Comparison of Active Vitamin D Compounds and a Calcimimetic in Mineral Homeostasis

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ABSTRACT

The differential effects between cinacalcet and active vitamin D compounds on parathyroid function, mineral metabolism, and skeletal function are incompletely understood. Here, we studied cinacalcet and active vitamin D compounds in mice expressing the null mutation for Cyp27b1, which encodes 25-hydroxyvitamin D-1α-hydroxylase, thereby lacking endogenous 1,25-dihydroxyvitamin D3 [1,25(OH)2D3]. Vehicle-treated mice given high dietary calcium had hypocalcemia, hypophosphatemia, and marked secondary hyperparathyroidism. Doxercalciferol and 1,25(OH)2D3 each normalized these parameters and corrected both the abnormal growth plate architecture and the diminished longitudinal bone growth observed in these mice. In contrast, cinacalcet suppressed serum parathyroid hormone (PTH) cyclically and did not correct the skeletal abnormalities and hypocalcemia persisted. Vehicle-treated mice given a “rescue diet” (high calcium and phosphorus, 20% lactose) had normal serum calcium and PTH levels; cinacalcet induced transient hypocalcemia and mild hypercalciuria. The active vitamin D compounds and cinacalcet normalized the increased osteoblast activity observed in mice with secondary hyperparathyroidism; cinacalcet, however, increased the number and activity of osteoclasts. In conclusion, cinacalcet reduces PTH in a cyclical manner, does not eliminate hypocalcemia, and does not correct abnormalities of the growth plate. Doxercalciferol and 1,25(OH)2D3 reduce PTH in a sustained manner, normalize serum calcium, and improve skeletal abnormalities.


Vitamin D and extracellular calcium ([Ca2+]e) play central roles in regulating systemic calcium homeostasis. [Ca2+]e activates a cation-sensing G-protein coupled receptor, the calcium-sensing receptor (CaSR), to modulate the concentrations of circulating parathyroid hormone (PTH) and to regulate renal calcium re-absorption.1 The activated form of vitamin D, 1,25-dihydroxyvitamin D [1,25(OH)2D] is synthesized via the action of the enzyme 25-hydroxyvitamin D-1α-hydroxylase [1α(OH)ase], which converts 25-hydroxyvitamin D to 1,25(OH)2D. This activated form of vitamin D acts predominantly on a nuclear vitamin D receptor (VDR) to modulate parathyroid function, mineral metabolism, and skeletal function.2 1,25(OH)2D synthesis is impaired in chronic kidney disease (CKD), at least in part because of suppression of the renal 1α(OH)ase by elevated serum phosphorus and/or serum fibroblast growth factor 23 (FGF-23), and in part because of loss of renal parenchyma. Consequently, low levels of 1,25(OH)2D develop in stage 3 CKD, leading to reduced calcium absorption.3 The resultant hypocalcemia and low 1,25(OH)2D can lead to the development of secondary hyperparathyroidism, mainly because of the fact that extracellular ionized calcium [Ca2+]e and 1,25(OH)2D regulate PTH biosyn-
thesis, with 1,25(OH)₂D predominantly suppressing PTH gene transcription⁴,⁵ and [Ca²⁺]₈, predominantly downregulating PTH mRNA translation.⁶,⁷ Both calcium⁸ and activated vitamin D⁹,¹⁰ coordinately regulate the growth of the parathyroid gland. In addition, increased [Ca²⁺]₈ by activating the CaSR, potently suppresses PTH secretion.¹¹ The ensuing secondary hyperparathyroidism, with disordered skeletal remodeling and mineralization defects, can contribute to the manifestations of renal osteodystrophy.

