucial role in skeletal development, and lack of the vitamin D receptor (VDR) or low vitamin D intake results in Rickets [4] [5].

Currently, active vitamin D analogues are used in several countries to treat patients with bone and mineral disorders associated with chronic renal disease or osteoporosis [6]. Interestingly, 1,25(OH)\textsubscript{2}D\textsubscript{3} has been demonstrated to promote osteoclastogenesis in co-cultures of osteoclast progenitor cells and osteoblastic cells [7]; in addition, 1,25(OH)\textsubscript{2}D\textsubscript{3} elevated receptor activator of nuclear factor kappa B ligand (RANKL), an essential cytokine for osteoclastogenesis, but inhibited expression of OPG, a decoy receptor of RANKL, in osteoblastic cells to promote osteoclast differentiation [8] [9]. In contrast, 1,25(OH)\textsubscript{2}D\textsubscript{3} was shown to inhibit osteoclast differentiation in osteoblastic cell-free culture systems: osteoclast formation induced by macrophage colony stimulating factor (M-CSF) and RANKL was inhibited in the presence of 1,25(OH)\textsubscript{2}D\textsubscript{3} [10] [11]. c-Fos protein, an essential transcription factor for osteoclast differentiation, or interferon beta (Ifn\beta), an inhibitor of osteoclastogenesis, was downregulated or elevated by 1,25(OH)\textsubscript{2}D\textsubscript{3}, respectively, in osteoclast progenitor cells [10] [11]. However, patients treated with a 1,25(OH)\textsubscript{2}D\textsubscript{3} prodrug, alfacalcidol, did not show inhibition of osteoclastic activity or increased bone mass, while patients treated with the vitamin D analogue ED71 exhibited significantly reduced osteoclast activities and increased bone mass [12].

Since postmenopausal osteoporosis is caused in part by estrogen-deficiency, treating of patients with estrogen is one option. However, continuous estrogen administration is associated with adverse effects such as uterine or mammary gland tumors or cardio-vascular disease [13]. Recently, we reported that hypoxia inducible factor 1 alpha (HIF1\textalpha) is required for osteoclast activation following estrogen-deficiency and for development of postmenopausal osteoporosis in animal models [14]. We found that in pre-menopausal mice, HIF1\textalpha activity in osteoclasts is continuously suppressed by estrogen but then HIF1\textalpha accumulate in osteoclasts following estrogen deficiency due to menopause, which in turn activates osteoclastic activity and promotes bone...
loss. Osteoclast specific HIF1α knockout or administration of a HIF1α inhibitor completely abrogated ovariectomy (OVX)-induced osteoclast activation and bone loss [14]. This study suggests that HIF1α could be a therapeutic target for postmenopausal osteoporosis.

Here, we show that HIF1α is a target of ED71 in vitro. HIF1α in osteoclasts was suppressed by ED71 but not by 1,25(OH)2D3. Since inhibition of osteoclast activity was seen in the patients treated with ED71 but not with 1,25(OH)2D3, this work confirms that HIF1α could be a target to treat postmenopausal osteoporosis patients.

Materials and Methods

Mice

C57BL/6 background wild-type mice were purchased from Sankyo Labo Service (Tokyo, Japan). VDR-deficient mice were established previously [4]. Animals were maintained under specific pathogen-free conditions in animal facilities certified by the Keio University School of Medicine animal care committee. All animal procedures were approved by the Keio University School of Medicine animal care committee.

Cell culture

To assess in vitro osteoclast formation, bone marrow cells isolated from H ifflox/flox or Ctsk Cre/H ifflox/flox mouse femurs and tibias were cultured for 72 hours in αMEM (Sigma-Aldrich Co., St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (FBS, JRH Biosciences Lenexa, KS, USA) and GlutaMax (Invitrogen Corp., Carlsbad, CA, USA) supplemented with M-CSF (50 ng/ml, Kyowa Hakko Kirin Co. Tokyo, Japan). Subsequently, adherent cells were collected and cultured under indicated conditions containing M-CSF (50 ng/ml), recombinant soluble RANKL (25 ng/ml, PeproTech Ltd., Rocky Hill, NJ, USA) using 1 × 10⁵ cells per well in 96-well plates. Osteoclastogenesis was evaluated by TRAP staining [15] [16]. Raw264.7 cells were maintained in DMEM (Sigma-Aldrich Co.) containing 10% heat-inactivated FBS (JRH Biosciences) and GlutaMax (Invitrogen

