The Vitamin D Receptor in Osteoblast-Lineage Cells Is Essential for the Proresorptive Activity of $1\alpha,25$(OH)$_2$D$_3$ In Vivo

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Abbreviations: $1\alpha,25$(OH)$_2$D$_3$, $1\alpha,25$-dihydroxyvitamin D$_3$; 25(OH)D$_3$, 25-hydroxyvitamin D$_3$; ANOVA, analysis of variance; Ca, calcium; Ctsk, cathepsin K; CTX-I, C-terminal crosslinked telopeptide of type I collagen; Cyp24a1, cytochrome P450 family 24 subfamily A member 1; FGF23, fibroblast growth factor 23; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; Ob-VDR-cKO, osteoblast-lineage-specific VDR conditional knockout; OPG, osteoprotegerin; Osx, osterix; RANKL, receptor activator of NF-κB ligand; RT-PCR, reverse transcriptase–polymerase chain reaction; TRAP, tartrate-resistant acid phosphatase; VDR, vitamin D receptor.

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Abstract

We previously reported that daily administration of a pharmacological dose of eldecalcitol, an analog of $1\alpha,25$-dihydroxyvitamin D$_3$ [1$\alpha,25$(OH)$_2$D$_3$], increased bone mass by suppressing bone resorption. These antiresorptive effects were found to be mediated by the vitamin D receptor (VDR) in osteoblast-lineage cells. Using osteoblast-lineage-specific VDR conditional knockout (Ob-VDR-cKO) mice, we examined whether proresorptive activity induced by the high-dose $1\alpha,25$(OH)$_2$D$_3$ was also mediated by VDR in osteoblast-lineage cells. Administration of $1\alpha,25$(OH)$_2$D$_3$ (5 μg/kg body weight/day) to wild-type mice for 4 days increased the number of osteoclasts in bone and serum concentrations of C-terminal crosslinked telopeptide of type I collagen (CTX-I, a bone resorption marker). The stimulation of bone resorption was concomitant with the increase in serum calcium (Ca) and fibroblast growth factor 23 (FGF23) levels, and decrease in body weight. This suggests that a toxic dose of $1\alpha,25$(OH)$_2$D$_3$ can induce bone resorption and hypercalcemia. In contrast, pretreatment of wild-type mice with neutralizing anti-receptor activator of NF-κB ligand; RT-PCR, reverse transcriptase–polymerase chain reaction; TRAP, tartrate-resistant acid phosphatase; VDR, vitamin D receptor.
NF-κB ligand (RANKL) antibody inhibited the 1α,25(OH)₂D₃-induced increase of osteoclast numbers in bone, and increase of CTX-I, Ca, and FGF23 levels in serum. The pre-treatment with anti-RANKL antibody also inhibited the 1α,25(OH)₂D₃-induced decrease in body weight. Consistent with observations in mice conditioned with anti-RANKL antibody, the high-dose administration of 1α,25(OH)₂D₃ to Ob-VDR-cKO mice failed to significantly increase bone osteoclast numbers, serum CTX-I, Ca, or FGF23 levels, and failed to reduce the body weight. Taken together, this study demonstrated that the proresorptive, hypercalcemic, and toxic actions of high-dose 1α,25(OH)₂D₃ are mediated by VDR in osteoblast-lineage cells.

**Key Words:** Proresorptive action, hypercalcemia, toxic action, osteoblast-lineage cells, VDR, Ob-VDR-cKO mice

Vitamin D₃ is an antirachitic factor produced by sunlight ultraviolet irradiation of 7-dehydrocholesterol in the skin (1, 2). In addition, vitamin D₃ is supplied via food intake (1, 2). Severe vitamin D deficiency causes hypocalcemia and insufficient calcification of osteoid, which result in rickets and osteomalacia (1, 2). Vitamin D₃ is hydroxylated at the C-25 position and converted to 25-hydroxyvitamin D₃ [25(OH)D₃] by the hepatic cytochrome P450 enzymes CYP2R1 and CYP27A1 (3, 4). The 25-hydroxylation of vitamin D₃ in the liver is rapid and not regulated. 25(OH)D₃ is then hydroxylated at the C-1α position by the renal 25(OH)D-1α-hydroxylase (CYP27B1), resulting in the generation of the biologically active form of vitamin D₃, 1,25-dihydroxyvitamin D₃ [1α,25(OH)₂D₃] (5). The expression of 1α-hydroxylase is negatively regulated by 1α,25(OH)₂D₃ itself, high dietary calcium (Ca), high dietary phosphorus (as “phosphate”), and fibroblast growth factor 23 (FGF23), a phosphaturic bone-derived hormone (6-10), and positively regulated by low dietary Ca and parathyroid hormone (6, 9-11). 1α,25(OH)₂D₃ produced in the kidneys is transported to the target organs via blood circulation (2). It then binds to the vitamin D receptor (VDR) and upregulates serum Ca levels through intestinal Ca absorption, renal tubular reabsorption of Ca, and bone resorption (2). Thus, 1α,25(OH)₂D₃ is widely recognized as a calcitropic hormone due to its tightly controlled renal production, blood-mediated delivery to the target tissues, and regulated actions through present VDR (2).

1α,25(OH)₂D₃ stimulates osteoclastic bone resorption in rat bone organ cultures (12). It can also stimulate osteoclast formation in coculture of mouse osteoblastic cells and hematopoietic cells (13). Osteoblastic cells express receptor activator of NF-xB ligand (RANKL) and osteoprotegerin (OPG), thereby regulating the generation of osteoclasts. 1α,25(OH)₂D₃ increases the RANKL/OPG ratio by upregulating RANKL and downregulating OPG gene transcription via VDR (14-16).

