INTRODUCTION

Vitamin D₃ was discovered as an anti-rachitic agent that improved bone mineralization. The biologically active metabolite of vitamin D₃, 1α,25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃), exerts a variety of actions on target tissues through the vitamin D receptor (VDR), a member of the nuclear receptor superfamily. VDR is expressed in specific cell types in various organs. Tissues expressing high levels of VDR, such as the intestines, kidneys, parathyroid glands, and bone, communicate with each other to regulate bone and mineral metabolism.

Therefore, it is not clear how much of the effect of vitamin D on bone is attributed to the direct actions of VDR in bone.

Three hormones, 1α,25(OH)₂D₃, parathyroid hormone (PTH), and fibroblast growth factor 23 (FGF23), regulate calcium and phosphate metabolism as an endocrine system. The production and clearance of these hormones are regulated by VDR and cell-surface receptors for PTH (PTHrP-R) and FGF23 (FGFR-Klotho complexes) in a ligand-dependent manner. 1α,25(OH)₂D₃ increases skeletal secretion of FGF23, which reduces 1α,25(OH)₂D₃ production in the kidneys by suppressing 1α-hydroxylase activity. In contrast, PTH enhances 1α,25(OH)₂D₃ production by stimulating 1α-hydroxylase activity in the kidneys, which in turn represses PTH transcription through VDR in the parathyroid glands. Addition, serum calcium levels per se directly regulate PTH secretion through the calcium-sensing receptor (CaSR).
receptor (CaSR) in the parathyroid glands.\(^1,^4\) Thus, several negative and positive feedback loops maintain serum concentrations of these hormones, calcium, and phosphate.\(^1,^5,^6\) Hence, a part of VDR-mediated actions in bone is included in the vitamin D-FGF23-PTH axis as the endocrine system.

The importance of VDR in bone has been validated by genetic studies of humans and mice.\(^10,^11\) VDR KO mice recapitulated rachitic skeletal phenotypes in patients with hereditary vitamin D-resistant rickets. Bone formation and mineralization were impaired in VDR KO mice.\(^12\) However, feeding VDR KO mice with a high-calcium diet abolishes rachitic changes in bone.\(^13\) These findings suggest that the positive effects of 1\(,\)25(OH)\(_2\)D\(_3\) on bone are a consequence of upregulation of intestinal calcium absorption.

Bone remodeling is a dynamic and highly coordinated process consisting of bone resorption by osteoclasts and bone formation by osteoblasts.\(^14\) Osteoporosis is a common disease caused by an imbalance of bone remodeling.\(^14,^15\) A bolus administration of 1\(,\)25(OH)\(_2\)D\(_3\) into mice induces osteoclast formation and hypercalcemia.\(^16\) Nevertheless, 1\(,\)25(OH)\(_2\)D\(_3\), 1\(,\)25(OH)\(_3\)D\(_3\), and 1\(,\)25(OH)(2\(-\)α,3\(-\)dihydroxypropyloxy)D\(_3\) (eldecalcitol [ELD]) have been used as therapeutic agents for osteoporosis to increase bone mass.\(^17\)\(^-\)\(^20\) ELD is a new derivative of 1\(,\)25(OH)\(_2\)D\(_3\) and not catalyzed to 1\(,\)25(OH)\(_2\)D\(_3\).\(^17\)\(^,^21\) ELD is more resistant to metabolic degradation via 24-hydroxylation than 1\(,\)25(OH)\(_2\)D\(_3\).\(^21\) These characteristics may explain that ELD can decrease more effectively the incidence of bone fracture and has more potent bone mass increasing activity than 1\(,\)25(OH)\(_2\)D\(_3\).\(^22\) We and others previously reported that ELD treatment increased bone mass by decreasing bone resorption.\(^17\)\(^-\)\(^20,^22\) Notably, these positive effects of ELD on bone are primarily caused by the suppression of bone resorption.\(^17\)\(^-\)\(^20,^22\) However, the cellular mechanisms underlying the antiresorptive properties of vitamin D compounds are poorly understood.

In the present study, we established two VDR cKO mouse lines in which VDR was ablated in osteoblast-lineage cells (Ob-VDR-cKO mice) and in osteoclasts (Ocl-VDR-cKO mice). Using these mouse lines, we examined the in vivo effects of ELD on bone resorption and bone formation. We found that VDR in osteoblast-lineage cells, but not osteoclasts, is critically involved in the ELD administration-induced increase in bone mass, and that VDR in osteoblast-lineage cells is involved in the regulation of serum calcium and FGF23 levels.