![Figure 1. 1,25(OH)2D3 is a more potent inhibitor of osteoclastogenesis in vitro than is ED71. (A, B and C) M-CSF-dependent osteoclast progenitor cells were isolated from wild-type mice and cultured in the presence of M-CSF (M, 50 ng/ml) + RANKL (R, 25 ng/ml) with or without indicated concentrations of ED71 or 1,25(OH)2D3 (1,25D) for 5 days. Cells were then stained with TRAP (A) and the number of multi-nuclear TRAP-positive cells was counted (B). Expression of Ctsk, NFATc1 and DC-STAMP, all of which are osteoclastic genes, was analyzed by realtime PCR (C). Expression of Blimp1, Bcl6 and Irf8 was analyzed by realtime PCR (D). Data represent mean expression of each relative to Actb ± SD (n = 5). *P<0.05; **P<0.01; ***P<0.001; NS, not significant. doi:10.1371/journal.pone.0111845.g001]
Corp.). For chemical treatment, cells were cultured in phenol red-free media containing 10% charcoal-stripped FBS (Thermo Fisher Scientific K.K., Yokohama, Japan), and treated with 1,25(OH)2D3 (Wako Pure Chemicals Industries, Osaka, Japan, 10−27 M) or ED71 (provided by Chugai Pharmaceutical Co., Ltd, Tokyo, Japan, 10−27 M). Hypoxic cultures was performed at 5% O2/5% CO2 using an INVIVO2 hypoxia workstation (Ruskin Technology Ltd., Bridgend, UK) according to manufacturer’s instruction.

Quantitative PCR analysis

Total RNA was isolated from bone marrow cultures using an RNeasy mini kit (Qiagen), and cDNA synthesis was done by using oligo (dT) primers and reverse transcriptase (Wako Pure Chemicals Industries). Quantitative PCR was performed using SYBR Premix ExTaq II reagent and a DICE Thermal cycler (Takara Bio Inc., Shiga, Japan), according to the manufacturer’s instructions. β-actin (Actb) expression served as an internal control. Primers for Nfatc1, Ctsk, DC-STAMP, Ifnβ and Actb were as follows.

Ctsk-forward: 5'-ACGGAGGCATTGACTCTGAAGATG-3'
Ctsk-reverse: 5'-GGAAGCACCAACGAGAGGAGAAAT-3'
NFATc1-forward: 5'-CAAGTCTCACCACAGGACTCA-3'
NFATc1-reverse: 5'-GCGTGAGAGGTTCATTCTCCAAG-3'
DC-STAMP-forward: 5'-TCCTCCATGAACAAACAGTC-3'

Figure 2. 1,25(OH)2D3 is more active in promoting c-Fos protein inhibition and Ifnβ induction in osteoclasts compared with ED71. (A and B) M-CSF-dependent osteoclast progenitor cells were isolated from wild-type mice and cultured in the presence of M-CSF alone (50 ng/ml) or M-CSF + RANKL (25 ng/ml) with or without 10−27 M ED71 or 1,25(OH)2D3 (1,25D) for 5 days. c-Fos protein was then assessed by western blot (A). Ifnβ expression was analyzed by realtime PCR (B). Data represent mean Ifnβ expression relative to that of Actb ± SD (n = 5). ***P<0.001.
doi:10.1371/journal.pone.0111845.g002

Figure 3. ED71 or 1,25(OH)2D3 activity requires the VDR. (A, B and C) M-CSF-dependent osteoclast progenitor cells were isolated from wild-type (WT) or VDR-deficient (VDR KO) mice and cultured in the presence of M-CSF alone (50 ng/ml) or M-CSF + RANKL (25 ng/ml) with or without indicated concentrations of ED71 or 1,25(OH)2D3 for 5 days. Cells were then stained with TRAP (A), and multi-nuclear TRAP-positive cells were counted (B). Expression of Ctsk, NFATc1 and DC-STAMP was assessed by realtime PCR (C). Data represent mean Ctsk, NFATc1 or DC-STAMP expression relative to that of Actb ± SD (n = 5).
doi:10.1371/journal.pone.0111845.g003
Fos protein was analyzed by western blot (represent mean ± SEM throughout the paper). All data are expressed as the mean ± SD.

VDR knockdown

Raw264.7 cells transduced with MISSION shRNA lentiviruses targeting the VDR or with lentiviruses harboring non-target control constructs (Sigma-Aldrich Co.) were generated according to the manufacturer’s instructions.

Statistical analyses

Statistical analyses were performed using an unpaired two-tailed Student’s t-test (*P<0.05; **P<0.01; ***P<0.005; NS, not significant, throughout the paper). All data are expressed as the mean ± SD.

