Inactivation of the 25-Hydroxyvitamin D 1α-Hydroxylase and Vitamin D Receptor Demonstrates Independent and Interdependent Effects of Calcium and Vitamin D on Skeletal and Mineral Homeostasis

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Running Title: 1α(OH)ase/VDR knockout mice

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Summary

We employed a genetic approach to determine whether deficiency of 1,25-dihydroxyvitamin D \([1,25(OH)_2D]\) and deficiency of the vitamin D receptor (VDR) produce the same alterations in skeletal and calcium homeostasis and whether calcium can subserve the skeletal functions of 1,25(OH)_2D and the VDR. Mice with targeted deletion of the 25-hydroxyvitamin D 1\(\alpha\)-hydroxylase (1\(\alpha\)(OH)ase\(^{-/-}\)) gene, the VDR gene, and both genes, were exposed to either: a high calcium intake, which maintained fertility but left mice hypocalcemic; this intake plus thrice weekly injections of 1,25(OH)_2D_3, which normalized calcium in the 1\(\alpha\)(OH)ase\(^{-/-}\) mice only; or a “rescue” diet, which normalized calcium in all mutants. These regimens induced different phenotypic changes thereby disclosing selective modulation by calcium and the vitamin D system. Parathyroid gland size and the development of the cartilaginous growth plate were each regulated by calcium and by 1,25(OH)_2D_3 but independent of the VDR. Parathyroid hormone secretion and mineralization of bone reflected ambient calcium levels rather than the 1,25(OH)_2D/VDR system. In contrast increased calcium absorption and optimal osteoblastogenesis and osteoclastogenesis were modulated by the 1,25(OH)_2D/VDR system. These studies indicate that the calcium ion and the 1,25(OH)_2D/VDR system exert discrete effects on skeletal and calcium homeostasis which may occur coordinately or independently.
INTRODUCTION

Vitamin D plays a major role in modulating calcium and skeletal homeostasis, and exerts a significant influence on the growth and differentiation of a variety of tissues (1-3). Vitamin D is absorbed from the diet and generated in skin by exposure to ultraviolet light. The secosteroid is transported in blood bound to vitamin D binding protein (4) and hydroxylated in the liver at the 25 position by a vitamin D 25-hydroxylase (CYP27) (5). The metabolite 25-hydroxyvitamin D is further hydroxylated at the 1α position to produce the active moiety, 1,25-dihydroxyvitamin D \([1,25(OH)_2D]\) (1-3). The enzyme catalyzing the production of 1,25(OH)_2D is 25-hydroxyvitamin D 1α-hydroxylase [1α(OH)ase or CYP27B1]. Both the cDNA and gene encoding this mitochondrial cytochrome P450 enzyme have been cloned from several species (6-12). A number of tissues can synthesize 1,25(OH)_2D but the kidney is the principal site generating the circulating hormone. The renal 1α(OH)ase is known to be tightly regulated by several factors including parathyroid hormone (PTH), calcium (Ca), phosphorus (P) and 1,25(OH)_2D per se (1-3). An alternate site of hydroxylation of 25-hydroxyvitamin D can be catalyzed by the enzyme 25-hydroxyvitamin D 24-hydroxylase [24(OH)ase or CYP24] yielding the metabolite 24,25-dihydroxyvitamin D (13). In target tissues, 1,25(OH)_2D is believed to exert most of its actions by binding to the vitamin D receptor (VDR), a member of the nuclear hormone receptor superfamily, and by regulating the transcription of vitamin D target genes (14). Nevertheless non-genomic effects of 1,25(OH)_2D have been reported in which 1,25(OH)_2D interacts with a putative membrane receptor, mediating the opening of calcium and chloride voltage-gated channels and activating MAP kinase (15).

We (16) and others (17) have previously reported a mouse model deficient in 1,25(OH)_2D by targeted ablation of the 1α(OH)ase gene (1α(OH)ase\(^{-/-}\)). After weaning, mice, fed
a diet of regular mouse chow, developed hyperparathyroidism, retarded growth and the skeletal abnormalities characteristic of rickets. These abnormalities mimic those described in the human genetic disorder vitamin D-dependent rickets type I [VDDR-I; also called pseudovitamin D-deficiency rickets (PDDR)] (18,19). Several laboratories have also reported mouse models with targeted ablation of the VDR gene (VDR\(^{-/-}\)) (20-22). These animals develop manifestations similar to those with \(1\alpha(\text{OH})\text{ase}\) ablation but also display alopecia. This constellation of abnormalities is observed in humans with VDR mutations in the inherited disorder vitamin D-dependent rickets type II [VDDR-II; also called hereditary vitamin D resistant rickets (HVDDR)] (23). Rescue of this phenotype has been successfully accomplished with a high-calcium, high-phosphorus, high-lactose diet administered for at least 1 month after weaning (22,24).

Consequently, it has been postulated that the major action of the VDR in skeletal growth, maturation and remodeling is its role in intestinal calcium absorption (25).

If \(1,25(\text{OH})_2\text{D}\) and VDR are both necessary and sufficient for the vitamin D endocrine system, then mutant animals deficient in either \(1,25(\text{OH})_2\text{D}\) or in VDR [\(1\alpha(\text{OH})\text{ase}\)^\(^+/-\) and VDR\(^{-/-}\), respectively] and the mutant animals deficient in both ligand and receptor [\(1\alpha(\text{OH})\text{ase}\)^\(^+/-\) VDR\(^{-/-}\)] should exhibit the same phenotypic alternations in mineral and skeletal homeostasis and should respond in the same way to alterations in dietary calcium. Furthermore, if the major role of the VDR in skeletal function is to increase extracellular fluid calcium by increasing intestinal absorption, as has been postulated (26), then the 3 mutant animals should also exhibit similar skeletal phenotypic changes as the serum calcium is altered. To test these hypotheses we mated heterozygous animals with deletion of the \(1\alpha(\text{OH})\text{ase}\) and the VDR and compared siblings that were homozygous for deletion of the genes encoding \(1\alpha(\text{OH})\text{ase}\), VDR and both genes. The use of the double mutants permitted us to explore if the elevated endogenous \(1,25(\text{OH})_2\text{D}\) levels seen
in VDR−/− mice might play a role in defining the phenotypes observed. We exposed these mutants to environmental conditions which would alter concentrations of the calcium ion or of the 1,25(OH)_{2}D_{3} ligand. The results demonstrate significant phenotypic differences that suggest discrete roles for the calcium ion and components of the 1,25(OH)_{2}D/VDR endocrine system in modulating mineral and skeletal homeostasis.
EXPERIMENTAL PROCEDURES