**Materials and Methods**

**Animals**

Osterix (Osx)-Cre\(^{\text{fl}}\)-\(^{\text{gr}}\) were purchased from the Jackson Laboratory.\(^23\) Cathepsin K-Cre Knock-in (Ctsk\(^{\text{Cre/+}}\)-Ctsk-Cre) mice were generated by the authors (TN, YY, and SK).\(^24\) Ctsk-Cre mice are established mice that exhibit highly specific expression of Cre in osteoclasts.\(^24\) VDR-floxed mice were generated by the authors (YY, YN, TN, and SK).\(^25\) The global VDR-KO (VDR\(^{\text{-/-}}\)) mice bearing a germline null mutation were generated by crossing VDR-floxed mice with CMV-Cre transgenic mice.\(^25\) Genotyping primers for the VGR gene (Fig. 1A, B) are listed in Supporting Table 1. To maintain the health of the global VDR KO mice, weaned global VDR KO mice were supplied minerals by feeding with a high-calcium diet (a standard diet [CLEA CE-2; Japan CLEA, Tokyo, Japan] supplemented with 2.0% calcium, 1.25% phosphorus, and 20% lactose). The other mice were fed a normal diet containing 1.1% calcium and 1.1% phosphorus, and 220 IU/100 g vitamin D\(_3\) (CLEA CE-2). For mating, one male and one or two female mice were housed in a cage (177 \(\times\) 285 \(\times\) 140 mm in size). Delivered pups were raised in the same cage until they were weaned. Then, the prepubertal mice were transferred to a different cage and housed by the same-gender siblings at maximum five mice per cage until they were subjected to ELD treatment. All mice were housed in a specific-pathogen-free facility in Matsumoto Dental University at 24°C \(\pm\) 2°C and 50% to 60% humidity with a 12-hour light/dark cycle, and were provided with sterilized water and diets ad libitum. Our facility is not approved by the Association for Assessment and Accreditation for Laboratory Animal Care.

**Administration of agents**

A solution of 20 ng/mL ELD (Chugai Pharmaceutical, Tokyo, Japan) in 0.1% ethanol/medium-chain triglyceride (Nissin Oillio, Tokyo, Japan) was prepared. The ELD solution was administered to 10-week-old male mice (50 ng/kg) daily by oral gavage for 4 weeks. Researchers knew what treatment was given to the mice. The mice were randomly assigned to either vehicle treatment group or ELD treatment group. Mice given ELD or the vehicle were double-labeled with tetracycline (20 mg/kg; Sigma, St. Louis, MO, USA) and calcine (10 mg/kg; Sigma) by subcutaneous injections at 5 days and 2 days, respectively, before being euthanized. During the entire treatment period, test animals were individually housed in a small cage (122 \(\times\) 192 \(\times\) 110 mm in size). Blood and tissue samples were collected at 24 hours after the last ELD treatment. Endpoint analysis of radiological bone morphometry and bone histomorphometry were performed in a blinded fashion.

**Cell cultures**

To prepare osteoclasts of each mouse, a procedure for purification of osteoclasts was carried out.\(^26\) Osteoclasts isolated from co-cultures of gender-mixed osteoblast-lineage cells of wild-type newborn mice and BM cells of each mouse aged 3 months (control: 2 males and 1 female; Ocl-VDR-cKO: 2 males and 1 female; n = 3 mice/group) were cultured in the presence of macrophage colony-stimulating factor (M-CSF) (Leukoprol; Kyowa Hakko Kirin, Tokyo, Japan) (1 \(\times\) 10\(^{4}\) IU/mL) for 12 hours in αMEM (Sigma) with 10% FBS (SAFC Biosciences, Lenexa, KS, USA). The cells were then treated with the vehicle or 1\(,\)25(OH)\(_2\)D\(_3\) (1 \(\times\) 10\(^{-8}\) M) for 30 hours to evaluate 24-hydroxylase mRNA expression.

**Radiological analysis of bone**

Left femurs were collected and fixed in 70% ethanol. Three-dimensional (3D) reconstructions in an upside-down position were obtained for measuring the trabecular bone volume/tissue volume (BV/TV), trabecular number (Tb.N), and trabecular thickness (Tb.Th) of distal femurs with micro-CT (μCT) (ScanXmate-A080; Comscan Tecnco, Yokohama, Japan) and integrated software (TBI/3D-BON; Ratoc System Engineering, Tokyo, Japan). The region of interest (ROI) for metaphyseal trabecular bone was set by hand to between 0.5 mm and 1.5 mm from the distal growth plate of femurs with deleted cortical shell. The distal femurs were scanned by 24-hour CT resolution, 250 μA current, and 28.5 keV energy. No density calibration was performed for the μCT. Tomographic measurements of BMD and cortical morphology were performed by peripheral quantitative CT (pQCT) (XCT Research SA; Stratec Medizintechnik, Birkenfeld, Germany). The bone was placed.
horizontally inside a tube and scanned in air using following conditions: 0.07 mm voxel size, 300 μA current, and 40 keV energy. Density calibration phantoms were performed separately in pQCT analysis. The scan line was adjusted using the scout view. The image analysis was carried out using integrated XCT 620C software (Stratec Medizintechnik). The metaphyseal pQCT slices of the distal femurs at 1.5 mm and 6 mm (0.46 mm slice thickness) from the growth plate were collected for the measurement of trabecular and cortical BMD, respectively. In pQCT and μCT analyses, one sample was placed in each scan.

Bone histomorphometry

Left tibias were fixed with ethanol, undecalcified, embedded in methyl methacrylate, frontally sectioned into 5-μm slices, and subjected to Villanueva bone staining. Histomorphometric analyses were performed by Ito Bone Histomorphometry Institute (Niigata, Japan). Standard bone histomorphometric nomenclature, symbols, and units, were used as described by the American Society for Bone and Mineral Research Histomorphometry Nomenclature Committee.