Active vitamin D compounds including 1,25(OH)₂D₃ (calcitriol)¹¹ and doxercalciferol [1αOHD₃, Hec-torol],¹² a vitamin D₃ analog that undergoes metabolic activation in vivo to form 1,25(OH)₂D₃, are used clinically to manage elevated PTH in CKD. The calcimimetic cinacalcet, an allosteric modulator of the CaSR, requires the presence of calcium ions to be effective¹³ and is used clinically to manage secondary hyperparathyroidism.¹⁴ Cinacalcet has also been approved for use in primary hyperparathyroidism due to parathyroid carcinoma.¹⁵ Recently, calcimimetics have been proposed as a potential adjunctive treatment for familial hypophosphatemic rickets, which is complicated by secondary hyperparathyroidism.¹⁶ However, the skeletal impact of calcimimetics is unclear. We therefore compared active vitamin D compounds and the calcimimetic, cinacalcet, in mice with targeted inactivation of Cyp27b1, the gene encoding the 1α(OH)ase enzyme, that lack the capacity to synthesize endogenous 1,25(OH)₂D. We exposed these mice [1α(OH)ase null mice] to two different environments: a high-calcium intake on which 1 to two different environments: a high-cal-

RESULTS

Serum Biochemistry and Parathyroid Gland Histology

Although vehicle-treated 1α(OH)ase null mice on the high-calcium intake were hypocalcemic and hypophosphatemic and had markedly increased serum PTH (Figure 1), doxercalciferol normalized serum calcium and serum phosphorus. 1,25(OH)₂D₃ similarly increased serum calcium (Figure 1A) but increased serum phosphorus more markedly than doxercalciferol (Figure 1C). Doxercalciferol and 1,25(OH)₂D₃ each dramatically reduced circulating concentrations of PTH (Figure 1D). With cinacalcet treatment, serum calcium remained low at 2 and 24 hours after dose administration (Figure 1A), and cyclic PTH fluctuations were observed (Figure 1D): that is, PTH levels decreased at 2 hours...
after dose administration, but rebounded by 24 hours, although not to vehicle-treated levels (Figure 1D). 1,25(OH)₂D₃ and doxercalciferol both decreased the size of the parathyroid glands below that seen in the vehicle-treated 1α(OH)ase null mice on a high-calcium diet (Figure 1E); parathyroid glands remained enlarged however in the cinacalcet-treated mice (Figure 1, D and E).

On the rescue diet, serum calcium, phosphorus, and PTH were normalized in null mice (Figure 1, A, C, and D, respectively). Cinacalcet treatment significantly reduced serum calcium after 2 hours [although not to the levels seen in the vehicle-treated 1α(OH)ase null mice on the high-calcium intake], but not after 24 hours (Figure 1A). Cinacalcet also increased 24-hour urine calcium (Figure 1B). Serum phosphorus levels were higher than those in the vehicle-treated controls (Figure 1C) 2 hours after cinacalcet administration but were not significantly elevated by 24 hours. Serum PTH levels fell at 2 hours, but were not significantly different from vehicle-treated levels after 24 hours (Figure 1D). Parathyroid glands also remained slightly increased in the cinacalcet-treated mice on a rescue diet (Figure 1E).

Mineralization of Bone and Cartilage
On the high-calcium intake, vehicle-treated 1α(OH)ase null mice had widened and distorted growth plates which were poorly mineralized (Figure 2A). Excess osteoid was also seen in the bone of the metaphysis (Figure 2B). Both 1,25(OH)₂D₃ and doxercalciferol normalized the size, structure, and mineralization of the growth plate, and reduced osteoid was seen (Figure 2). In contrast, widened and poorly mineralized growth plates were seen after cinacalcet treatment along with abundant osteoid lining trabeculae (Figure 2). On the rescue diet, the width of the growth plate in the 1α(OH)ase null mice was largely restored in vehicle-treated mice and mineralization of bone was normalized (Figure 2). After cinacalcet treatment, the growth plate remained widened; however, mineralization of bone markedly improved (Figure 2).

Compared with the double labeling seen in wild-type mice, altered deposition of calcein, a fluorochrome that binds newly calcified matrix, was seen in the bone of vehicle-treated 1α(OH)ase null mice on a high-calcium intake compared with the double labeling seen in wild-type mice (Figure 3A). Calcein labeling was similarly but somewhat less markedly abnormal in cinacalcet-treated null animals (Figure 3A). In contrast, discrete double labels were easily evident in trabecular bone of 1,25(OH)₂D₃ and doxercalciferol-treated 1α(OH)ase null mice, similar to that observed in wild-type mice (Figure 3A), and the mineral apposition rate (MAR) (Figure 3C) and bone formation rate (BFR/BS) (Figure 3C) in these groups was not significantly different from those of wild-type mice (Figure 3B). In null mice on a rescue diet, the MAR was markedly improved in the vehicle-treated and cinacalcet-treated mice (Figure 3B) and the BFR/BS was not significantly different between the vehicle-treated and cinacalcet-treated mice (Figure 3C).