Derivation of 1α(OH)ase and VDR double null mice—The derivation of the two parental strains of 1α(OH)ase<sup>−/−</sup> mice and VDR<sup>−/−</sup> mice by homologous recombination in embryonic stem cells was previously described by Panda et al (16) and Li et al (20), respectively. VDR<sup>−/−</sup> mice were a generous gift of Dr. Marie Demay, Massachusetts General Hospital, Boston, MA. Briefly, a neomycin resistance gene was inserted in place of exon VI, VII and VIII of the mouse 1α(OH)ase gene replacing both the ligand binding and heme binding domains. RT-PCR of renal RNA from homozygous 1α(OH)ase<sup>−/−</sup> mice confirmed that no 1α(OH)ase mRNA is expressed from this allele (16). A neomycin resistance gene was inserted in place of exon III of the mouse VDR gene replacing the second zinc finger of the DNA binding domain. RT-PCR of intestinal and renal RNA from homozygous VDR<sup>−/−</sup> mice confirmed a truncated mRNA is expressed from this allele (20). Mice heterozygous for the null 1α(OH)ase allele and mice heterozygous for the VDR allele were fertile (16,20). VDR<sup>+/−</sup> mice were mated with 1α(OH)ase<sup>+/−</sup> mice and offspring heterozygous at both loci were then mated to one another to generate pups homozygous for both 1α(OH)ase and VDR null alleles [1α(OH)ase<sup>−/−</sup>VDR<sup>−/−</sup>]. Lines were maintained by mating 1α(OH)ase<sup>−/−</sup>VDR<sup>−/−</sup> males and 1α(OH)ase<sup>+/−</sup>VDR<sup>+/−</sup> females. These mice were maintained on a mixed genetic background with contributions from C57BL/6J and BALB/c strains. To enhance fertility of females, all breeders were maintained on a high calcium diet containing 1.5% calcium in the drinking water and autoclaved chow containing 1% calcium, 0.85% phosphorus, 0% lactose and 2.2 units of vitamin D per gram (Ralston Purina Co., St. Louis, MO).

In vivo experiments—All animal experiments were carried out in compliance with and approval by the Institutional Animal Care and Use Committee. Mutant mice and control littermates were maintained in a virus- and parasite-free barrier facility and exposed to a 12h/12h light/dark cycle.
At 21 days of age, wild-type (WT) and mutant mice were weaned onto one of three different regimens and maintained on these until sacrifice at 4 months of age: (1) the high-calcium diet described above; (2) this same high-calcium diet plus thrice weekly intraperitoneal injections of 1,25(OH)2D3, 0.0625 µg per mouse (27); or (3) a “rescue diet” (TD96348 Teklad, Madison, WI) of γ-irradiated chow containing 2% calcium, 1.25% phosphorus, 20% lactose and 2.2 units vitamin D per gram.

Genotyping of mice—Genomic DNA was isolated from tail fragments by standard phenol-chloroform extraction and isopropanol precipitation (16). To determine the genotype at both the 1α(OH)ase and VDR loci, four PCRs were conducted for each animal. To test for the presence of the wild-type 1α(OH)ase allele, DNA was amplified with forward primer 5’AGACTGCACTCCACTCTGAG 3’ and reverse primer 5’ GTTTCCTACACGGATGTCTC 3’. For the neomycin gene the primers were neo-F 5’ ACAACAGACAATCGGCTGCTC 3’ and neo-R 5’ CCATGGGTCACGCAGATAC 3’. The wild type VDR allele was detected using forward primer 5’ CTGCCCTGCTCCACAGTCCTT 3’ and reverse primer 5’ CGAGACTCTCCAATGTGAAC 3’. The disrupted VDR allele was assayed using the neo forward primer 5’ GCTGCTCTGATGCCAGTTGGTTC 3’ and a neo reverse primer 5’ GCAGACTCTCCAATGTGAAGC 3’. PCR conditions were thirty cycles for all: 1α(OH)ase allele, 94°C for 1 min; 58°C for 1 min; and 72°C for 1 min; VDR and disrupted VDR allele, 94°C for 1 min; 65°C for 1 min; and 72°C for 1 min; and neomycin 94°C for 1 min; 60°C for 1 min, and 72°C for 1 min.

RT-PCR—RNA was isolated from mouse kidney and long bones, using Trizol reagent (Invitrogen Inc) according to the manufacturer’s protocol. The forward and the reverse primers used for amplification of the mouse 1α(OH)ase mRNA were 5’
GCAGAGGCTCCGAAGTCTTC 3’ and 5’ TGTCTGGGACACGGGAATTC 3’ and 24(OH)ase mRNA were 5’ ACCGTGGACAGAAGCATA 3’ and 5’ AAATCCAGAGCGTGCTGCCTG 3’. The forward and the reverse primers for core binding factor a I (Cbfa I) mRNA were 5’ GTGACACCGTGTCAGCAAG 3’ and 5’ GGAGCAGAAGGTTGAC 3’. For receptor activator of NF-κB ligand (RANKL) mRNA mRNA the forward and the reverse primers were 5’ CACACCTCACCATCAATGCTGC 3’ and 5’ GAAGGGTGGACACCTGAATGC 3’. The forward and the reverse primers for GAPDH mRNA used as a loading control were 5’ CATGGAGAAGGCTGGGGCTC 3’ and 5’ CACTGACACGTGAC 3’. The conditions for thirty-two cycles of PCR reactions were 94°C for 1 min; 58°C for 1 min, and 72°C for 1 min.

Biochemical and hormone analyses—Serum calcium and alkaline phosphatase were determined by autoanalyzer (Beckman Synchron 67; Beckman Instruments). Serum 1,25(OH)2D3 was measured by radioimmunoassay (ImmunoDiagnostic Systems, Bolden, UK) and intact PTH was measured by a 2-site immunoradiometric assay (Immunotopics, San Clemente, CA).

Skeletal Radiography—Femurs were removed and dissected free of soft tissue. Contact radiographs were taken using a Faxitron model 805 (Faxitron Contact, Faxitron, Germany) radiographic inspection system (22-kV voltage and 4-min exposure time). Eastman Kodak Co. (Kodak, Rochester, NY) X-Omat TL film was used and processed routinely.