On the contrary, long-term treatment with pharmacological doses of active vitamin D₃ drugs, as well as sustained overexpression of VDR in mature osteoblast-lineage cells, has been demonstrated to increase bone mass by suppressing bone resorption (17-25). Due to this efficacy, 3 vitamin D₃ analogs, 1α(OH)D₃ (alfacalcidol), 1α,25(OH)₂D₃ (calcitriol), and 1α,25(OH)₂β-(3-hydroxy-propyloxy) D₃ (eldecalcitol), have been approved for the treatment of osteoporosis in Japan (17, 20, 23). Alfacalcidol undergoes 25-hydroxylation in the liver and acts as 1α,25(OH)₂D₃, whereas eldecalcitol functions as a hormonally active vitamin D₃ by directly binding VDR (26, 27). Eldecalcitol is shown to inhibit bone resorption and stimulate formation in an ovariectomized rat model of osteoporosis (18). Transgenic overexpression of VDR in mature osteoblast-lineage cells also decreased bone resorption and increased formation under physiological conditions (24, 25). The inhibition of bone resorption by alfacalcidol was suggested to be due to decreasing the pool of osteoclast precursors (19). In Phase 2 clinical studies on osteoporosis in 2005, treatment with eldecalcitol for 12 months significantly increased bone mineral density and reduced urinary levels of N-terminal crosslinked telopeptide of type I collagen, a bone resorption marker, in a dose-dependent manner (28). Therefore, it has been confirmed both experimentally and clinically that long-term treatment with pharmacological doses of active vitamin D₃ analogs suppresses bone resorption and results in a net increase in bone mass.

To evaluate the effects of eldecalcitol on bone resorption and formation, we administered a pharmacological dose of eldecalcitol (50 ng/kg of body weight/day) daily for 4 weeks to C57BL/6 male mice aged 9 weeks (29). We confirmed that eldecalcitol administration increased bone mass by suppressing bone resorption via downregulation of the RANKL/OPG ratio in bone tissues without affecting the number of osteoclast precursors (29). To clarify which type of VDR-expressing cells preferentially regulated the eldecalcitol-induced increase in bone mass, we generated VDR-expressing (osteoblast and osteocyte)-specific VDR conditional...
knockout [Ob-VDR-cKO, osterix (Osx)-Cre^{Tg0}; VDR^{fl/0}] and osteoclast-specific VDR-cKO [Ocl-VDR-cKO, cathepsin (Ctsk)^{Cre+}; VDR^{fl/0}] mice (30). Administration of eldecalcitol for 4 weeks neither suppressed bone resorption nor increased bone mass in Ob-VDR-cKO mice (30). In contrast, it suppressed bone resorption and subsequently increased bone mass in Ocl-VDR-cKO mice and control mice (30). These results suggested that VDR in osteoblast-lineage cells, but not enterocytes, renal cells, or osteoclasts, primarily mediates the eldecalcitol and other bioactive vitamin D-induced increase in bone mass (14, 15, 24, 25, 29, 30).

In the present study, we examined (i) whether administration of large amounts of 1α,25(OH)₂D₃ increases bone resorption in vivo, and if so, (ii) whether the proresorptive effects are mediated by VDR in osteoblast-lineage cells. High-dose administration of 1α,25(OH)₂D₃ to normal mice stimulated osteoclastic bone resorption with increasing serum Ca levels, intact FGF23 levels, and reducing body weight. The proresorptive effects were not observed in neutralizing anti-RANKL antibody-treated mice or Ob-VDR-cKO mice. Concomitantly, the hypercalcemic and weight loss effects of 1α,25(OH)₂D₃ were attenuated in both anti-RANKL antibody-treated mice and Ob-VDR-cKO mice. Taken together, our study suggests that in vivo administration of a toxic dose of 1α,25(OH)₂D₃ promotes bone resorption via VDR in osteoblast-lineage cells. We also discuss the physiological roles of VDR in osteoblast-lineage cells.

Materials and Methods

Animals

C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). Osx-Cre^{Tg0} (C57BL/6 genetic background) were purchased from the Jackson Laboratory (Bar Harbor, ME) (31). VDR-floxed mice (C57BL/6 genetic background) were generated by the authors (Y.Y., Y.N., and S.K.) (32). Mice were fed a standard diet containing 0.8% ground) were generated by the authors (Y.Y., Y.N., and S.K.) (31). VDR-floxed mice (C57BL/6 genetic background) were purchased from the Jackson Laboratory (Bar Harbor, Japan). Osx-Cre Tg/0 (C57BL/6 genetic background) were purchased from Japan SLC (Shizuoka, Japan). Femora were fixed with 4% paraformaldehyde and decalcified in 10% EDTA (pH 7.3) for 3 weeks at 4 °C. Then, the specimens were dehydrated in a graded series of ethanol solutions, embedded in paraffin, and cut into 4-µm-thick sections. Double staining for methyl green and tartrate-resistant acid phosphatase staining

Administration of drugs

A stock solution of 1α,25(OH)₂D₃ (Fujifilm Wako, Osaka, Japan) dissolved in ethanol was diluted in propylene glycol at a 1:9 ratio. 1α,25(OH)₂D₃ (0, 1, 5, 10, or 20 µg/kg of body weight/day) was administered daily for 4 days subcutaneously to 8-week-old male C57BL/6 mice. Anti-RANKL antibody (catalog No. 47104001, clone OYC1, Oriental Yeast, Tokyo, Japan) (33, 34) or control IgG (R&D systems, Minneapolis, MN) (5 mg/kg) was subcutaneously injected once to 8-week-old male C57BL/6 mice 4 days prior to 1α,25(OH)₂D₃ or vehicle treatment for 4 days. Eight-week-old Ob-VDR-cKO (Osx-Cre^{Tg0}; VDR^{fl/0}) or control (Osx-Cre^{Tg0}; VDR^{fl/+}) mice were treated with 1α,25(OH)₂D₃ (5 µg/kg of body weight/day) or vehicle for 4 days. In this Ob-VDR-cKO mouse experiment, we used both sexes, because the regulation of calcium-phosphate homeostasis and bone metabolism by 1α,25(OH)₂D₃ does not appear to be gender-dependent (1, 2). Mice were euthanized with ether 24 hours after the last 1α,25(OH)₂D₃ injection, and blood and tissue samples were collected. Mice were treated according to the institutional (Matsumoto Dental University) ethical guidelines for animal experimentation and safety.

Serum biochemistry

Ca and phosphorus concentrations in serum were measured by the Calcium E-test kit (Fujifilm Wako) and the Phospha-C test kit (Fujifilm Wako), respectively. Serum C-terminal crosslinked telopeptide of type I collagen (CTX-I) concentrations were measured using the RatLaps EIA kit (catalog No. AC-06F1, Immunodiagnostic Systems, Boldon, UK) (35). Intact FGF23 levels were measured using the FGF-23 ELISA kit (catalog No. CY-4000, Kainos Laboratories, Tokyo, Japan) (36).