Results

1,25(OH)_{2}D_{3} inhibits osteoclastogenesis more potently than does ED71 in vitro

Since treatment with ED71, a vitamin D3 analogue, inhibits osteoclast activity and increases bone mineral density more effectively than does the pro-1,25(OH)_{2}D_{3} agent, alfacalcidol [12], we asked whether ED71 inhibited osteoclastogenesis more effectively than 1,25(OH)_{2}D_{3} (1,25D) in vitro (Fig. 1). To do so, we isolated osteoclast progenitor cells from wild-type mice and cultured them in the presence of M-CSF and RANKL, with or without ED71 or 1,25(OH)_{2}D_{3}. We then evaluated osteoclastogenesis by counting multi-nuclear TRAP-positive osteoclasts and examining expression of osteoclastic genes (Fig. 1A-D). Indeed, ED71 significantly inhibited osteoclast differentiation based on both TRAP and gene expression analysis, while 1,25(OH)_{2}D_{3} was more effective in inhibiting osteoclastogenesis than was ED71 in vitro (Fig. 1A and B). Expression of osteoclast differentiation markers such as Cathepsin K (Csk), nuclear factor of activated T cells 1 (NFATc1) and dendritic cell specific transmembrane protein (DC-STAMP) was more significantly inhibited by 1,25(OH)_{2}D_{3} than by ED71 treatment (Fig. 1C). Induction of B lymphocyte-inducible maturation protein 1 (Blimp1) followed by suppression of B cell lymphoma 6 and interferon regulatory factor 8 (Irf8) is reportedly required for osteoclastogenesis [14,17,18]. We found that treatment of osteoclast progenitors with 1,25(OH)_{2}D_{3} elicited more robust inhibition of Blimp1 and activation of Bcl6 and Ifnβ than did treatment with ED71 (Fig. 1D), suggesting that 1,25(OH)_{2}D_{3} is more potent in inhibiting osteoclastogenesis induced by M-CSF and RANKL than ED71.

1,25(OH)_{2}D_{3} reportedly inhibits osteoclast differentiation induced by M-CSF and RANKL by inhibiting c-Fos protein expression in vitro [10]. We found that, by western blot, c-Fos protein was induced by RANKL, and ED71 did not suppress c-Fos protein in osteoclasts as effectively as did 1,25(OH)_{2}D_{3} (Fig. 2A). Although 1,25(OH)_{2}D_{3} reportedly inhibits osteoclastogenesis induced by M-CSF and RANKL via Ifnβ induction in vitro [11], we found that, unlike 1,25(OH)_{2}D_{3}, ED71 did not induce Ifnβ expression in osteoclasts (Fig. 2B).

The VDR is required for both 1,25(OH)_{2}D_{3} and ED71 activity on osteoclasts

Since 1,25(OH)_{2}D_{3} and ED71 activities differ in osteoclasts, we utilized vitamin D receptor (VDR)-deficient mice to test whether
both compounds act on osteoclasts via the VDR (Fig 3). Osteoclast progenitors were isolated from wild-type and VDR-deficient mice and cultured in the presence of M-CSF and RANKL, with or without 1,25(OH)2D3 or ED71 (Fig 3). Inhibitory effects of either 1,25(OH)2D3 or ED71 on osteoclast differentiation were not seen in VDR-deficient osteoclasts (Fig 3A and B). Similarly, inhibition of the expression of osteoclastic genes Ctsk, NFATc1 and DC-STAMP seen following 1,25(OH)2D3 or ED71 treatment was absent in osteoclasts lacking the VDR (Fig 3C).

Moreover, decreased c-Fos protein and elevated Ifnβ expression seen following treatment with 1,25(OH)2D3 or ED71 were abrogated in VDR-deficient osteoclasts (Fig 4A and B), supporting the idea that both compounds act on osteoclasts via the VDR.