Serum biochemistry

Calcium and phosphorus concentrations in serum were determined by a Calcium E-test kit (Wako, Osaka, Japan) and a Phospho-C test kit (Wako). Serum concentrations of PTH were measured using a mouse PTH1–84 ELISA kit (Immunotopics, San Clemente, CA, USA). Serum FGF23 concentrations were quantified by a FGF23 ELISA kit (KAINOS Laboratories, Japan).
Tokyo, Japan). Serum tartrate-resistant acid phosphatase 5b (TRAP5b) activities were measured using a mouse TRAP assay kit (Immunodiagnostic Systems, Boldon, UK). Serum C-terminal cross-linked teleopeptide of type I collagen (CTX) concentrations were determined using a RatLaps EIA kit (Immunodiagnostic Systems). Serum procollagen type I N-terminal propeptide (P1NP) concentrations were measured using a rat/mouse P1NP EIA kit (Immunodiagnostic Systems). Serum 1α,25(OH)2D levels were determined using a 1,25-dihydroxyvitamin D ELISA kit (IBL international, Hamburg, Germany). ELD was not measurable in the 1α,25(OH)2D assay (data not shown).

**Real-time RT-PCR**

Tissue samples were collected, immediately soaked in TRIzol (Invitrogen, Carlsbad, CA, USA), and homogenized with TissueLyser II (Qiagen, Venlo, Netherlands). Total RNA was extracted with a Purelink RNA mini kit (Invitrogen). First-strand cDNA was synthesized from total RNA with an oligo (dT)12–18 primer (Invitrogen) and ReverTra Ace reverse transcriptase (ToYoBo, Osaka, Japan) according to the manufacturers’ protocols. For preparation of bone samples, tibias were isolated and the epiphysis and adherent soft tissues were cut away and rubbed off with Kimwipe papers. The cleaned tibias containing bone marrow were subjected to the total RNA extraction. Real-time RT-PCR for the quantification of mRNA expression was performed using the Fast SYBR Green (Applied Biosystems, Waltham, MA, USA) and StepOnePlus System (Applied Biosystems). The following temperature profile was used: 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. Each expression level was calculated using a relative standard curve. Gene expression was normalized to Gapdh. Mouse primers for Vdr, 24-hydroxylase (Cyp24a1), Fgf23, osteoprotegerin (Opg), receptor activator of NF-κB ligand (Rankl), M-csf, IL-34, Sost, Gapdh, phosphate-regulating neutral endopeptidase, X-linked (Phex), and dentin matrix protein-1 (Dmp-1), are listed in Supporting Table 2.

**Immunohistochemical staining**

Harvested tissues from 3-month-old male mice were fixed in 4% paraformaldehyde in PBS, processed with serial dehydration, embedded in paraffin, and sectioned into 4-μm slices. Tibias were decalcified with 10% EDTA for 2 weeks prior to serial dehydration. The sections were subjected to staining for VDR (αVDR mAb, Clone D2K6W; Cell Signaling Technology, Danvers, MA, USA). VDR protein was visualized with αratIgG-HRP and a Tyramide Signal Amplification kit (PerkinElmer, Waltham, MA, USA). Nuclei were stained with Hoechst (Thermoscientific, Waltham, MA, USA). Images were obtained using a confocal laser scanning microscope (LSM 510; Carl Zeiss). Images were reconstructed from four slices of z-stacks with a step size of 1.15 μm using ZEN lite software (Carl Zeiss).

**Real-time RT-PCR**

Images were obtained using Axioplan-2 imaging microscope (Carl Zeiss, Oberkochen, Germany) and hematoxylin. Images were obtained using an Axioplan-2 scanning microscope (LSM 510; Carl Zeiss). Images were reconstructed from four slices of z-stacks with a step size of 1.15 μm using ZEN lite software (Carl Zeiss).

**Immunohistochemical staining**

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**Statistics**

The statistical analysis was performed using GraphPad Prism 7 statistical software (GraphPad Software, San Diego, CA, USA). The data obtained were analyzed by the D’agostino-Pearson test (n ≥ 8) or the Shapiro-Wilk test (n < 8) to assess normal distribution. To compare two groups, equality of the two variances was assessed using an F-test. When the data sets met both the test requirements for distribution and variance, a Student’s t test was used. When the data did not meet one of the test requirements, non-parametric, Mann-Whitney U test was used. Actually, some data sets of the Ob-VDR-cKO line exhibited non-normal distribution (Fig. 3G, 4E [Rankl/Opg], 5C; Supporting Fig. 1E). Other data sets had unequal variances with normal distribution (Fig. 4E [Opg], 5B; Supporting Fig. 1D). These data were analyzed by Mann-Whitney U test. To compare multiple groups, one-way ANOVA with the Dunnett’s post hoc test or the Tukey’s post hoc test was performed; p < 0.05 was considered statistically significant. All data are expressed as the mean ± SE. Medians and interquartile ranges of each group are shown in Supporting Tables 3 and 4.

**Study approval**

All animal studies were conducted in accordance with the guidelines for studies with laboratory animals of the Matsumoto Dental University Experimental Animal Committee.