Tartrate-resistant acid phosphatase staining

Femora were fixed with 4% paraformaldehyde and decalcified in 10% EDTA (pH 7.3) for 3 weeks at 4 °C. Then, the specimens were dehydrated in a graded series of ethanol solutions, embedded in paraffin, and cut into 4-µm-thick sections. Double staining for methyl green and tartrate-resistant acid phosphatase (TRAP) was performed. TRAP-positive osteoclasts were detected as described previously (37). The number of osteoclasts per millimeter of trabecular bone surface was measured using Image J software (National Institutes of Health, Bethesda, MD). Images were obtained using Plan-Neofluor 5x/0.15 and Plan-Neofluor 40x/0.75 objectives (Carl Zeiss, Oberkochen, Germany) on a microscope (Axioplan 2 imaging; Carl Zeiss) with a digital camera (AxioCamHRc, Carl Zeiss). Images were captured using AxioVision software (Carl Zeiss). Figures were constructed using Photoshop (Adobe, San Jose, CA).
Real-time reverse transcriptase–polymerase chain reaction

Tissue samples were collected, immediately soaked in TRIzol (Thermo Fisher Scientific, Waltham, MA), and homogenized with TissueLyser II (Qiagen, Hilden, Germany). Total RNA was extracted using the Purelink RNA mini kit (Thermo Fisher Scientific). First-strand cDNA was synthesized from total RNA with the oligo (dT)12-18 primer (Thermo Fisher Scientific) and ReverTra Ace reverse transcriptase (ToYoBo, Osaka) according to the manufacturer’s protocols. For preparation of bone samples, tibiae were isolated, and the epiphysis and adherent soft tissues were cut away and rubbed off with Kimwipe papers. The cleaned tibiae containing bone marrow were subjected to total RNA extraction. Real-time reverse transcriptase–polymerase chain reaction (RT-PCR) for the quantification of mRNA expression was performed using the Fast SYBR Green and StepOnePlus System (Thermo Fisher Scientific). The following temperature profile was used: 95 °C for 20 seconds, followed by 40 cycles of 95 °C for 3 seconds and 60 °C for 30 seconds. Each gene expression level was calculated using a relative standard curve. Gene expression was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (Gapdh). Mouse primers for Rankl, Opg, Ctsk, Cyp24a1, and Gapdh (Hokkaido System Science, Sapporo, Japan) are listed in Table 1.

Table 1. Primers Used in Real-time RT-PCR

| Gene   | Forward                      | Reverse                      |
|--------|------------------------------|------------------------------|
| Gapdh  | 5’-TGTGTCCGTCGTGGATCTGA-3’   | 5’-TTGCTGTGTAAGTCCGGAGG-3’   |
| Rankl  | 5’-CATGTCGACCTTCAGAACC-3’    | 5’-CAGGCTCCTCCAGGCAATGA-3’   |
| Opg    | 5’-CATGAGGTTTCCTCGACAGCC-3’  | 5’-ACAGCCAGTGCACATTGGTTTA-3’ |
| Ctsk   | 5’-CCAGCCAGAGGGCT-3’         | 5’-CTTTCCGTCGGTCTGATAC-3’    |
| Cyp24a1| 5’-CGTCCCCATTGCGCTTGTG-3’    | 5’-TCTTTGATTTGGGGTGA-3’      |

Statistical analysis

The statistical analysis was performed using the GraphPad Prism 8 statistical software (GraphPad Software, San Diego, CA). All data except for Fig. 1A are presented as the mean ± standard deviation (SD) of at least 4 mice. To compare 2 groups with normal distribution and equal variances, 2-tailed Student t test was used to assess significance. For analysis of 2 groups whose datasets do not have normal distribution or equal variances, Mann-Whitney U test was used. To compare multiple groups, 1-way analysis of variance (ANOVA) with the Tukey post hoc test was performed. A P value of < 0.05 was considered significant.

Results

A pilot time-course experiment was performed to identify the optimal duration of 1α,25(OH)2D3 administration for the induction of hypercalcemia. 1α,25(OH)2D3 was injected at a daily dosage of 1 μg/kg of body weight to male and female C57BL/6 mice, whose sera were collected from tail vein at day 0, 1, 2, and 3, and by heart puncture at day 4 under ether anesthesia. At day 4, the mice injected with 1α,25(OH)2D3 were euthanized, because they looked exhausted. Daily injection of 1α,25(OH)2D3 caused daily increases of serum Ca levels throughout the 4-day follow-up period (Fig. 1A). To determine the in vivo optimal dose of 1α,25(OH)2D3 for the induction of bone resorption, 8-week-old male C57BL/6 mice were injected daily with different amounts of 1α,25(OH)2D3 (1, 5, 10, or 20 μg/kg of body weight/day) for 4 days and their sera were collected (Fig. 1B). Injection of 1α,25(OH)2D3 dose-dependently increased serum Ca levels (Fig. 1C). It did not significantly increase serum phosphorus levels, which varied greatly in each data set. Of note, 1 mouse in each group treated with ≥5 μg/kg of body weight/day exhibited severe hyperphosphatemia (>15 mg/dL) (Fig. 1D). Serum concentrations of intact FGF23, an osteocyte and osteoblast-derived key hormone in the calcium-phosphate homeostasis (7, 8, 38, 39), were drastically increased by 1α,25(OH)2D3 injection (Fig. 1E). Serum concentrations of C-terminal telopeptide of type I collagen (CTX-I), a bone resorption marker, were also upregulated by injection of 1α,25(OH)2D3 in a dose-dependent manner (Fig. 1F). The 1α,25(OH)2D3 injection dose-dependently reduced body weight (Fig. 1G). These results suggested that the administration of large amounts of 1α,25(OH)2D3 is toxic and stimulates bone resorption. One mouse died in the 10 μg/kg and in the 20 μg/kg of body weight/day group during the experiment. These mice were not included in Fig. 1. Therefore, we selected 5 μg/kg of body weight/day for 4 days as the optimal dose and period for further analyses of the proresorptive (i.e., toxic) action.