Western blot analysis—Proteins were extracted from long bones and quantitated by a protein assay kit (Bio-Rad, Mississauga, Ontario, Canada). Protein samples (30 µg) were fractionated by SDS-PAGE and transferred to PVDF membrane. Immunoblotting was carried out using monoclonal antibodies against Runx2/Cbfa I (MBL International, Woburn, MA, USA) and
tubulin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Bands were visualized using the ECL chemiluminescence detection method (Amersham).

**Histology**—Thyroparathyroidal tissue, femurs and tibiae were removed and fixed in PLP fixative (2% paraformaldehyde containing 0.075M lysine and 0.01M sodium periodate) overnight at 4°C and processed histologically as previously described (28). The proximal ends of the tibiae were decalcified in ethylene-diamine tetra-acetic acid (EDTA) glycerol solution for 5-7 days at 4°C. Decalcified tibiae and other tissues were dehydrated and embedded in paraffin after which 5 µm sections were cut on a rotary microtome. The sections were stained with hematoxylin and eosin (H&E) or histochemically for collagen, alkaline phosphatase activity (ALP), or tartrate resistant acid phosphatase (TRAP) activity as described below. Alternatively, undecalcified tibiae were embedded in LR White acrylic resin (London Resin Company Ltd., London, UK) and 1-µm sections were cut on an ultramicrotome. These sections were stained for mineral with the von Kossa staining procedure and counterstained with toluidine blue.

**Immunohistochemical staining for aggrecan and RANKL** — The cartilage matrix protein, aggrecan, and the transcription factor, RANKL, were determined by immunohistochemistry as described previously (28). Briefly, rabbit antiseraum to bovine aggrecan (R130, courtesy of Dr. A.R. Poole, Shriners Hospital, Montreal, Canada) or affinity purified goat polyclonal antibody raised against a peptide mapping at the carboxy terminus of RANKL (C-20, Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) were applied to de-waxed paraffin sections overnight at room temperature. As a negative control, the pre-immune serum was substituted for the primary antibody. After washing with high salt buffer (50 mM Tris-HCl, 2.5% NaCl, 0.05% Tween 20, pH 7.6) for 10 min at room temperature followed by two 10-min washes with PBS, the sections were incubated with secondary antibody (biotinylated goat anti-rabbit IgG or
biotinylated rabbit antigoat IgG, Sigma), washed as before and processed using the Vectastain ABC-AP kit (Vector Laboratories, Inc.). Red pigmentation to demarcate regions of immunostaining was produced by a 10- to 15-min treatment with Fast Red TR/Naphthol AS-MX phosphate (Sigma, containing 1 mM levamisole as endogenous alkaline phosphatase inhibitor). The sections were then washed with distilled water, counterstained with methyl green, and mounted with Kaiser’s glycerol jelly.

Histochemical staining for collagen, alkaline phosphatase (ALP) and tartrate resistant acid phosphatase (TRAP) — Total collagen was detected in paraffin sections using a modified method of Lopez-De Leon and Rojkind (29). De-waxed sections were exposed to 1% sirius red in saturated picric acid for 1 h. After washing with distilled water, the sections were counterstained with haematoxylin, and mounted with Biomount medium.

Enzyme histochemistry for ALP activity was performed as previously described (30,31). Briefly, following preincubation overnight in 1% magnesium chloride in 100 mm tris-maleate buffer (pH 9.2), de-waxed sections were incubated for 2 hours at room temperature in a 100 mM tris-maleate buffer containing naphthol AS-MX phosphate (0.2mg/ml, Sigma) dissolved in ethylene glycol monomethyl ether (Sigma) as substrate, and fast red TR (0.4mg/ml, Sigma) as a stain for the reaction product. After washing with distilled water, the sections were counterstained with Vector methyl green nuclear counterstain (Vector laboratories), and mounted with Kaiser's glycerol jelly.

Enzyme histochemistry for TRAP was performed using a modification of a previously described protocol (32). De-waxed sections were pre-incubated for 20 minutes in buffer containing 50 mM sodium acetate and 40 mM sodium tartrate at pH 5.0. Sections were then incubated for 15 minutes at room temperature in the same buffer containing 2.5 mg/ml naphthol
AS-MX phosphate (Sigma) in dimethylformamide as substrate, and 0.5mg/ml fast garnet GBC (Sigma) as a color indicator for the reaction product. After washing with distilled water the sections were counterstained with methyl green and mounted in Kaiser’s glycerol jelly.

Double calcein labeling—Double calcein labeling was performed by intra-peritoneal injection of mice with 10µg calcein/g body weight (C-0875, Sigma) at 10 days and 3 days prior to sacrifice. Bones were harvested and embedded in LR White acrylic resin described as above. Serial sections were cut and the freshly cut surface of each section was viewed and imaged using fluorescence microscopy. The double calcein labeled width of cortex and trabeculae was measured using Northern Eclipse image analysis software v6.0 (Empix Imaging Inc., Mississauga, ON) and the mineral apposition rate (MAR) was calculated as the interlabel width/labeling period.

Computer-assisted image analysis—After H&E staining or histochemical staining of sections from six mice of each genotype on each dietary regimen, images of fields were photographed with a Sony digital camera. Images of micrographs from single sections were digitally recorded using a rectangular template, and recordings were processed using Northern Eclipse image analysis software (28,33). To measure the size of the parathyroid glands, the border of the glands were traced on micrographs of H&E stained sections and traced areas of parathyroid glands were recorded automatically by Northern Eclipse image analysis software. For measuring the width of growth plates of tibiae, the distances between the proximal (epiphyseal) and distal (metaphyseal) sides of the growth plate were traced on micrographs of H&E stained sections and traced distances were recorded automatically by Northern Eclipse image analysis software. For determining the trabecular bone volume relative to the total volume (BV/TV) in collagen stained sections, the osteoid volume relative to the bone volume (OV/BV) in von Kossa stained sections,
ALP positive area and intensity (summary total gray) in ALP histochemical stained sections, and
the number and size of osteoclasts in TRAP histochemical stained sections, thresholds were set
using green and red channels. The thresholds were determined as described previously (28). The
trabecular volume was measured in the metaphyseal region from 0.5 mm below the distal
(metaphyseal) side of the growth plate to 1.5 mm towards the diaphysis and ALP and TRAP
parameters were measured in the fields of metaphyseal regions.