**Results**

Deletion of the VDR gene in osteoblast-lineage cells

We generated Ob-VDR-cKO (VDR+/–; Osx-Cre+/–) mice by crossing VDR-floxed mice(25) with Osterix-Cre (Osx-Cre) transgenic mice.(23) The efficiency of osteoblast lineage-specific VDR inactivation in Ob-VDR-cKO mice was evaluated by DNA, RNA, and protein levels (Fig. 1). Genomic DNA extracted from major target tissues of vitamin D was subjected to PCR analysis (Fig. 1A, B). A 338-bp amplicon indicating correct excision of the floxed VDR exon was observed in the calvaria and long bone, but not in the duodenum, skin, or kidneys (Fig. 1B). Real-time RT-PCR analysis revealed that the expression levels of Vdr mRNA in the duodenum, skin, and kidneys were comparable between VDR+/–; Osx-Cre+/– mice (control) and Ob-VDR-cKO mice (Fig. 1C). In contrast, the expression of Vdr mRNA in bone was much lower in Ob-VDR-cKO mice than in control mice (Fig. 1C; also see Fig. 3A).

Immunohistochemical analysis showed that VDR protein was expressed in intestinal epithelial cells, hair follicle keratinocytes and kidney tubular cells similarly in both control and Ob-VDR-cKO mice, but not in the global VDR-KO mice (Fig. 1D). In bone tissues, VDR protein was expressed in osteoblasts and osteocytes, but was not appreciably in osteoclasts or hypertrophic chondrocytes in control mice (Fig. 1E–I). Hematopoietic cells in bone marrow (BM) hardly expressed VDR protein (Fig. 1E, F, H). VDR protein in bone largely disappeared in Ob-VDR-cKO mice.
Thus, the establishment of osteoblast lineage-specific ablation of VDR was confirmed in Ob-VDR-cKO mice.

Bone phenotypes and responses to ELD in Ocl-VDR-cKO mice

Both 1α,25(OH)2D3 and ELD were reported to suppress M-CSF and RANKL-induced osteoclast differentiation in vitro.30,31 This suggests that though the levels of VDR in bone marrow cells and osteoclasts are quite low, vitamin D compounds may directly act on osteoclasts in vivo. We then generated Ocl-VDR-cKO (VDRfl/fl; CtskCre/+) mice by crossing VDR-flxed mice with Cathepsin K-Cre Knock-in (Ctsk-Cre) mice(24) (Fig. 2). The functional impairment of VDR in Ocl-VDR-cKO mice was confirmed by assessing 1α,25(OH)2D3-induced transcription of 24-hydroxylase (Cyp24a1) in osteoclasts generated from BM cells of Ocl-VDR-cKO mice (Fig. 2A). Appreciable expression of VDR in osteoclasts was not observed in immunohistochemistry, but the level of 24-hydroxylase mRNA was up-regulated by 1α,25(OH)2D3 in osteoclasts prepared from VDR+/+; CtskCre/+ (control) mice (Fig. 2A). Such upregulation, however, was markedly blunted in osteoclasts from Ocl-VDR-cKO mice (Fig. 2A). Femurs and tibias of control and Ocl-VDR-cKO mice were subjected to radiographic and histomorphometric analysis (Fig. 2B–F; Tables 1 and 2; Supporting Table 3). The 3D μCT images of distal femurs revealed that Ocl-VDR cKO mice had no obvious abnormalities in bone tissues (Fig. 2B). Then, ELD (50 ng/kg body weight) was orally administered every day to control and Ocl-VDR cKO mice for 4 weeks. The μCT analysis revealed that ELD treatment increased trabecular bone mass similarly in control and Ocl-VDR cKO mice (Fig. 2B). Peripheral quantitative CT (pQCT) analysis showed that the ELD treatment increased trabecular bone volume/bone surface (BFR/BS) in proximal tibias determined by histomorphometry. Mice were double-labeled with tetracycline and calcine. n = 5 mice per group. Values represent the mean ± SE. *p < 0.05; **p < 0.001; by two-tailed Student’s t test except for C (one-way ANOVA followed by Dunnett’s test) and F (one-tailed Student’s t test); compared to vehicle.
Table 1. Trabecular Parameters of Distal Femurs in Control and Ocl-VDR-cKO Mice

| µCT analysis | Control (n = 5 per group) | Ocl-VDR-cKO (n = 8 per group) |
|--------------|---------------------------|-------------------------------|
|              | Vehicle                   | ELD                           | Vehicle                   | ELD                           |
| Tb.N (mm⁻¹)  | 4.5 ± 0.4                 | 5.8 ± 0.2*                    | 5.3 ± 0.4                 | 5.9 ± 0.4*                    |
| Tb.Th (mm)   | 25.8 ± 1.2                | 28.7 ± 0.2                    | 25.8 ± 0.8                | 27.8 ± 1.1                    |
| Tb.Sp (mm)   | 204 ± 18                  | 144 ± 6.3 """"                | 197 ± 17                  | 143 ± 10 """"                |

Values are mean ± SE.
* p < 0.05 (versus vehicle, two-tailed Student’s t-test)
** p < 0.05 (control group: vehicle, Dunnett’s adjustment for multiple comparisons).