A single pre-injection of neutralizing anti-RANKL antibody (OYC1) (34) into mice was shown to consistently suppress bone resorption even after administration of parathyroid hormone daily for 2 weeks (33). When mice are pretreated with anti-RANKL antibody,
α,25(OH)2D3-induced bone resorption should be observed. Therefore, a single dose of anti-RANKL antibody or control IgG (5 mg/kg of body weight) was administered to male C57BL/6 mice, and 4 days later, 1α,25(OH)2D3 (5 μg/kg of body weight/day) was daily administered for 4 days. At day 4, femora, tibiae, kidneys, and blood were collected from these mice (Fig. 2A). In control IgG-pretreated mice, a number of TRAP-positive osteoclasts were observed along the bone surface (Fig. 2B and 2C). When 1α,25(OH)2D3 was administered to these mice, an increased number of osteoclasts was observed. Pretreatment with anti-RANKL antibody markedly reduced osteoclast numbers in the vehicle-treated mice (Fig. 2B and 2C). Administration of 1α,25(OH)2D3 hardly induced osteoclasts in bone tissues in anti-RANKL antibody-pretreated mice (Fig. 2B, C). Consistent with this, 1α,25(OH)2D3 significantly increased serum CTX-I levels in control IgG-pretreated mice, but not in anti-RANKL antibody-pretreated mice (Fig. 2D). These results suggested that a toxic dose of 1α,25(OH)2D3 can induce bone resorption in vivo.

Figure 1. Dose-response effects of 1α,25(OH)2D3 on serum parameters. (A) Time-course changes of serum calcium (Ca) levels in a pilot test at a dosage of 1 μg/kg of body weight/day. The vehicle-treated group consisted of 1 male and 2 female mice. The 1α,25(OH)2D3-treated group consisted of 1 female and 3 male mice. (B) The experimental schedule for the determination of an optimal dose. Eight-week-old male C57BL/6 mice were administered different doses of 1α,25(OH)2D3 (1, 5, 10, or 20 μg/kg of body weight/day) daily for 4 days. Mice were euthanized at day 4 to collect blood samples, and then subjected to measurements of serum Ca (C), phosphorus (P) (D), intact FGF23 (E), CTX-I (F) levels, and body weight (G). Values represent the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001; by 2-tailed Student t test; compared with the vehicle-treated (-) group. Groups sharing a letter are not significantly different (P > 0.05, 1-way ANOVA with post hoc Tukey test). b-bc, c-bc; not significant. Groups having different letters are significantly different (P < 0.05).
Next, we compared serum biochemical parameters and mRNA expression in bone and kidneys between control IgG-pretreated and anti-RANKL antibody-pretreated mice. Administration of 1α,25(OH)2D3 significantly increased serum Ca levels in control IgG-pretreated mice. The 1α,25(OH)2D3 administration also increased serum Ca levels in anti-RANKL antibody-pretreated mice, but the extent of the increase was smaller (~50%) than that observed in control IgG-pretreated mice (Fig. 3A). Serum phosphorus levels were not significantly increased by 1α,25(OH)2D3 administration in control IgG-pretreated or anti-RANKL antibody-pretreated mice. Of note, the 2 of 4 1α,25(OH)2D3-treated mice in the control IgG-pretreated group exhibited severe hyperphosphatemia (>15 mg/dL), whereas 1α,25(OH)2D3 did not induce hyperphosphatemia in any of the anti-RANKL antibody-pretreated mice (Fig. 3B). Upregulation of RANKL mRNA expression by 1α,25(OH)2D3 in bone was observed in both control and anti-RANKL antibody-pretreated mice (Fig. 3C). Expression levels of OPG mRNA in bone were not altered by 1α,25(OH)2D3 (Fig. 3D). On the other hand, upregulated expression of cathepsin K (Ctsk) mRNA, a marker enzyme of osteoclasts, in bone was observed in control mice, but not in anti-RANKL
antibody-pretreated mice (Fig. 3E). Cyp24A1, an enzyme that hydroxylates C-24 of 1α,25(OH)₂D₃ and 25(OH)D₃, is induced by 1α,25(OH)₂D₃ in the target tissues through VDR (1, 2). The increase in renal Cyp24A1 expression by 1α,25(OH)₂D₃ was comparable between control mice and anti-RANKL pretreated mice (Fig. 3F). This suggested...
(i) that 1α,25(OH)₂D₃ stimulates osteoclastogenesis through the upregulation of RANKL expression in bone tissues, and (ii) that the inhibition of 1α,25(OH)₂D₃-induced osteoclastogenesis by anti-RANKL antibody can reduce the hypercalcemic effects of 1α,25(OH)₂D₃.

To examine whether the proresorptive effects of a toxic dose of 1α,25(OH)₂D₃ were mediated by VDR in osteoblast-lineage cells, we evaluated bone resorption in 1α,25(OH)₂D₃-treated Ob-VDR-cKO mice. 1α,25(OH)₂D₃ (5 μg/kg of body weight/day) was administered daily for 4 days to control (Osx-Cre⁺/⁻; VDR⁺/⁺) mice and Ob-VDR-cKO (Osx-Cre⁺/⁻; VDR⁻/⁻) mice of both sexes, and their femora, tibiae, and blood were collected (Fig. 4A).

TRAP-positive osteoclasts were similarly observed in both control and Ob-VDR-cKO mice treated with the vehicle (Fig. 4B, C). Administration of 1α,25(OH)₂D₃ markedly increased the number of TRAP-positive osteoclasts in the femora of control mice, but not in those of Ob-VDR-cKO mice (Fig. 4B, C). Injection of 1α,25(OH)₂D₃ increased serum CTX-I levels in control mice, but not in Ob-VDR-cKO mice (Fig. 4D). Therefore, osteoclast formation induced by 1α,25(OH)₂D₃ was mediated by VDR in osteoblast-lineage cells. We then analyzed serum biochemical parameters and mRNA expression of genes related to osteoclastogenesis in bone tissues of control and Ob-VDR-cKO mice. Administration of 1α,25(OH)₂D₃...
significantly increased serum Ca levels in control mice, but not in Ob-VDR-cKO mice (Fig. 5A). Serum phosphorus levels were not significantly increased by 1α,25(OH)₂D₃ in both control and Ob-VDR-cKO mice. Of note, 2 of 4 1α,25(OH)₂D₃-treated control mice exhibited severe hyperphosphatemia (>30 mg/dL), whereas 1α,25(OH)₂D₃ did not induce hyperphosphatemia in any of the Ob-VDR-cKO mice (Fig. 5B). Upregulation of RANKL mRNA expression by 1α,25(OH)₂D₃ was observed in bone of control mice, but not in bone of Ob-VDR-cKO mice (Fig. 5C). Expression levels of OPG mRNA in bone were not altered by 1α,25(OH)₂D₃ (Fig. 5D). Consistent with the changes in