**HIF1α is a target of ED71 but not 1,25(OH)2D3 in osteoclasts**

Next, we asked whether HIF1α is a target of ED71 in osteoclasts (Fig 5). Interestingly, we found that in cultured osteoclasts, HIF1α protein levels were suppressed by ED71 but not by 1,25(OH)2D3 (Fig 5A). In contrast, Hif1α mRNA expression in osteoclasts was not inhibited by either treatment (Fig 5B), suggesting that ED71 suppresses HIF1α at the protein level as demonstrated by estrogen treatment [14]. To determine if the VDR is required for ED71-mediated HIF1α protein suppression in osteoclasts, we generated two independent VDR knockdown Raw264.7 lines using shVDR‑1 and shVDR‑2 as well as a control (shControl) line (Fig 5C) and then treated cells with ED71 or 1,25(OH)2D3 (Fig 5D). HIF1α protein suppression by ED71 seen in control cells was abrogated in both VDR knockdown lines, suggesting that HIF1α protein suppression by ED71 is VDR-dependent. We then isolated osteoclast progenitors from Ctsk Cre/Hif1aFlox/Flox mice, cultured them in normoxic conditions to suppress HIF1α protein in osteoclasts (Fig 5E), and treated cells with or without ED71 or 1,25(OH)2D3 (Fig 5E). ED71 treatment effectively inhibited osteoclast differentiation, even in HIF1α-suppressed cells, suggesting that ED71 likely targets factors other than HIF1α protein in osteoclasts (Fig 5E). However, ED71 was less effective than 1,25(OH)2D3 in inhibiting osteoclastogenesis in HIF1α-suppressed cells (Fig 5E).

**Discussion**

Postmenopausal osteoporosis treatment is required to prevent disruption of daily activity or adverse outcomes due to fragile fractures. Among anti-osteoporosis agents, anti-bone resorptive or bone-forming agents include bisphosphonates, selective estrogen receptor modulator (SERM), ED71 and denosumab, or teriparatide, respectively. Strong inhibition of osteoclastic activity beyond physiological levels by bisphosphonates frequently causes adverse effects such as osteonecrosis of the jaw or severely suppressed bone turnover (SSBT) [19] [20]. Meanwhile, teriparatide treatment is limited to less than two years in order to prevent development of tumors, particularly osteosarcoma.

Recently, we showed that HIF1α protein accumulation in osteoclasts following estrogen-deficiency was accompanied by osteoclast activation and bone loss in mice [14]. Either osteoclast-specific HIF1α conditional knockout or wild-type mice administered a HIF1α inhibitor were protected from...
OVX-induced osteoclast activation and bone loss. Moreover, HIF1α inhibition did not interfere with physiological osteoclast activities [14]. Thus, blocking HIF1α pharmacologically could represent an ideal treatment for postmenopausal osteoporosis, as it could target pathologically-activated osteoclasts without altering physiological osteoclastogenesis required for bone turnover. In this study, we found that both ED71, which is used as therapeutic agents for postmenopausal osteoporosis therapy, inhibits HIF1α protein expression. Indeed, patients treated with ED71 exhibit reduced osteoclastic activity and increased bone mass without adverse effects such as osteopenia [12], jaw osteonecrosis or SSBT, as seen in treated bisphosphonate-treated patients [19] [20].

Bone is a target tissue of vitamin D, and indeed, VDR was identified in osteoblasts [21–24]. In contrast, it is controversial whether the VDR is expressed in osteoclasts, with some authors reporting expression [25–28] and others not [21,23,29,30]. Recently, Wang et al. demonstrated that the VDR is not expressed in multi-nuclear osteoclasts using immunohistochemistry of EGTA-decalcified adult mouse bones [21]. In addition, direct effects of 1,25(OH)2D3 have been demonstrated in osteoclasts and osteoclast progenitors [10] [11], and here we report that these effects are VDR-dependent (Fig. 3). Taken together, these studies suggest that extremely low levels of the VDR in osteoclasts may be sufficient to transduce vitamin D signals.

ED71 and 1,25(OH)2D3 have been demonstrated to inhibit osteoclast-bone resorption activity by reducing expression of the sphingosine-1-phosphate receptor 2 (S1PR2) in circulating osteoclast precursor cells and blocking the migration of these cells to the bone surface by S1P; although differences in pharmacological action between ED71 and 1,25(OH)2D3 were not demonstrated [31]. Here, we observed that, although 1,25(OH)2D3 was more potent than ED71 in inhibiting osteoclastogenesis induced by M-CSF and RANKL in vitro, HIF1α inhibition in osteoclasts was specific to ED71. We also found that ED71 inhibited osteoclastogenesis even in HIF1α-suppressed cells, suggesting that ED71 likely targets factors other than HIF1α protein in osteoclasts. However, ED71 was less effective than 1,25(OH)2D3 in inhibiting osteoclastogenesis in HIF1α-suppressed cells, which contrasts with observations seen in patients where the effect of ED71 on osteoclastogenesis is superior to that of 1,25(OH)2D3 [12]. The cause of this difference remains to be elucidated, but the difference of potential activity to target HIF1α-protein in osteoclasts explains, at least in part, this difference. In addition, it is possible that ED71 inhibits osteoclastogenesis through effects on different cell types. Further investigations are needed to define molecular actions of vitamin D3 analogues on bone metabolism. Nonetheless, HIF1α inhibition could serve as an index to assess osteoclastogenesis in vitro when developing anti-osteoporosis agents. Moreover, our study indicates that targeting HIF1α could constitute an effective treatment for osteoporosis, one that would not interfere with physiological bone turnover.