(Tables 1; Supporting Table 3). We previously reported that ELD treatment decreased osteoclast number (N.Oc/BS) and bone formation rate (BFR/BS). N.Oc/BS and BFR/BS were suppressed by ELD treatment similarly in both genotypes (Fig. 2E, F; Supporting Table 3). Responses to ELD in other histomorphometric parameters, such as osteoblast number (N.Ob/BS), mineral apposition rate (MAR), and double-labeled surface (dLS/BS), and single-labeled surface (sLS/BS) were similar between control and Ocl-VDR-cKO mice (Table 2; Supporting Table 3). These results suggest that VDR in osteoclasts does not play an important role for normal bone homeostasis and vitamin D–induced increase in bone mass.

Bone phenotypes and responses to ELD in Ob-VDR-cKO mice

We then investigated the role of VDR in osteoblast-lineage cells for bone metabolism using Ob-VDR-cKO mice treated with ELD or the vehicle. The bone Vdr mRNA level in Ob-VDR-cKO mice was markedly lower than that in control mice (Fig. 3A). ELD administration did not change the expression levels of VDR in both genotypes. The 3D µCT and pQCT data of the vehicle-treated groups indicated that VDR ablation in osteoblast-lineage cells affected neither BMD nor radiographic morphology (Fig. 3B–G; Supporting Table 4). However, ELD treatment increased trabecular bone mass in control but not Ob-VDR-cKO mice (Fig. 3B). ELD treatment also increased trabecular BMD by approximately 20% in control but not Ob-VDR-cKO mice (Fig. 3C; Supporting Table 4). The cortical BMD, thickness, and area measured by pQCT were comparable among all groups (Supporting Fig. 1A–C). The pQCT data showed that marrow area was slightly decreased and cortical area/total area was slightly increased by ELD treatment in control mice (Supporting Fig. 1D, E). The morphometric analysis using µCT revealed that parameters of trabecular bone structure, such as BV/TV, Tb.N, and Tb.Th, were significantly increased and Tb.Sp was significantly decreased by ELD administration in control but not Ob-VDR-cKO mice (Fig. 3D–G; Supporting Table 4). These results suggest that VDR in osteoblast-lineage cells is largely dispensable for homeostatic control of bone structure, but is essential for the ELD-induced increase in bone mass.

ELD treatment slightly increased serum calcium levels in control but not Ob-VDR-cKO mice (Fig. 3H). Serum phosphorus levels were not altered by the ELD treatment in both genotypes (Fig. 3I). Serum PTH concentrations were significantly down-regulated by the ELD treatment in both control and Ob-VDR-cKO mice (Fig. 3J). Serum levels of 1α,25(OH)₂D were comparable between control and Ob-VDR-cKO mice, though the levels were similarly suppressed by ELD treatment in both genotypes (Fig. 3K). FGF23 levels of serum and mRNA in bone were significantly lower in Ob-VDR-cKO mice than in control mice (Fig. 3L). The FGF23 levels were upregulated by ELD treatment, but the upregulation was markedly blunted in Ob-VDR-cKO mice. These results suggest that VDR in osteoblast-lineage cells is involved in FGF23 production.

Bone resorption and formation in response to ELD in Ob-VDR-cKO mice

Effects of ELD treatment on bone resorption were also assessed histologically and biochemically. TRAP-positive cells were similarly observed in both control and Ob-VDR-cKO mice given the vehicle (Fig. 4A, B; Supporting Table 4). N.Oc/BS was reduced by approximately 40% by the ELD treatment in control but not Ob-VDR-cKO mice (Fig. 4A, B; Supporting Table 4). Circulating bone resorption markers, TRAP5b and CTx, were significantly decreased by ELD treatment in control but not Ob-VDR-cKO mice (Fig. 4C, D; Supporting Table 4). We then examined the effects of ELD on mRNA expression of osteoclastogenesis-related factors in bone (Fig. 4E). RANKL and M-CSF are cytokines responsible for osteoclastogenesis. OPG is a decoy receptor of RANKL, which inhibits osteoclastogenesis by blocking the RANKL-RANK interaction. IL-34 plays a key role in osteoclastogenesis in M-CSF-deficient op/op mice, because IL-34 and M-CSF share the same receptor, c-Fms. ELD treatment failed to affect the expression of RANKL, M-CSF, and IL-34 mRNA (Fig. 4E).

Table 2. Histomorphometric Parameters of Tibial Trabecular Bones in Control and Ocl-VDR-cKO Mice

|              | Control (n = 5 per group) | Ocl-VDR-cKO (n = 5 per group) |
|--------------|---------------------------|-------------------------------|
|              | Vehicle                   | ELD                           | Vehicle                   | ELD                           |
| N.Ob/BS (mm⁻¹) | 13.5 ± 2.3                | 9.7 ± 0.4                     | 16.3 ± 2.5                | 9.9 ± 0.9*                   |
| MAR (mm/day) | 1.35 ± 0.09               | 1.34 ± 0.04                   | 1.43 ± 0.04               | 1.25 ± 0.03*                 |
| dLS/BS (%)   | 20.7 ± 3.6                | 12.0 ± 2.1                    | 22.4 ± 1.9                | 16.3 ± 2.0                   |
| sLS/BS (%)   | 19.9 ± 1.1                | 15.7 ± 1.0*                   | 21.8 ± 1.8                | 16.4 ± 2.5                   |