Figure 5. Effects of osteoblast-lineage-specific ablation of VDR on 1α,25(OH)₂D₃-induced changes in serum Ca and P, and mRNA expression in bone and kidneys. Results were obtained from the same experiments as Fig. 4. Serum levels of Ca (A) and P (B) and intact FGF23 (G) were measured. mRNA expression levels of RANKL (C), OPG (D), and Ctsk (E) in tibiae, and Cyp24A1 in kidneys (F) were quantified by real-time RT-PCR. Results of serum intact FGF23 levels (G) obtained from the same experiments as Fig. 2 were also shown for a close comparison between the effects of pre-treatment with αRANKLmAb and those of osteoblast-lineage-specific ablation of VDR. Values represent the mean ± SD. *P < 0.05; by 2-tailed Mann-Whitney U test; compared with the vehicle-treated (-) group. Groups sharing a letter are not significantly different (P > 0.05, 1-way ANOVA with post hoc Tukey test). Groups having different letters are significantly different (P < 0.05).
osteoclast numbers and RANKL expression, upregulated expression of Ctsk mRNA by 1α,25(OH)₂D₃ in bone was observed in control mice, but not in Ob-VDR-cKO mice (Fig. 5E). Expression of renal Cyp24A1 mRNA was increased by 1α,25(OH)₂D₃, similarly in both control and Ob-VDR-cKO mice (Fig. 5F). These observations suggested that in vivo administration of a toxic dose of 1α,25(OH)₂D₃ can induce osteoclast formation through VDR-mediated control of gene expression in osteoblast-lineage cells. Serum levels of intact FGF23 were several-hundred-fold increased by 1α,25(OH)₂D₃ administration in the control (control IgG and control) groups. 1α,25(OH)₂D₃ increased serum FGF23 levels a hundred times in anti-RANKL antibody-pretreated mice, although the pretreatment with anti-RANKL antibody attenuated the increase of FGF23 levels induced by 1α,25(OH)₂D₃ administration. Notably, 1α,25(OH)₂D₃ failed to significantly increase serum FGF23 levels in Ob-VDR-cKO mice, indicating that production of FGF23 is tightly regulated by VDR in osteoblast-lineage cells (Fig. 5G).

Lastly, we assessed the relationship between body weight loss and bone resorption induced by 1α,25(OH)₂D₃. Administration of 1α,25(OH)₂D₃ (5 μg/kg of body weight/day for 4 days) significantly reduced the body weight in control IgG-pretreated mice and anti-RANKL antibody-pretreated mice. The reduction induced by 1α,25(OH)₂D₃ was greatly attenuated in anti-RANKL antibody-pretreated mice (Fig. 6A). Administration of 1α,25(OH)₂D₃ significantly reduced the body weight in control mice, but not in Ob-VDR-cKO mice (Fig. 6B). To take a closer look at the relationship between the % changes in body weight and serum Ca levels, all data of these parameters in this study were subjected to a correlation plot analysis (Fig. 6C). The correlation squared (R²) was 0.76, and the slope was −2.85 (P < 0.0001), indicating that the body weight loss was sufficiently explained by hypercalcemia. These results suggested that inhibition of bone resorption can prevent 1α,25(OH)₂D₃ toxicity.

Discussion

We demonstrated (i) that high-dose administration of 1α,25(OH)₂D₃ can stimulate bone resorption and (ii) that this stimulation is due to VDR in osteoblast-lineage cells. Administration of 1α,25(OH)₂D₃ (5 μg/kg of body weight/day for 4 days) to control mice increased osteoclast numbers, serum Ca, serum intact FGF23, and serum CTX-I levels. When 1α,25(OH)₂D₃ was administered to mice pretreated with anti-RANKL antibody, the 1α,25(OH)₂D₃-induced bone resorption was suppressed and the increase in serum Ca was attenuated. Ob-VDR-cKO mice did not exhibit the 1α,25(OH)₂D₃-induced increase in these bone resorption-related parameters. A high variability in serum phosphorus concentrations induced by 1α,25(OH)₂D₃ administration was probably due to osteoclastic bone resorption, which enhanced release of phosphate and Ca from bone. Subsequently, enhancement of urinary phosphate excretion likely occurred due to 1α,25(OH)₂D₃-induced increase in serum intact FGF23 levels. FGF23 has been discovered to be a phosphaturic hormone that suppresses phosphate reabsorption in renal proximal tubules (40, 41). Simultaneous enhancement of bone resorption and FGF23 production seemed to fluctuate serum phosphorus levels. This fluctuation was not observed in anti-RANKL antibody-pretreated mice or Ob-VDR-cKO mice. The present study revealed that a toxic dose of 1α,25(OH)₂D₃ promotes bone resorption and this toxic action is mediated by VDR in osteoblast-lineage cells in vivo. It is well established that 1α,25(OH)₂D₃ can stimulate osteoclast formation in coculture of mouse osteoblastic cells and hematopoietic cells through VDR expressed in osteoblast-lineage cells (42). Taken together, we conclude that 1α,25(OH)₂D₃ plays a proresorptive role via VDR in osteoblast-lineage cells in vitro and in vivo (Fig. 7).

Long-term administration of a pharmacological dose of eldecalcitol as well as alfacalcidol suppressed bone resorption and increased bone mass (17-23, 28-30). Transgenically overexpressed VDR in mature osteoblast-lineage cells in mice also increased bone mass by suppressing bone resorption (24, 25). These antiresorptive effects were not observed in Ob-VDR-cKO mice (30). Thus, the proresorptive action and the antiresorptive action of bioactive vitamin D are mediated by VDR in osteoblast-lineage cells. How does VDR in osteoblast-lineage cells select the mode of action? Increases in bone resorption by 1α,25(OH)₂D₃ are shown to require a large dose and relatively short treatment period of around 4 days. In contrast, inhibition of bone resorption by bioactive vitamin D needs lower doses (=pharmacological doses) and a long treatment period of more than 4 weeks. This long and modest stimulation with eldecalcitot and stimulation by overexpressed VDR in mature osteoblast-lineage cells are shown to reduce the RANKL/OPG ratio in bone tissues (25, 29, 30). It is well known that the RANKL/OPG ratio is regulated by factors other than 1α,25(OH)₂D₃, such as parathyroid hormone and various cytokines (16). Long-term administration of a pharmacological dose of bioactive vitamin D may regulate the secretion and expression of such regulators of bone resorption via VDR in osteoblast-lineage cells.