Acknowledgments

This work was supported by a grant-in-aid for Scientific Research. We thank Prof. M. Suematsu and Dr. Y.A. Minamishima for technical support in performing hypoxic culture. We also thank Dr. Shigeaki Kato for providing VDR-deficient mice.

Author Contributions

Conceived and designed the experiments: HM M. Matsumoto YT TM. Performed the experiments: YS YM SY M. Morita. Analyzed the data: HK EK AF WH TT RW KM. Contributed reagents/materials/analysis tools: TK. Wrote the paper: TM.

References

1. Report of a WHO Study Group (1994) Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. World Health Organ Tech Rep Ser 10: 1–129.
2. Ettinger B, Pressman A, Sklarin P, Bauer DC, Cauley JA, et al. (1998) Associations between low levels of serum estradiol, bone density, and fractures among elderly women: the study of osteoporotic fractures. J Clin Endocrinol Metab 83: 2239–2243.
3. Sakuma M, Endo N, Hagiwara H, Harada A, Matsu Y, et al. (2011) Serum 25-hydroxyvitamin D status in hip and spine-fracture patients in Japan. J Orthop Sci 16: 418–423.
4. Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, et al. (1997) Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. Nat Genet 16: 391–396.
5. Winzenberg T, Jones G (2013) Vitamin D and bone health in childhood and adolescence. Calcif Tissue Int 92: 140–150.
6. Plum LA, DeLuca HF (2010) Vitamin D, disease and therapeutic opportunities. Nat Rev Drug Discov 9: 941–953.
7. Takahashi N, Akatou T, Usagawa N, Sasaki T, Yamaguchi A, et al. (1983) Osteoblastic cells are involved in osteoclast formation. Endocrinology 123: 2600–2602.
8. Yasuda H, Shimna N, Nakagawa N, Yamaguchi K, Kinoshita M, et al. (1998) Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci U S A 95: 3597–3602.
9. Miyamoto T, Suda T (2003) Differentiation and function of osteoclasts. Keio J Med 52: 1–7.
10. Takacs-Hinz, Sugiura A, Uchiyama Y, Katagiri N, Okazaki M, et al. (2006) c-Fos protein as a target of anti-osteoclastic action of vitamin D, and synthesis of new analogs. J Clin Invest 116: 528–535.
11. Saki S, Takashii H, Matsuoka K, Kaneko H, Furukawa K, et al. (2009) I-Alpha, 25-hydroxyvitaminD3 inhibits osteoclastogenesis through IFN-beta dependent NFATc1 suppression. J Bone Miner Metab 27: 643–652.
12. Matsutomo T, Ito M, Hayashi Y, Hirota T, Tanigawara Y, et al. (2011) A new active vitamin D3 analog, edelcalcitol, prevents the risk of osteoporotic fractures—a randomized, active comparator, double-blind study. Bone 49: 605–612.
26. Mee AP, Hoyland JA, Braidman IP, Freemont AJ, Davies M, et al. (1996) Demonstration of vitamin D receptor transcripts in actively resorbing osteoclasts in bone sections. Bone 18: 295–299.
27. Menaa C, Barsony J, Reddy SV, Cornish J, Candy T, et al. (2000) 1,25-Dihydroxyvitamin D3 hyposensitivity of osteoclast precursors from patients with Paget’s disease. J Bone Miner Res 15: 228–236.
28. Langub MC, Reinhardt TA, Horst RL, Malluche HH, Koszewski NJ (2000) Characterization of vitamin D receptor immunoreactivity in human bone cells. Bone 27: 383–387.
29. Boivin G, Mesguich P, Pike JW, Bouillon R, Meunier PJ, et al. (1987) Ultrastructural immunocytochemical localization of endogenous 1,25-dihydroxyvitamin D3 and its receptors in osteoblasts and osteocytes from neonatal mouse and rat calvaria. Bone Miner 3: 125–136.
30. Merke J, Klaus G, Hugel U, Waldherr R, Ritz E (1986) No 1,25-dihydroxyvitamin D3 receptors on osteoclasts of calcium-deficient chicken despite demonstrable receptors on circulating monocytes. J Clin Invest 77: 312–314.
31. Kikuta J, Kawamura S, Oishi F, Shirazaki M, Sakai S, et al. (2013) Sphingosine-1-phosphate-mediated osteoclast precursor monocyte migration is a critical point of control in antiresorptive action of active vitamin D. Proc Natl Acad Sci U S A 110: 7009–7013.