Bone marrow cell cultures —Primary bone marrow cell cultures were performed as previously
described (34). Tibiae and femurs of 4-month-old mice fed a rescue diet were removed under
aseptic conditions, and bone marrow cells were flushed out with Dulbecco’s modified Eagle’s
minimal essential medium containing 10% fetal calf serum, 50 µg/ml ascorbic acid, 10 mM β-
glycerophosphate, and 10⁻⁸ M dexamethasone. Cells were dispersed by repeated pipetting, and a
single cell suspension was achieved by forcefully expelling the cells through a 22-gauge syringe
needle. 10⁶ bone marrow cells were cultured in 55-cm² petri dishes in 10 ml of the above-
mentioned medium. The medium was changed every 4 days. The non-adherent cells containing
hematopoietic elements were removed by gently pipetting when the medium was changed for the
first time. Cultures were maintained for 18 days. At the end of the culture period cells were
washed with PBS, fixed with PLP fixative, and then stained. For determination of total colonies
formed, cells were first washed in borate buffer (10 mM; pH 8.8) and then stained with 1%
methylene blue (wt/vol) in borate buffer for 30 min at room temperature. Cells were then washed
three times in borate buffer alone and left to dry before the number of colonies was quantitated
by image analysis as described. For determination of mineralized colonies, cells were exposed to
a solution of Alizarin Red S, pH 6.2 (1 mg/ml), for 30 min at room temperature, after which the
colonies were gently washed under running water and left to dry. After each staining, culture
plates were photographed over a light box with a Sony charge-coupled device camera. Images were analyzed using Northern Eclipse image analysis software. The data were imported to a spreadsheet program and processed as previously described (34).

Statistical Analysis—Data from image analysis are presented as means ± SEM. Statistical comparisons were made using a two-way ANOVA, with P< 0.05 being considered significant.
RESULTS

*Genotypic Selection of Mutant Mice*—Representative PCR profiles used for genotyping the mutant mice are shown in Fig. 1a. The neomycin cassette replaced the second zinc finger of the DNA binding domain of the VDR (upper panel) and replaced both the substrate binding and heme binding domains of the 1α(OH)ase enzyme (lower panel) in the VDR\(^{-/-}\) and 1α(OH)ase\(^{-/-}\) mice respectively.

Expression of 1α(OH)ase and 24(OH)ase genes—The 1α(OH)ase gene was expressed at higher levels in the VDR\(^{-/-}\) mice than in wild-type mice when animals received a high calcium intake (Fig. 1b, left panel); this was not reduced by administering exogenous 1,25(OH)\(_{2}\)D\(_{3}\) to these animals (Fig. 1b, right panel) but was reduced by eliminating hypocalcemia with the rescue diet (Fig. 1b, middle panel).

Expression of the 24(OH)ase gene was reduced in all mutant mice on the high calcium diet (Fig. 1b, upper panel) but was restored to wild-type levels in 1α(OH)ase\(^{-/-}\) mice receiving exogenous 1,25(OH)\(_{2}\)D\(_{3}\) (Fig. 1b, lower panel). On the rescue diet, 24(OH)ase gene expression was restored to normal or near normal (Fig. 1b, middle panel).

*Biochemistry*—On the high calcium intake, serum 1,25(OH)\(_{2}\)D\(_{3}\) levels were undetectable in 1α(OH)ase\(^{-/-}\) mice and the 1α(OH)ase\(^{-/-}\)VDR\(^{-/-}\) mice but were 9-fold elevated in the VDR\(^{-/-}\) mice (Fig. 1c) consistent with the increased 1α(OH)ase activity and with diminished 1,25(OH)\(_{2}\)D clearance as a result of decreased 24(OH)ase expression in VDR\(^{-/-}\) mice. On the rescue diet, 1,25(OH)\(_{2}\)D\(_{3}\) levels fell toward the normal range of wild-type mice in VDR\(^{-/-}\) mice in which 1α(OH)ase fell and 24(OH)ase rose from their respective levels in corresponding hypocalcemic animals. With 1,25(OH)\(_{2}\)D\(_{3}\) treatment, serum 1,25(OH)\(_{2}\)D\(_{3}\) concentrations in 1α(OH)ase\(^{-/-}\) mice were not significantly different from wild-type but remained highly elevated in VDR\(^{-/-}\) mice;
furthermore, in the \(1\alpha(\text{OH})\text{ase}^{-/}\text{VDR}^{-/}\) mice, which had reduced 24(OH)ase expression, concentrations of 1,25(OH)\(_2\)D\(_3\) rose sharply to a level approaching that of VDR\(^{-/}\) mice.

On the high calcium intake, all mutant animals were hypocalcemic (Fig. 1d), but when the rescue diet was administered, mean serum calcium levels in all mutant mice rose to wild-type levels. With exogenous 1,25(OH)\(_2\)D\(_3\) treatment serum calcium rose to wild-type levels in \(1\alpha(\text{OH})\text{ase}^{-/}\) mice, but both the VDR\(^{-/}\) mice and the \(1\alpha(\text{OH})\text{ase}^{-/}\text{VDR}^{-/}\) mice remained significantly hypocalcemic.

All the hypocalcemic mutant mice had markedly elevated serum PTH concentrations on the high calcium intake (Fig. 1e) and these animals were also hypophosphatemic (Fig. 1f). Serum alkaline phosphatase levels, most likely reflecting osteoblast stimulation, paralleled the PTH levels (Fig. 1g). In mutant animals on the rescue diet, serum PTH concentrations as well as serum phosphorus and alkaline phosphatase, all returned to the wild-type range (Fig. 1e-g). Treatment with exogenous 1,25(OH)\(_2\)D\(_3\) normalized serum PTH, phosphorus and alkaline phosphatase concentrations in \(1\alpha(\text{OH})\text{ase}^{-/}\) mice but all parameters remained abnormal in VDR\(^{-/}\) mice and \(1\alpha(\text{OH})\text{ase}^{-/}\text{VDR}^{-/}\) mice (Fig. 1e-g).