Some studies suggested that vitamin D₃ analogs selectively recruit VDR coactivators for transcription (43, 44).
However, we saw just similar effects on bone resorption between eldecalcitol and 1α,25(OH)2D3 at a pharmacological dose (29) and also at a toxic dose of 5 μg/kg of body weight/day for 4 days (serum Ca: 9.3 ± 0.52 mg/dL [vehicle, n = 4], 16.3 ± 0.98a mg/dL [eldecalcitol, n = 5], 15.4 ± 0.91a mg/dL [1α,25(OH)2D3, n = 4]), *p < 0.0001; serum CTX-I: 17.9 ± 5.5 ng/ml [vehicle, n = 4], 146 ± 59.7a ng/mL (eldecalcitol, n = 5), 110 ± 44.3a ng/mL [1α,25(OH)2D3, n = 4], *p < 0.05, 1-way ANOVA with Tukey test). Coactivator complexes recruited by toxic doses of 1α,25(OH)2D3 and eldecalcitol may be different from those by pharmacological doses and this is one of the possibilities for the opposite actions. Elucidation of the mechanism by which VDR in osteoblast-lineage cells exert biphasic and opposite effects on bone resorption currently remains as an unresolved and valuable challenge (Fig. 7).
Bioactive vitamin D regulates serum Ca levels. Does VDR in osteoblast-lineage cells play important roles in this central function in physiology? Vitamin D-deficient/insufficient animals and humans, as well as global VDR-KO mice, exhibit hypocalcemia and rickets/osteomalacia (2, 45-47). We previously analyzed VDR expression in bone tissues by immunohistochemistry and found that VDR was expressed exclusively in osteoblasts and osteocytes among bone cells, and was limited in chondrocytes, osteoclasts, and bone marrow cells (30). Although VDR expression was almost completely diminished in bone of Ob-VDR-cKO mice, no notable abnormalities were found in bone formation, bone resorption, serum Ca levels, or overall health of these mice in homeostasis (30). Late-osteoblast-lineage (mature osteoblast and osteocyte)-specific VDR-cKO mice also showed no abnormalities in the bone phenotype, and serum Ca levels (48). Therefore, 1α,25(OH)₂D₃-VDR signaling in extraskeletal tissues is essential for Ca homeostasis. Global VDR-KO mice do not exhibit abnormalities before weaning (3 weeks of age) and develop rickets or osteomalacia after weaning (47). A high-Ca diet can cure rickets/osteomalacia in global VDR-KO mice (49, 50). Transgenic expression of VDR in the intestine in global VDR-KO mice normalized intestinal Ca absorption and mineralization of bone (51). These findings suggest that 1α,25(OH)₂D₃ has evolved as a hormone to stimulate Ca absorption from the intestine. 1α,25(OH)₂D₃ increases reabsorption of Ca in distal tubules in kidneys (50, 52, 53). In global VDR-KO mice, renal Ca reabsorption is suppressed, and consequently, their Ca excretion to urine is increased (50). Thus, bioactive vitamin D also regulates renal Ca reabsorption. VDR in osteoblast-lineage cells was unlikely involved in such renal and intestinal regulation of serum Ca levels in homeostasis (Fig. 7).

Long-term administration of a pharmacological dose of eldecalcitol to mice slightly but significantly increased serum Ca levels (29, 30). This mild hypercalcemic effect of eldecalcitol was also not observed in Ob-VDR-cKO mice (30). Thus, VDR in osteoblast-lineage cells may function for regulation of serum Ca levels and bone resorption specifically when bioactive vitamin D is overloaded by administration (Fig. 7).

1α,25(OH)₂D₃ did not enhance bone resorption in mice pretreated with anti-RANKL antibody, as that was assessed by osteoclast numbers and CTX-I. However, 1α,25(OH)₂D₃ increased serum Ca levels in these mice but not in Ob-VDR-cKO mice. What is the reason for this? If this hypercalcemic action was caused by effects of 1α,25(OH)₂D₃ on intestine or kidney, it was surprising that 1α,25(OH)₂D₃ did not increase serum Ca levels in Ob-VDR-cKO mice. We consider that the difference in 1α,25(OH)₂D₃-induced FGF23 production between anti-RANKL antibody-pretreated mice and Ob-VDR-cKO is the reason for the presence or absence of the hypercalcemic action. FGF23 is recently reported to promote Ca reabsorption in renal distal tubules through the transient receptor potential cation channel subfamily V member 5 (TRPV5) in a VDR-independent manner (38). FGF23/VDR-double KO mice on rescue diet showed more severe renal Ca wasting than VDR-KO mice on rescue diet (38). Consistently, mice with conditional deletion of FGF receptor 1 (a receptor for FGF23) in the distal tubule exhibit renal Ca wasting and reduced trabecular bone mineral density (54). Therefore, we speculate that sufficiently increased FGF23 by 1α,25(OH)₂D₃ administration enhanced renal Ca reabsorption through FGF receptor 1 in distal tubules and contributed to the increase of serum Ca levels in control mice and anti-RANKL-pretreated mice.

Then, the result of FGF23 levels raises another question about smaller increases in serum FGF23 levels by 1α,25(OH)₂D₃ administration in anti-RANKL-pretreated mice than in control IgG-pretreated mice. Quinn et al (39) reported that not only high serum phosphorus levels but also high serum Ca levels increased FGF23 production in bone. However, the best correlation between serum Ca and phosphorus and serum FGF23 levels was found between serum FGF23 levels and the Ca × phosphorus product. This means FGF23 synthesis is highly interrelated to both serum phosphorus and Ca levels. The relationship between the Ca × phosphorus product and serum FGF23 levels was not linear but quadratic. This quadratic relationship is likely to be the reason for the smaller increases in FGF23 levels in anti-RANKL-pretreated mice, as the extent of increases in serum Ca levels by 1α,25(OH)₂D₃ administration was
smaller in anti-RANKL-pretreated mice than in control IgG-pretreated mice.