Assessment of Bone Turnover
Osteoblast surfaces of bone and serum osteocalcin levels were increased in vehicle-treated null mice on a high-calcium intake, compared with those in wild-type mice (Figure 4, A and B). Osteoblast surfaces and osteoblast activity as indicated by serum osteocalcin were reduced to normal in all treated animals after secondary hyperparathyroidism was reduced or eliminated (Figure 4, A and B). In null mice on a rescue diet, elimination of the secondary hyperparathyroidism also resulted in a normalized osteoblast surface, and osteocalcin levels were not different from those in wild-type mice (Figure 4, A and B). In 1α(OH)ase null mice on a high-calcium intake, osteoclast numbers were increased after cinacalcet treatment relative to wild-type mice and to the vehicle-treated control. Serum TRAP5b levels generally paralleled these changes (Figure 4, C and D). On a rescue diet, cinacalcet treatment also increased osteoclast numbers significantly compared with vehicle-treated 1α(OH)ase null mice, and compared with wild-type mice. TRAP5b levels were increased in parallel with the increases in osteoclasts in the calcified matrix null animals on a rescue diet.
Growth and Mineral Density of Long Bones

On a rescue diet, radiolucrency (Figure 5A) and length of bones improved in the vehicle-treated null mice but the femurs remained slightly shorter than the femurs of wild-type mice (Figure 5B). Cinacalcet treatment resulted in more radiolucent and shorter bones (Figure 5, A and B). Bones of cinacalcet-treated 1α(OH)ase null mice on a rescue diet also displayed lower BMD than the vehicle-treated mice and the wild-type mice (Figure 5C), but this appeared to reflect primarily increased bone resorption.

MicroCT images confirmed the reduced mineralization of secondary ossification centers in the epiphyses and the reduction of mineralized trabeculae in the metaphyses of femurs of vehicle-treated 1α(OH)ase null mice on a high-calcium diet (Figure 6). Reduced mineralization of the epiphyses was also seen with cinacalcet but a modest increase in mineralized trabeculae was observed (Figure 6). In contrast, both 1,25(OH)2D3 and doxercalci
erol produced marked improvement in mineralization in the epiphyses and an increase in mineralized trabeculae (Figure 6).

In the vehicle-treated and cinacalcet-treated 1α(OH)ase−/− mice on a rescue diet, mineralization of the epiphyses also improved compared with the vehicle-treated 1α(OH)ase−/− mice on a high-calcium intake, but there were reduced trabeculae in cinacalcet-treated animals on a rescue diet, reflecting the increased bone resorption (Figure 6).

DISCUSSION

In the 1α(OH)ase null mice on a high-calcium intake, both 1,25(OH)2D3 and doxercalci
erol produced marked improvement in circulating PTH levels and parathyroid gland size. Both compounds increased serum phosphorus levels on this normal phosphorus, 0% lactose diet, but at the dose used only 1,25(OH)2D3 induced hyperphosphatemia. Whether this observation represents a systematic difference between the two active vitamin D compounds is unclear and whether there are differential effects on phosphate absorption and/or differential potency of these two sterols in their capacity to increase the phosphaturic hormone PFG-23 will require further study.