*Parathyroid Gland Size in Wild-Type and Mutant Mice*—Although the parathyroid glands in the VDR\(^{-/}\) mice were enlarged on the high calcium intake they were even larger in the \(1\alpha(\text{OH})\text{ase}^{-/}\) mice and the \(1\alpha(\text{OH})\text{ase}^{-/}\text{VDR}^{-/}\) mice (Fig. 2a and 2d). On the rescue diet, parathyroid gland size decreased into the normal range in the normocalcemic VDR\(^{-/}\) mice but still remained significantly enlarged in the two normocalcemic but 1,25(OH)\(_2\)D\(_3\) deficient models i.e., the \(1\alpha(\text{OH})\text{ase}^{-/}\) mice and the \(1\alpha(\text{OH})\text{ase}^{-/}\text{VDR}^{-/}\) mice (Fig. 2b and 2d). After 1,25(OH)\(_2\)D\(_3\) treatment parathyroid gland size normalized in the \(1\alpha(\text{OH})\text{ase}^{-/}\) animals but not in the two VDR deficient models i.e., the VDR\(^{-/}\) and the double mutant that remained hypocalcemic.
**Skeletal Alterations**—On the high calcium intake femoral size was significantly reduced in all hypocalcemic mutant models reflecting decreased long bone growth (Fig. 3a). On the rescue diet, femoral size of all 3 mutant models remained slightly decreased but approached that of the wild-type (Fig. 3b). Exogenous 1,25(OH)\_2D\_3 treatment only improved femoral growth of the 1\(\alpha\)(OH)ase\(^{-/}\) mice and not that of the VDR\(^{-/}\) or the double mutants (Fig. 3c).

The cartilaginous growth plates were enlarged and distorted in all three mutant models on the high calcium intake, but these alterations were most pronounced in the 1\(\alpha\)(OH)ase\(^{-/}\) mice and the 1\(\alpha\)(OH)ase\(^{-/}\)VDR\(^{-/}\) mice (Fig. 4a and 4d) and less pronounced in the VDR\(^{-/}\) animals. The growth plate of the VDR\(^{-/}\) mice appeared normal on the rescue diet but still remained substantially altered in the 1\(\alpha\)(OH)ase\(^{-/}\) and double mutant mice (Figure 4b and 4d). Treatment with exogenous 1,25(OH)\_2D\_3 normalized the growth plate of the 1\(\alpha\)(OH)ase\(^{-/}\) mice but not that of the VDR\(^{-/}\) or the double mutant (Fig. 4c and 4d).

Trabecular bone volume (BV/TV) was increased in all three hypocalcemic mutant models on a high calcium intake (Fig. 5a and 5d) but after 4 months on a rescue diet, trabecular bone volume was reduced in all three mutants below the levels in wild-type animals (Fig. 5b and 5d). In contrast, treatment with 1,25(OH)\_2D\_3 normalized trabecular bone volumes in the 1\(\alpha\)(OH)ase\(^{-/}\) mice but not in the other two models (Fig. 5c and 5d).

In the hypocalcemic mice on the high calcium intake, osteoid volume reflecting unmineralized bone matrix was increased in all mutant animals in both trabecular and cortical bone (Fig. 6a and b). When hypocalcemia was eliminated by the rescue diet, osteoid volume was reduced to that of wild-type in all mutants. In contrast, exogenous 1,25(OH)\_2D\_3 treatment normalized osteoid only in the 1\(\alpha\)(OH)ase\(^{-/}\) mice (Fig. 6a and b). Mineral apposition rate (MAR) was increased in all 3 mutants on the high calcium intake and was reduced below normal in all 3
mutants on the rescue diet. MAR was normalized by 1,25(OH)₂D₃ treatment only in 1α(OH)ase⁻/⁻ mice but remained elevated in the VDR⁻/⁻ and 1α(OH)ase⁻/⁻VDR⁻/⁻ mice (Fig. 6c and d).

Osteoblast levels were significantly elevated in all 3 mutants with secondary hyperparathyroidism (Fig. 7a and d) on the high calcium intake and Cbfa I mRNA and protein expression in bone were also increased (Fig. 7e and f). Osteoblasts were significantly reduced in the mutants on the rescue diet, to levels below those in wild-type mice (Fig. 7b and d) and Cbfa I mRNA or protein were also reduced (Fig. 7e and f). After exogenous 1,25(OH)₂D₃ treatment, only the 1α(OH)ase⁻/⁻ mice normalized their osteoblast levels whereas these remained elevated in the VDR⁻/⁻ and the double mutants (Fig. 7c and d).

To determine the cause of the reduced osteoblasts in the mutants on the rescue diet primary bone marrow cultures were examined for the capacity to formed osteogenic colonies from marrow progenitors. Reduced bone forming colonies were observed in the marrow of all 3 mutants on the rescue diet compared with wild-type controls (Fig. 8).

TRAP-positive osteoclast numbers were not significantly different from levels in wild-type animals in any of the mutants exposed to any of the dietary manipulations (Fig. 9). However, the average size of the TRAP-positive osteoclasts was reduced in all 3 mutants on the high calcium intake. On the rescue diet, TRAP-positive osteoclast size was no different in the mutant mice than in wild-type mice. After 1,25(OH)₂D₃ treatment, however, only the 1α(OH)ase⁻/⁻ mice normalized and average osteoclast size remained below wild-type in the VDR⁻/⁻ and double mutants (Fig. 9c and d).

Expression of RANKL mRNA was diminished in bone of the mutants compared to wild-type bone, when the animals were on a high calcium intake (Fig. 10a). RANKL protein in osteoblastic cells was also found to be low in all three mutants on the high calcium intake (Fig. 10b).
10b, e and f) and in the two mutants treated with 1,25(OH)₂D₃ that were deficient in VDR (Fig. 10d, e and f). On the rescue diet, RANKL mRNA (Fig. 10a and protein (Fig. 10c, e and f) were markedly below wild-type levels.
DISCUSSION

We have analyzed mouse mutants which harbor deletions of the genes encoding the 1α(OH)ase, the VDR or both genes and subjected them to three different nutritional regimens. The analysis of the double mutants allowed us to assess which phenotypic differences seen in these animals compared to the VDR−/− mice might be due to the elevated endogenous 1,25(OH)2D of the VDR−/− mice. This would indicate that the 1,25(OH)2D functions in the absence of an intact VDR. The high calcium intake facilitated breeding of the mutant mice, notably the double mutants, in view of the fact that both 1α(OH)ase−/− and VDR−/− animals display reduced fertility, at least in part because of severe hypocalcemia (35). Nevertheless, in the absence of dietary lactose serum calcium concentrations remained sub-normal in mutants fed the high calcium intake. The lactose in the rescue diet facilitates calcium entry across the intestine independent of the 1,25(OH)2D/VDR system although the mechanism is unclear. Serum calcium was normalized on the rescue diet in the absence of 1,25(OH)2D, or of the VDR, or of both 1,25(OH)2D and VDR, and so was not due to a VDR-independent action of 1,25(OH)2D. In the absence of lactose, endogenous elevated 1,25(OH)2D could not normalize calcium in VDR−/− mice and exogenous 1,25(OH)2D3 could only normalize calcium in the 1α(OH)ase−/− mice which retained an intact VDR. Consequently, these results confirm the importance of both 1,25(OH)2D and the VDR in intestinal calcium absorption. Indeed, 1,25(OH)2D-dependent increases in gut absorption of calcium (36) and in intestinal calbindin D9k are well documented and effects on the duodenal epithelial calcium channels have recently been reported (37).