Daily administration of a toxic dose of 1α,25(OH)₂D₃ reduced the body weight proportionally to the increase of serum Ca levels. In addition, 1α,25(OH)₂D₃ administration suppressed locomotor activity of the mice; however, the body weight loss and decrease in locomotor activity by 1α,25(OH)₂D₃ were absent in anti-RANKL antibody-pretreated mice and Ob-VDR-cKO mice. This suggests that bone resorption is related to vitamin D toxicity (hypervitaminosis D). We are currently investigating the relationship between the inhibition of bone resorption and hypervitaminosis D using anti-RANKL antibody and Ob-VDR-cKO mice. Future studies are expected to reveal new roles of VDR in osteoblast-lineage cells and the mechanism by which inactivation of VDR signaling in osteoblast-lineage cells prevents the toxic action of vitamin D.

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References

1. DeLuca HF. Vitamin D: historical overview. Vitam Horm. 2016;100:1-20.
2. Bouillon R, Marcocci C, Carmeliet G, et al. Skeletal and extraskeletal actions of vitamin D: current evidence and outstanding questions. Endocr Rev. 2019;40(4):1109-1151.
3. Zhu JG, Ochalek JT, Kaufmann M, Jones G, Deluca HF. CYP2R1 is a major, but not exclusive, contributor to 25-hydroxyvitamin D production in vivo. Proc Natl Acad Sci U S A. 2013;110(39):15650-15655.
4. Guo YD, Strugnell S, Back DW, Jones G. Transfected human liver cytochrome P-450 hydroxylates vitamin D analogs at different side-chain positions. Proc Natl Acad Sci U S A. 1993;90(18):8668-8672.
5. St-Arnaud R, Messerlian S, Moir JM, Omdahl JL, Glorieux FH. The 25-hydroxyvitamin D 1-alpha-hydroxylase gene maps to the pseudovitamin D-deficiency rickets (PDDR) disease locus. J Bone Miner Res. 1997;12(10):1552-1559.
6. Murayama A, Takeyama K, Kitanaka S, et al. Positive and negative regulations of the renal 25-hydroxyvitamin D3 1alpha-hydroxylase gene by parathyroid hormone, calcitonin, and 1alpha,25(OH)₂D₃ in intact animals. Endocrinology. 1999;140(5):2224-2231.
7. Shimada T, Hasegawa H, Yamasaki Y, et al. FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. J Bone Miner Res. 2004;19(3):429-433.
8. Shimada T, Kakitani M, Yamasaki Y, et al. Targeted ablation of Fgf23 demonstrates an essential physiological role of FGF23 in phosphate and vitamin D metabolism. J Clin Invest. 2004;113(4):561-568.
9. Kaufmann M, Lee SM, Pike JW, Jones G. A high-calcium and phosphate rescue diet and VDR-expressing transgenes normalize serum vitamin D metabolite profiles and renal Cyp27b1 and Cyp24a1 expression in VDR null mice. Endocrinology. 2015;156(12):4388-4397.
10. Meyer MB, Benkusky NA, Kaufmann M, et al. A kidney-specific genetic control module in mice governs endocrine regulation of the cytochrome P450 gene Cyp27b1 essential for vitamin D₃ activation. J Biol Chem. 2017;292(42):17541-17558.
11. Miao D, He B, Lanske B, et al. Skeletal abnormalities in Pth-null mice are influenced by dietary calcium. Endocrinology. 2004;145(4):2046-2053.
12. Raiss L, Trummel CL, Holick MF, Deluca HF. 1,25-dihydroxycholecalciferol: a potent stimulator of bone resorption in tissue culture. Science. 1972;175(4023):768-769.
13. Takahashi N, Akatsu T, Udagawa N, et al. Osteoblastic cells are involved in osteoclast formation. Endocrinology. 1988;123(5):2600-2602.
14. Nakamichi Y, Udagawa N, Suda T, Takahashi N. Mechanisms involved in bone resorption regulated by vitamin D. J Steroid Biochem Mol Biol. 2018;177:70-76.
15. Nakamichi Y, Takahashi N, Udagawa N, Suda T. Osteoclastogenesis and vitamin D. In: Feldman D, eds. Vitamin D. 4th ed. Cambridge: Academic Press; 2018:309-317.
16. Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev. 1999;20(3):345-357.
17. Orimo H, Shiraki M, Hayashi Y, et al. Effects of 1 alpha-hydroxyvitamin D₃ on lumbar bone mineral density and vertebral fractures in patients with postmenopausal osteoporosis. Calcif Tissue Int. 1994;54(4):370-376.
18. Shiraiishi A, Takeda S, Masaki T, et al. Alfacalcidol inhibits bone resorption and stimulates formation in an ovariectomized rat model of osteoporosis: distinct actions from estrogen. J Bone Miner Res. 2000;15(4):770-779.
19. Shibata T, Shira-Ishi A, Sato T, et al. Vitamin D hormone inhibits osteoclastogenesis in vivo by decreasing the pool of osteoclast precursors in bone marrow. J Bone Miner Res. 2002;17(4):622-629.
20. Matsumoto T, Takano T, Saito H, Takahashi F. Vitamin D analogs and bone: preclinical and clinical studies with eldecalcitol. Bonekey Rep. 2014;3:513.
21. Takeda S, Smith SY, Tamura T, et al. Long-term treatment with eldecalcitol (1α, 25-dihydroxy-26-(3-hydroxypropyloxy) vitamin D₃) suppresses bone turnover and leads to prevention...
of bone loss and bone fragility in ovariectomized rats. *Calci Tissue Int.* 2015;96(1):45-55.

22. Smith SY, Doyle N, Boyer M, Chouinard L, Saito H. Eldelcalcitol, a vitamin D analog, reduces bone turnover and increases trabecular and cortical bone density, mass, and strength in ovariectomized cynomolgus monkeys. *Bone.* 2013;57(1):116-122.

23. Matsumoto T, Ito M, Hayashi Y, et al. A new active vitamin D₃ analog, eldelcalcitol, prevents the risk of osteoporotic fractures—a randomized, active comparator, double-blind study. *Bone.* 2011;49(4):610-612.

24. Gardiner EM, Baldock PA, Thomas GP, et al. Increased formation and decreased resorption of bone in mice with elevated vitamin D receptor in mature cells of the osteoblastic lineage. *Faseb J.* 2000;14(13):1908-1916.