Values are mean ± SE.
* p < 0.05 (versus vehicle; two-tailed Student’s t-test).
Fig. 3. Bone phenotypes and responses to ELD in Ob-VDR-cKO mice. (A) Real-time RT-PCR analysis of Vdr mRNA expression in long bones prepared from male control and Ob-VDR-cKO mice treated with ELD (50 ng/kg/day) or the vehicle for 4 weeks. $n = 5$ mice per group. (B) μCT of femurs in control and Ob-VDR-cKO mice. 3D reconstructed sagittal views (upper panels) and horizontal views (lower panels) of distal femurs. Bar = 0.5 mm. (C) Trabecular BMD of distal femurs measured by pQCT. $n = 10$ mice per group. (D) Trabecular BV/TV, (E) trabecular bone number (Tb.N), (F) trabecular thickness (Tb.Th), and (G) trabecular separation (Tb.Sp) in distal femurs were measured by μCT. $n = 7$ mice per group. Serum levels of (H) calcium ($n = 13$ mice per group), (I) phosphorus ($n = 9$ mice per group), and (J) PTH ($n = 9$ mice per group). (K) Serum levels of 1α,25(OH)2D ($n = 7$ mice per group). (L) Serum FGF23 levels (left, $n = 13$ mice per group) and Fgf23 mRNA expression in long bones (right, $n = 5$ mice per group). Values represent the mean ± SE. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; by two-tailed Student’s $t$ test except for C (one-way ANOVA followed by Dunnett’s test), G (nonparametric, one-tailed Mann-Whitney $U$ test); compared to vehicle; K (one-way ANOVA followed by Tukey’s test).
increased the expression of Opg mRNA, thereby decreasing the Rankl/Opg ratio in control mice (Fig. 4E). These results suggest that VDR in osteoblast-lineage cells is required for suppression of bone resorption by ELD treatment.

Effects of ELD treatment on bone formation were assessed by dynamic histomorphometry and serum biochemical parameters (Fig. 5A–H; Supporting Table 4). No obvious differences in the morphology, distribution, number, or function of osteoblasts were observed between control or Ob-VDR-cKO mice given the vehicle (Fig. 5A–G). Histological examination using Villanueva bone staining can identify the bone phenotype between the mature mineralized bone (unstained) and osteoid (purplish red) (Fig. 5A). ELD administration reduced the number of active osteoblasts with cuboidal shape (Fig. 5A, B, arrows) and osteoid surfaces (Fig. 5A, C, purplish red) in control but not Ob-VDR-cKO mice (Fig. 5A–C; Supporting Table 4). N.Ob/BS was reduced approximately 50% by ELD treatment in control but not Ob-VDR-cKO mice (Fig. 5B; Supporting Table 4). We then estimated bone formation by double-fluorescence labeling of the mineralized matrix (Fig. 5D–H). MAR was not affected by ELD treatment in both control and Ob-VDR-cKO mice (Fig. 5E; Supporting Table 4). dLS/BS, sLS/BS, and BFR/BS were significantly reduced by the ELD treatment in control mice (Fig. 5F–H; Supporting Table 4). No appreciable changes in these parameters were observed by ELD administration in Ob-VDR-cKO mice. Coincidently, the serum indicator for bone formation, P1NP, was reduced significantly by the ELD treatment in control mice only (Fig. 5I; Supporting Table 4). The expression of Sost mRNA encoding sclerostin, an inhibitor of the Wnt signaling pathway, was upregulated by the ELD treatment in control but not Ob-VDR-cKO mice (Fig. 5J; Supporting Table 4). We estimated bone formation by double-fluorescence labeling of the mineralized matrix (Fig. 5D–H). MAR was not affected by ELD treatment in both control and Ob-VDR-cKO mice (Fig. 5E; Supporting Table 4). dLS/BS, sLS/BS, and BFR/BS were significantly reduced by the ELD treatment in control mice (Fig. 5F–H; Supporting Table 4). No appreciable changes in these parameters were observed by ELD administration in Ob-VDR-cKO mice. Coincidently, the serum indicator for bone formation, P1NP, was reduced significantly by the ELD treatment in control mice only (Fig. 5I; Supporting Table 4). The expression of Sost mRNA encoding sclerostin, an inhibitor of the Wnt signaling pathway, was upregulated by the ELD treatment in control but not Ob-VDR-cKO mice (Fig. 5J). We then estimated bone formation by double-fluorescence labeling of the mineralized matrix (Fig. 5D–H). MAR was not affected by ELD treatment in both control and Ob-VDR-cKO mice (Fig. 5E; Supporting Table 4). dLS/BS, sLS/BS, and BFR/BS were significantly reduced by the ELD treatment in control mice (Fig. 5F–H; Supporting Table 4). No appreciable changes in these parameters were observed by ELD administration in Ob-VDR-cKO mice. Coincidently, the serum indicator for bone formation, P1NP, was reduced significantly by the ELD treatment in control mice only (Fig. 5I; Supporting Table 4). The expression of Sost mRNA encoding sclerostin, an inhibitor of the Wnt signaling pathway, was upregulated by the ELD treatment in control but not Ob-VDR-cKO mice (Fig. 5J). Phex and Dmp-1 are early osteocyte markers and are regarded as upstream factors in suppressing FGF23 expression. The expression of Phex and Dmp-1 mRNA was comparable among all groups (Fig. 5K, L). Taken