In the hypocalcemic mice on the high calcium intake, secondary hyperparathyroidism (based on serum PTH and alkaline phosphatase concentrations) appeared more severe in the 1α(OH)ase−/− and 1α(OH)ase−/− VDR−/− mice than in VDR−/− mice, i.e., in the mutants with
1,25(OH)₂D deficiency. Nevertheless, when serum calcium was raised by the rescue diet, normalization of the elevated serum PTH concentrations occurred in all mutants. In \( \alpha \text{OHase}^{-/-} \) mice treated with exogenous 1,25(OH)₂D₃, in whom mean serum calcium levels were normalized, circulating PTH concentrations fell into the normal range but PTH levels remained increased in 1,25(OH)₂D₃-treated VDR⁻/⁻ and in \( \alpha \text{OHase}^{-/-} \text{VDR}^{-/-} \) mice whose ambient calcium levels remained low. Consequently, the ambient calcium concentration appears to suppress secretion of PTH independently of the 1,25(OH)₂D/VDR system.

Increased parathyroid gland size was present in all hypocalcemic mutants and remained elevated in the \( \alpha \text{OHase}^{-/-} \) and double mutants on the rescue diet when they were no longer hypocalcemic. However, parathyroid gland size was normalized in the VDR⁻/⁻ animals on the rescue diet when these animals were no longer hypocalcemic. In these mice, the elevated endogenous circulating 1,25(OH)₂D levels may have contributed, even in the absence of an intact VDR, to the reduction in gland size. Administration of exogenous 1,25(OH)₂D₃ normalized parathyroid gland size in the \( \alpha \text{OHase}^{-/-} \) mice but not in the VDR⁻/⁻ mutants or double mutants where hypocalcemia persisted. Previous studies have demonstrated an in vivo role for extracellular calcium (38) in parathyroid cell growth via the calcium sensing receptor and previous in vitro studies have also suggested a role for 1,25(OH)₂D (39). Our studies confirm both these actions in vivo and demonstrate the co-operative nature of the calcium ion and 1,25(OH)₂D in exerting this effect.

The cartilaginous growth plate was enlarged and distorted with a widened hypertrophic zone in all three mutants while they were hypocalcemic but the growth plate abnormalities were more pronounced in the \( \alpha \text{OHase}^{-/-} \) and the \( \alpha \text{OHase}^{-/-} \text{VDR}^{-/-} \) mutants both of which had undetectable serum 1,25(OH)₂D than in the VDR⁻/⁻ animals which had markedly elevated
endogenous serum 1,25(OH)₂D. When hypocalcemia was eliminated on the rescue diet the
growth plate including the widened hypertrophic zone of the VDR⁻/⁻ mice normalized as
previously described (25) but still remained considerably enlarged in the 1α(OH)ase⁻/⁻ mice, and
in the 1α(OH)ase⁻/⁻/VDR⁻/⁻ mice. As also reported by others (36), exogenous 1,25(OH)₂D₃
normalized the growth plate of 1α(OH)ase⁻/⁻ mice. However, it did not normalize the growth
plate of the double mutant where hypocalcemia persisted. Consequently, both ambient calcium
and 1,25(OH)₂D, even in the absence of an intact VDR, appear to exert a major effect on the
cartilaginous growth plate and are co-operatively required for its normal development. The
persistent abnormality of the growth plate in normocalcemic 1α(OH)ase⁻/⁻ mice on the rescue diet
has also been noted by others (40) and has been observed in VDR/Retinoid X Receptor (RXR)
gamma double null mutant mice (41). Consequently, this supports the possibility that
1,25(OH)₂D interacts with a novel nuclear receptor in chondrocytes which heterodimerizes with
RXR gamma (41).

Bone volume and osteoblast numbers were increased in the mutant animals with
secondary hyperparathyroidism as has been previously reported in studies with the 1α(OH)ase⁻/⁻
(16,17) and the VDR⁻/⁻ models (20,21). These increases most likely reflect the well characterized
“anabolic” activity of PTH (42). Consistent with this observation, increases were observed in the
transcription factor Cbfa I/Runx2, which is known to be essential for osteoblastic differentiation
during embryogenesis and is also required for the anabolic effect of PTH in postnatal animals
(43). The increased bone volume was however associated with increased osteoid volume in the
animals with secondary hyperparathyroidism. In those animals who received a high calcium
intake, serum calcium levels, although decreased, were higher than we and others have
previously reported in mutant mice fed a normal calcium intake (16,17,20,21). Osteoid volumes
were therefore lower than in previous reports. In addition mineral apposition could be detected and was, in fact, elevated in the mice with secondary hyperparathyroidism. The mineral apposition rate fell as PTH levels fell reflecting the reduced bone formation. Unmineralized osteoid was no greater than in wild-type mice when hypocalcemia and hypophosphatemia were eliminated i.e., in 1,25(OH)2D3-treated 1α(OH)ase−/− mice, but also in all mutants on the rescue diet. Consequently once extracellular calcium and phosphorus levels are normalized mineralization of osteoid does not appear to require the 1,25(OH)2D/VDR system.

Despite greatly increased PTH levels, osteoclast numbers in these mutants with deficiency of the 1,25(OH)2D/VDR system were not significantly increased above levels observed in normocalcemic vitamin D-replete wild-type controls whose PTH levels were normal. Bone turnover in the mutant animals was therefore uncoupled and osteoclast numbers in the mutants could be considered inappropriately low. We did find, in the present studies, that average osteoclast size was decreased in the mutants with secondary hyperparathyroidism on the high calcium diet and in the 1,25(OH)2D3 treated VDR−/− and double mutants. Furthermore, levels of RANKL, the transcription factor stimulated by 1,25(OH)2D3 which is required for the normal differentiation and maintenance of osteoclasts (44) were also decreased in the mutants. Consequently, the 1,25(OH)2D/VDR system appears necessary for maximal PTH-induced osteoclast production and the increase in bone volume appeared to reflect enhanced osteoblastic activity due to PTH that was dissociated from an increase in bone resorption.