25. Baldock PA, Thomas GP, Hodge JM, et al. Vitamin D action and regulation of bone remodeling: suppression of osteoclastogenesis by the mature osteoblast. *J Bone Miner Res.* 2006;21(10):1618-1626.

26. Fukushima M, Suzuki Y, Toira Y, Matsunaga I, Ochi K, Nagano H. Metabolism of 1α-hydroxyvitamin D₃ to 1α,25-dihydroxyvitamin D₃ in perfused rat liver. *Biochem Biophys Res Commun.* 1975;66(2):632-638.

27. Ono Y. Multifunctional and potent roles of the 3-hydroxypropoxy group provide eldecalcitol’s benefit in osteoporosis treatment. *J Steroid Biochem Mol Biol.* 2014;139:88-97.

28. Matsumoto T, Miki T, Haging H, et al. A new active vitamin D, ED-71, increases bone mass in osteoporotic patients under vitamin D supplementation: a randomized, double-blind, placebo-controlled clinical trial. *J Clin Endocrinol Metab.* 2005;90(9):5031-5036.

29. Harada S, Mizoguchi T, Kobayashi Y, et al. Daily administration of eldelcalcitol (ED-71), an active vitamin D analog, increases bone mineral density by suppressing RANKL expression in mouse trabecular bone. *J Bone Miner Res.* 2012;27(2):461-473.

30. Nakamichi Y, Udagawa N, Horibe K, et al. VDR in Osteoblast-lineage cells primarily mediates vitamin D treatment-induced increase in bone mass by suppressing bone resorption. *J Bone Miner Res.* 2017;32(6):1297-1308.

31. Rodda SJ, McMahon AP. Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. *Development.* 2006;133(16):3231-3244.

32. Yamamoto Y, Yoshizawa T, Fukuda T, et al. Vitamin D receptor in osteoblasts is a negative regulator of bone mass control. *Endocrinology.* 2013;154(3):1008-1020.

33. Furuya Y, Mori K, Ninomiya T, et al. Increased bone mass in mice after single injection of anti-receptor activator of nuclear factor-kappaB ligand-neutralizing antibody: evidence for bone anabolic effect of parathyroid hormone in mice with few osteoclasts. *J Biol Chem.* 2011;286(42):37023-37031.

34. RRID:AB_2861189, https://scicrunch.org/resolver/AB_2861189

35. RRID:AB_2801265, https://scicrunch.org/resolver/AB_2801265

36. RRID:AB_2782966, https://scicrunch.org/resolver/AB_2782966

37. Sato M, Nakamichi Y, Nakamura M, et al. New 19-nor-20(2S)-1α,25-dihydroxyvitamin D₃ analogs strongly stimulate osteoclast formation both in vivo and in vitro. *Bone.* 2007;40(2):293-304.

38. Andrukhova O, Smorodchenko A, Egerbacher M, et al. FGF23 promotes renal calcium reabsorption through the TRPV5 channel. *EMBO J.* 2014;33(3):229-246.

39. Quinn SJ, Thomsen AR, Pang JL, et al. Interactions between calcium and phosphorus in the regulation of the production of fibroblast growth factor 23 in vivo. *Am J Physiol Endocrinol Metab.* 2013;304(3):E310-E320.

40. The ADHR Consortium. Autosomal dominant hypophosphatemic rickets is associated with mutations in FGF23. *Nat Genet.* 2000;26(3):345-348.

41. Yamashita T, Konishi M, Miyake A, Inui K, Itoh N. Fibroblast growth factor (FGF)-23 inhibits renal phosphate reabsorption by activation of the mitogen-activated protein kinase pathway. *J Biol Chem.* 2002;277(31):28265-28270.

42. Takeda S, Yoshizawa T, Nagai Y, et al. Stimulation of osteoclast formation by 1,25-dihydroxyvitamin D requires its binding to vitamin D receptor (VDR) in osteoblastic cells: studies using VDR knockout mice. *Endocrinology.* 1999;140(2):1005-1008.

43. Takeyama K, Masuhiro Y, Faseb J. Vitamin D receptor with transcriptional coactivators by a vitamin D analog. *Mol Cell Biol.* 1999;19(2):1049-1055.

44. Issa LL, Leong GM, Sutherland RL, Eisman JA. Vitamin D analogue-specific recruitment of vitamin D receptor coactivators. *J Bone Miner Res.* 2002;17(5):879-890.

45. Bouillon R, Carmeliet G, Verlinden L, et al. Vitamin D and human health: lessons from vitamin D receptor null mice. *Endocr Res.* 2008;29(6):726-776.

46. Thacher TD, Clarke BL. Vitamin D insufficiency. *Mayo Clin Proc.* 2011;86(1):50-60.

47. Yoshizawa T, Handa Y, Uematsu Y, et al. Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. *Nat Genet.* 1997;16(4):391-396.

48. Lieben L, Masuyama R, Torrekens S, et al. Normocalcemia is maintained in mice under conditions of calcium malabsorption by vitamin D-induced inhibition of bone mineralization. *J Clin Invest.* 2012;122(5):1803-1815.

49. Amling M, Priemel M, Holzmann T, et al. Rescue of the skeletal phenotype of vitamin D receptor-ablated mice in the setting of normal mineral ion homeostasis: formal histomorphometric and biomechanical analyses. *Endocrinology.* 1999;140(11):4982-4987.

50. Song Y, Kato S, Fleet JC. Vitamin D receptor (VDR) knockout mice reveal VDR-dependent regulation of intestinal calcium absorption and ECaC2 and calbindin D9k mRNA. *J Nutr.* 2003;133(11):374-380.

51. Xue Y, Fleet JC. Intestinal vitamin D receptor is required for normal calcium and bone metabolism in mice. *Gastroenterology.* 2009;136(4):1317-27, e1.

52. Burnatowska MA, Harris CA, Sutton RA, Seely JF. Effects of vitamin D on renal handling of calcium, magnesium and phosphate in the hamster. *Kidney Int.* 1985;27(6):864-870.

53. Kumar R, Tebben PJ, Thompson JR. Vitamin D and the kidney. *Arch Biochem Biophys.* 2012;523(1):77-86.

54. Han X, Yang J, Li L, Huang J, King G, Quarles LD. Conditional deletion of Fgfr1 in the proximal and distal tubule identifies distinct roles in phosphate and calcium transport. *PLoS One.* 2016;11(2):e0147845.