**Fig. 4.** Responses of bone resorption to ELD in Ob-VDR-cKO mice. (A) TRAP staining of tibial sections from male control and Ob-VDR-cKO mice treated with ELD (50 ng/kg/day) or the vehicle for 4 weeks. Sections were counterstained with methyl green. Bar = 100 μm. (B) N.Oc/BS in proximal tibias determined by histomorphometry. n = 5–7 mice per group. Serum (C) TRAP5b activity and (D) CTx concentrations. n = 13 mice per group. (E) Real-time RT-PCR analysis for mRNA expression of osteoclastogenesis-related factors (Opg, Rankl, M-csf, IL-34) in long bones. The ratio of Rankl/Opg (middle) was calculated in each mouse and the data of each vehicle-treated group was set as 1. n = 5 mice per group. Values represent the mean ± SE. *p < 0.05, **p < 0.01; by two-tailed Student’s t test except for E (nonparametric, one-tailed Mann-Whitney U test); compared to vehicle.
Fig. 5. Responses of bone formation to ELD in Ob-VDR-cKO mice. (A) Villanueva bone staining of trabecular bone in tibias of male control and Ob-VDR-cKO mice treated with ELD (50 ng/kg/day) or the vehicle for 4 weeks. Bar = 50 μm. (B) N.Ob/BS and (C) osteoid surface/bone surface (OS/BS) in proximal tibias determined by histomorphometry. n = 5–7 mice per group. (D) Tetracycline and calcein-labels of trabecular bones in proximal tibias. Bar = 100 μm. (E) Mineral apposition rate (MAR), (F) double-labeled surface/bone surface (dLS/BS), (G) single-labeled surface/bone surface (sLS/BS), and (H) BFR/BS in proximal tibias determined by histomorphometry. n = 5–7 mice per group. (I) Serum concentrations of procollagen type I N-terminal propeptide (P1NP). n = 13 mice per group. Real-time RT-PCR analysis of (J) Sost, (K) Phex, and (L) Dentin matrix protein-1 (Dmp-1) mRNA expressions in long bones. n = 5 mice per group. Values represent the mean ± SE. *p < 0.05, **p < 0.01; by two-tailed Student's t test except for B, C (nonparametric, two-tailed Mann-Whitney U test), and G (one-way ANOVA followed by Dunnett's test); compared to vehicle.
together, these results suggest that ELD treatment increases bone mass by suppressing osteoclastic bone resorption through osteoblast-lineage VDR.

Discussion

Not only osteoblast-lineage cells, but also osteoclasts and their precursors were shown to be the targets of 1α,25(OH)2D3. It was also reported that 1α,25(OH)2D3 acted on hypertrophic chondrocytes and promoted endochondral ossification. By using a specific and sensitive antibody, we showed here that VDR is expressed preferentially in osteoblasts and osteocytes in bone tissues. Immunohistochemical signals of VDR were faint in osteoclasts, hematopoietic cells, and several differentiation stages of chondrocytes. Osteoblast-specific deletion of the VDR gene largely abolished VDR immunoreactivity in bone. These results suggest that osteoblast-lineage cells are the major target cells of 1α,25(OH)2D3 in bone. Although immunohistochemical signals were faint in osteoclasts in vivo, we have shown that 24-hydroxylase in osteoclasts was induced by 1α,25(OH)2D3. These results suggest that VDR in bone cells other than osteoblast-lineage cells plays a role in bone formation and resorption under certain conditions such as osteoporosis, osteoarthritis, or bone metastasis.

In spite of the anti-rachitic activity of vitamin D in vivo, 1α,25(OH)2D3 inhibited mineralization in cultures of osteoblast-lineage cells. 1α,25(OH)2D3 was also reported to stimulate or inhibit the production of type I collagen by osteoblast-lineage cells in culture. These observations suggest that 1α,25(OH)2D3 directly controls differentiation of osteoblast-lineage cells and mineralization of bone. The present study, however, showed that Ob-VDR cKO mice have no discernible defects in bone formation, mineralization, or serum P1NP levels in homeostasis. In agreement with our results, two different osteoblast-lineage VDR cKO mouse lines utilizing Col1a1-Cre and Dmp1-Cre mice showed normal bone formation. Collectively, these findings suggest that 1α,25(OH)2D3 is unlikely to be a positive regulator for bone formation. The positive effect of 1α,25(OH)2D3 on bone mineralization in vivo appears to be mediated by VDR in extraskeletal tissues such as the intestines, kidneys, and parathyroid glands.