The increase in the thickness of the cartilaginous growth plate in the mutants may also have been contributed to by impaired growth plate remodeling i.e., impaired resorption of hypertrophic chondrocytes by chondroclasts/osteoclasts at the chondro-osseous junction. This has been previously noted in VDR−/− mutants (26). It has been reported that in co-cultures in vitro
using wild-type spleen cells but osteoblasts from VDR−/− mice, 1,25(OH)₂D₃ was unable to
stimulate osteoclast production whereas PTH and other bone resorbing agents could (45).
Consequently the “normal level” of osteoclasts seen in our in vivo models in the absence of the
1,25(OH)₂D/VDR system may have reflected the action of PTH and other local bone resorbers.
The more exuberant osteoclastic response generally expected with sustained increases in PTH of
the magnitude seen in our mutants (42) may reflect increased production of 1,25(OH)₂D and its
action via the VDR in skeletal cells when these factors are not limiting.

Another striking finding in all three mutants of the 1,25(OH)₂D/VDR system was the
reduction in osteoblast numbers, mineral apposition rate and bone volume below levels in wild-
type mice which occurred on the rescue diet. This was associated with decreased production of
mineralized colonies ex vivo providing further evidence that osteogenesis was impaired. The
issue of whether vitamin D directly induces bone formation has been controversial (46).
However, our observation in animals with normal calcium levels but deficient vitamin D indicate
that the presence of an intact 1,25(OH)₂D/VDR system may be necessary for baseline bone
formation. Such findings were not previously reported in 1α(OH)ase−/− and VDR−/− mice fed a
rescue diet and may represent differences in the duration of exposure to this diet post-weaning
i.e., about 100 days in our study versus approximately 39 days (40) and 54 days (26),
respectively, in previous studies. Consequently, bone formation may be more vitamin D-
dependent as the animals age. The failure of elevated endogenous 1,25(OH)₂D in the VDR−/−
mice to alleviate the reduction in bone volume, suggests that both 1,25(OH)₂D and the VDR are
required for baseline bone formation. Our results are therefore consistent with a physiologic
anabolic role for endogenous 1,25(OH)₂D and the VDR in vivo.
In summary, our studies show that the calcium ion and the 1,25(OH)₂D/VDR system may exert physiological effects *in vivo* independently or in concert. Thus, mineralization of bone and inhibition of elevated PTH secretion appear mainly dependent on ambient calcium levels rather than the presence of the 1,25(OH)₂D/VDR system whereas optimal intestinal absorption of calcium, maximal increases in PTH-induced osteoclastogenesis and maintenance of osteoblastogenesis seem dependent on a functional 1,25(OH)₂D/VDR system. In contrast, the calcium ion and 1,25(OH)₂D in the absence of an intact VDR may act in concert to regulate parathyroid gland size and the normal development of the cartilaginous growth plate. Finally, our studies also show that the vitamin D system is required for both anabolic and catabolic effects on the skeleton, therefore mimicking the dual functions of parathyroid hormone.
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The abbreviations are: Cbfa I, core binding factor a I; 1,25(OH)$_2$D, 1,25-dihydroxyvitamin D; 1α(OH)ase, 25-hydroxyvitamin D-1α-hydroxylase; PTH, parathyroid hormone; Ca, calcium; P, Phosphorus; 24(OH)ase, 25-hydroxyvitamin D-24-hydroxylase; VDR, vitamin D receptor; VDDR-I, vitamin D-dependent rickets type I; PDDR, pseudovitamin D-deficiency rickets; VDDR-II, vitamin D-dependent rickets type II; VDDR, hereditary vitamin D resistant rickets; RANKL, receptor activator of nuclear factor kappa B ligand; RT-PCR, reverse transcriptase-polymerase chain reaction; ALP, alkaline phosphatase; TRAP, tartrate resistant acid phosphatase.
FIGURE LEGENDS

FIG. 1 Genotyping of mice, expression of 1α(OH)ase and 24(OH)ase genes and serum chemistry. (a) Representative PCR profiles used for genotyping the mutant mice were obtained as described in Experimental Procedures. A neomycin cassette (neo) replaced exon III of the VDR gene (upper panels) and exon VI, VII and VIII of the 1α(OH)ase gene (bottom panels). (b) Comparison of 1α(OH)ase and 24(OH)ase expression in kidney of wild-type (WT), 1α(OH)ase−/−, VDR−/−, and 1α(OH)ase−/−VDR−/− mice fed a high calcium diet; a rescue diet; or a high calcium diet with 1,25(OH)2D3 administration as described in Experimental Procedures. Specific 1α(OH)ase and 24(OH)ase products were amplified from the tissue RNAs by RT-PCR. The GAPDH was used as a loading control. (c) Serum calcium, (d) 1,25(OH)2D3, (e) PTH, (f) phosphorus and (g) alkaline phosphatase (ALP) were determined in wild-type (WT), 1α(OH)ase−/−, VDR−/−, and 1α(OH)ase−/−VDR−/− mice fed a high calcium diet; a rescue diet; or a high calcium diet with 1,25(OH)2D3 administration as described in Experimental Procedures. Each value is the mean ± SE of determinations in 5 mice of the same genotype. *, P<0.05; **, P<0.01; ***, P<0.001 compared with wild-type on the same diet.

FIG. 2 Parathyroid gland size. Representative micrographs of parathyroid glands (arrows) and adjacent thyroid tissue of wild-type (WT), 1α(OH)ase−/−, VDR−/−, and 1α(OH)ase−/−VDR−/− littermates fed (a) a high calcium diet, (b) a rescue diet, or (c) a high calcium diet with 1,25(OH)2D3 injections. (d) Sections were stained with hematoxylin and eosin, bar=100 µm. Parathyroid gland sizes (areas) were determined by computer-assisted image analysis as described in Experimental Procedures and are presented as the mean ± SE in 6 mice of the
same genotype on the same diet. *, P<0.05, **, P<0.01; ***, P<0.001 compared with wild-type on the same diet.

**FIG. 3 Radiographs and femoral lengths.** Representative contact radiographs of the femurs of wild-type (WT), 1α(OH)ase−/−, VDR−/−, and 1α(OH)ase−/−VDR−/− mice fed a high calcium diet, a rescue diet, or a high calcium diet with 1,25(OH)2D3 administration. Right panels show the quantitation of femoral length from each mutant model fed each of the 3 diets. Each value is the mean ± SE of determinations in 4 mice of the same genotype on the same diet. ***, P<0.001 relative to wild-type on the same diet.

**FIG. 4 Size of the cartilaginous growth plates.** Representative micrographs of the proximal end of the tibia of wild-type (WT), 1α(OH)ase−/−, VDR−/−, and 1α(OH)ase−/−VDR−/− mice fed (a) a high calcium diet, (b) a rescue diet, or (c) a high calcium diet with 1,25(OH)2D3 administration. Sections were stained with hematoxylin and eosin, bar=100 µm. The width of the cartilaginous growth plate in the mutants on the different diets was determined as described in Materials and Methods and is shown in (d). Each value is the mean ± SE of determinations in 6 mice of the same genotype on the same diet. *, P<0.05, ***, P<0.001 relative to wild-type on the same diet.

**FIG. 5 Bone volume.** Representative histology of the upper half of the tibia of wild-type (WT), 1α(OH)ase−/−, VDR−/−, and 1α(OH)ase−/−VDR−/− mice fed either (a) a high calcium diet; (b) a rescue diet; or (c) a high calcium diet with 1,25(OH)2D3 injections. Sections were stained for collagen, bar=400 µm. The trabecular bone volume (d) was determined as described in Experimental Procedures and is presented as a percent of the tissue volume [BV/TV (%)] for each mutant on
each diet. Each value is the mean ± SE of determinations in 6 animals of the same genotype *, P<0.05; ***, P<0.001 compared with wild-type on the same diet.

**FIG. 6 Osteoid volume and mineral apposition rate.** Osteoid volume was determined in undecalcified von Kossa stained sections as described in Experimental Procedures and is presented as a percent of bone volume [OV/BV (%)] of trabeculae (a) and of cortex (b) in wild-type (WT), 1α(OH)ase−/−, VDR−/−, and 1α(OH)ase−/−VDR−/− mice fed a high calcium diet; a rescue diet; or a high calcium diet with 1,25(OH)2D3 injections. Mineral apposition rate (MAP) of trabeculae (c) and cortex (d) of the same animals was also determined as described in Experimental Procedures. Each value is the mean ± SE of determinations in 6 animals of the same genotype on the same diet. **, P<0.01; ***, P<0.001 relative to wild-type mice on the same diet.

**FIG. 7 Alkaline phosphatase and Cbfa I expression.** Representative micrographs of tibial sections, stained for alkaline phosphatase (ALP) activity, from wild-type (WT), 1α(OH)ase−/−, VDR−/−, and 1α(OH)ase−/−VDR−/− littermates fed (a) a high calcium diet, (b) a rescue diet or (c) a high calcium diet with 1,25(OH)2D3 injections. The bar=100 µm. The ALP positive area as a percent of the tissue area and the relative intensity of the ALP positivity (summary total gray of ALP positive production) were determined in the metaphyseal regions as described in Experimental Procedures for each mutant on each diet shown in the left and right panels respectively of section d. Each value is the mean ± SE of determinations in 6 animals of the same genotype. ***, P<0.001 relative to wild-type mice on the same diet. RT-PCR (e) and Western blots (f) of long bone extracts for expression of Cbfa I. GAPDH and tubulin were used as loading
controls for RT-PCR and Western blots, respectively. Lane 1: WT; Lane 2: 1α(OH)ase−/−; Lane 3: VDR−/−; Lane 4: 1α(OH)ase−/−VDR−/−.

**FIG. 8 Bone marrow cell cultures and mineralized colony formation.** Bone marrow cells from wild-type (WT), 1α(OH)ase−/−, VDR−/−, and 1α(OH)ase−/−VDR−/− littermates fed a rescue diet were cultured in osteogenic differentiation medium for 18 days and examined for total number of colonies (total CFU-f) by methylene blue staining (a) and for mineralized colonies (CFU-fob) by alizarin red S staining (b). Quantification by image analysis (c) as described in Experimental Procedures. Values are the mean ± SE of triplicate determinations from 3 replicate experiments. *, P<0.05 and *** P<0.001 versus wild-type controls.

**FIG. 9 Acid phosphatase staining.** Representative micrographs of sections stained histochemically for tartrate-resistant acid phosphatase activity (TRAP) of the tibial metaphysis of wild-type (WT), 1α(OH)ase−/−, VDR−/−, and 1α(OH)ase−/−VDR−/− littermates fed (a) a high calcium diet, (b) a rescue diet, or (c) a high calcium diet with 1,25(OH)2D3 injections. The bar=200 µm. The number of TRAP positive osteoclasts per field of tissue and the average size of TRAP-positive osteoclasts are shown in the left and right panels respectively of section d. Each value is the mean ± SE of determinations in 6 animals of the same genotype. **, P<0.01 and *** P<0.001 relative to wild-type mice on the same diet.

**Fig. 10 Expression of RANKL.** RT-PCR (a) was performed on bone extracts for expression of mRNA encoding RANKL as described in Experimental Procedures. GAPDH was employed as a loading control. Lane 1: WT; Lane 2: 1α(OH)ase−/−; Lane 3: VDR−/−; Lane 4: 1α(OH)ase−/−VDR−/−.
Representative micrographs (b, c and d) of sections stained by immunohistochemistry for RANKL of tibial metaphysis of wild-type (WT), 1α(OH)ase−/−, VDR−/−, and 1α(OH)ase−/−VDR−/− littermates fed a high calcium diet (b), a rescue diet (c), or a high calcium diet with 1,25(OH)2D3 injections (d). The RANKL positive tissue area and RANKL staining intensity (summary total gray) are shown in panels e and f, respectively. Each value is the mean ± SE of determinations in 6 animals of the same genotype. *, P<0.05 and *** P<0.001 relative to wild-type mice on the same diet.
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Inactivation of the 25-hydroxyvitamin D-1alpha-hydroxylase and vitamin D receptor demonstrates independent and interdependent effects of calcium and vitamin D on skeletal and mineral homeostasis

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