Long-term treatment with 1α,25(OH)2D3, as well as ELD, suppressed bone formation in control mice. This suppressive effect on bone formation appears to be an indirect action of 1α,25(OH)2D3 and ELD, that is, a consequence of coupling bone resorption to bone formation. Our laboratory has recently proposed that sclerostin may be a key mediator for the coupling process. OPG KO mice exhibit stimulated bone formation coupled with excessive bone resorption and coincidentally show abrogated expression of sclerostin. When excessive bone resorption occurring in OPG KO mice was normalized by the treatment with bisphosphonates or anti-RANKL neutralizing antibody, expression of sclerostin was regained and the increased bone formation returned to the normal level. Indeed, ELD treatment increased Sost mRNA expression in bone of control but not Ob-VDR cKO mice. However, ELD failed to increase Sost mRNA expression in primary cultures of osteoblast-lineage cells (Supporting Fig. 2), suggesting that the upregulation of Sost mRNA expression in vivo was mediated by factors other than ELD. These findings suggest that the suppression of bone formation by long-term treatment with 1α,25(OH)2D3 or ELD is due to the consequences of coupling (Fig. 6). Studies are ongoing in our laboratory to elucidate the mechanism by which bone resorption suppresses Sost mRNA expression in osteocytes.

Suppression of bone resorption induced by long-term treatment with ELD occurred through VDR in osteoblast-lineage cells but not in osteoclasts (Fig. 6). Among osteoclastogenesis-related factors (RANKL, OPG, M-CSF, and IL-34) examined, Opg expression was increased by the ELD treatment in control but not Ob-VDR cKO mice. The RANKL/OPG ratio is believed to be one of the most important factors that regulate osteoclastogenesis in vivo. Actually, ELD treatment decreased the RANKL/OPG ratio in control mice. However, we could not reproduce in vitro downregulation of the RANKL/OPG ratio in cultures of osteoblast-lineage cells. Therefore, the change of the RANKL/OPG ratio by the ELD treatment does not appear to be a direct action of VDR on OPG or RANKL promoters. Bone microenvironment and endocrine systems may be required for appropriate osteoclastogenesis. Expression of RANKL is upregulated by PTH, as well as 1α,25(OH)2D3. The reduced PTH levels by the ELD treatment may decrease the RANKL/OPG ratio in osteoblast-lineage cells of control mice. Besides RANKL and OPG, several factors produced by osteoblast-lineage cells, such as semaphorin 3A, Wnt5A, and EphB4, modulated differentiation and function of osteoclasts. Expression of these factors may be changed by the vitamin D treatment. Further studies are required to assess the possible involvement of such modulators in the actions of vitamin D on bone resorption.

In this study, we showed genetic evidence that VDR in osteoblast-lineage cells has a pivotal role for FGF23 production. Upregulation of serum levels of FGF23 by ELD treatment was much greater in control than Ob-VDR cKO mice. Nevertheless,
serum phosphate levels were unchanged in control and Ob-VDR-cKO mice treated with ELD or the vehicle. PTH, the production of which is regulated by FGF23 and vitamin D, is also known to reduce serum phosphate levels by decreasing renal phosphate reabsorption. Therefore, the vitamin D-FGF23–PTH interaction may be regulated to maintain phosphate homeostasis.

There are four limitations in our study. The first is the limitation of statistical assessment. Unequal variances and non-normal distribution were observed in some data sets. That is probably due to an insufficient number of samples. The second is radiological assessment. Due to our insufficient skills for the μCT analysis, the morphology of cortical bone was evaluated using pQCT. Biomechanical data of cortical bone were not shown in this study. The third is immunohistochemistry. VDR was clearly detected in osteoblasts and osteocytes but not other cells in bone. However, it is possible that cells expressing VDR at levels below the detection threshold respond to ELD. The fourth is experiments using cKO mice. Ob-VDR-cKO mice expressed VDR in bone at 10% transcript level of control mice. This may be due to the incomplete deletion of VDR in osteoblast-lineage cells or VDR expression by bone cells other than osteoblast-lineage cells. In addition, VDR expression in tissues that did not examine may be downregulated in Ob-VDR-cKO mice. Therefore, we admit the possibility that some of our conclusion may include overestimation or underestimation of roles of VDR in osteoblast-lineage cells and other bone cells. Further experiments using different VDR cKO lines would be essential to exclude this possibility.

This study raises a new question about mechanisms underlying the lack of calcemic effect of ELD in Ob-VDR-cKO mice. Bone formation was suppressed by ELD in control mice but not in Ob-VDR-cKO mice. Accordingly, consumption of blood calcium for bone formation may be decreased by ELD treatment in control mice and leads to mild hypercalcemia in control mice only. Dysregulation of calcemic action and FGF23 production in Ob-VDR-cKO mice may be related to different responsiveness to ELD in sclerostin expression and the RANKL/OPG ratio from control mice (Fig. 6). An unbiased approach, comparison of metabolome between the two genotypes treated with ELD, would be required for understanding the overall mechanisms of the ELD actions.

In conclusion, VDR in osteoblast-lineage cells is a key mediator of ELD actions for increasing bone mass. 1α,25(OH)2D3 and ELD act on VDR in multiple cell types in extraskeletal tissues as well and possibly induce unfavorable side effects. There are clinically and experimentally used agents that are delivered specifically to bone. The development of a method for bone-specific delivery of active derivatives of vitamin D will bring a promising strategy for prevention and treatment of osteoporosis.

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Authors’ roles: YN, NU, and NT designed the studies, analyzed data, and wrote the manuscript. YN, NU, KH, TM, and AH conducted experiments and acquired data. YY and SK provided VDR-foxed and the global VDR KO mice. TN and SK provided Ctsk-Cre mice. TS edited the manuscript.

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