The effects of 1,25(OH)2D3 and of doxercalci
erol on the
Active vitamin D compounds and cinacalcet normalize osteoblast activity but cinacalcet, and not active vitamin D compounds, increases osteoclasts. Bone turnover is shown: in wild-type (WT) mice; in 1α(OH)ase null mice [1α(OH)ase −/−] on a high-calcium intake (h) treated with vehicle, 1,25(OH)2D3, doxercalciferol, and cinacalcet; in 1α(OH)ase null mice on a rescue diet (r) treated with vehicle, and cinacalcet. (A) Osteoblast surface relative to bone perimeter (N.Oc/bone perimeter (mm)); (B) serum osteocalcin concentrations; (C) osteoclast numbers relative to bone surface (OB.S/BS); (D) serum concentrations of TRAP5b. Each value is the mean ± SEM. Number of replicates for OB.S/BS from left to right are as follows: 4 for WT, 5 for vehicle, 5 for 1,25(OH)2D3, 5 for doxercalciferol, 5 for cinacalcet, 7 for vehicle, and 6 for cinacalcet. Number of replicates for osteocalcin from left to right are as follows: 5 for WT, 3 for vehicle, 4 for 1,25(OH)2D3, 5 for doxercalciferol, 3 for cinacalcet, 6 for vehicle, and 5 for cinacalcet. Number of replicates for N.Oc/bone perimeter from left to right are as follows: 3 for WT, 4 for vehicle, 7 for 1,25(OH)2D3, 5 for doxercalciferol, 5 for cinacalcet, 8 for vehicle, and 12 for cinacalcet. Number of replicates for serum TRAP5b from left to right are as follows: 4 for WT, 3 for vehicle, 3 for 1,25(OH)2D3, 3 for doxercalciferol, 3 for cinacalcet, 3 for vehicle, and 4 for cinacalcet. *P < 0.05 relative to WT mice; **P < 0.01 relative to WT mice; ***P < 0.001 relative to WT mice; *P < 0.05 relative to vehicle-treated 1α(OH)ase null mice on a high-calcium intake; **P < 0.01 relative to vehicle-treated 1α(OH)ase null mice on a high-calcium intake; ***P < 0.001 relative to vehicle-treated 1α(OH)ase null mice on a high-calcium intake; ∼P < 0.05 relative to vehicle-treated 1α(OH)ase null mice on a rescue diet. Osteoblast numbers and activity were increased in bone of vehicle-treated null animals on a rescue diet and serum FGF-23 levels in response to cinacalcet to determine if this hormone may be modified directly by the calcimimetic of a mild hypoparathyroid state over a portion of the 24-hour period. In recent studies we have reported an inverse relationship between bone formation and serum FGF-23 levels in the parathyroid glands likely stemmed from both direct inhibitory actions of these compounds on parathyroid growth and PTH production, as well as from indirect effects due to increased serum calcium activating the CaSR. In contrast, although cinacalcet significantly reduced circulating PTH concentrations, the results were biphasic and less pronounced than the reductions produced by 1,25(OH)2D3 or doxercalciferol. The lesser effectiveness of cinacalcet in reducing PTH in this model likely reflects both the potency and the pharmacokinetics of cinacalcet as well as the absence of any direct inhibitory action of active vitamin D. A recent communication of the reduced effectiveness of cinacalcet relative to active vitamin D in other rodent models supports the current observations. Calcimimetics have been reported to reduce parathyroid hyperplasia, but parathyroid gland size was not significantly decreased by cinacalcet in the 1α(OH)ase null mice on a high-calcium intake, in contrast to the effects produced by the active vitamin D compounds. Furthermore, the significant hypocalcemia observed in the vehicle-treated 1α(OH)ase null mice was not improved by the use of cinacalcet, suggesting that the CaSR, although expressed in the gastrointestinal tract, is not capable of substituting for vitamin D-mediated intestinal calcium transport.

When 1α(OH)ase null mice were maintained on a rescue diet, animals became normocalcemic and normophosphatemic and had normal PTH levels. The normalization of these blood analytes likely reflects the capacity of the 20% lactose in the rescue diet to facilitate vitamin D–independent calcium and phosphorus absorption in the gut. Treatment of these animals with cinacalcet induced modest hypocalcemia and hypercalcemia, reflecting the reduced renal calcium re-absorption consequent to stimulation of the renal CaSR. Although serum PTH was decreased at 2 hours, at 24 hours PTH was not significantly different than vehicle-treated levels. The elevated serum phosphorus in these animals at 2 hours but not at 24 hours likely reflects the induction by the calcimimetic of a mild hypoparathyroid state over a portion of the 24-hour period. In recent studies we have reported an inverse relationship between bone formation and serum FGF-23 levels in the 1α(OH)ase null mice. It will be of importance in future studies to measure FGF-23 levels in response to cinacalcet to determine if this hormone may be modified directly by the calcimimetic.

Osteoblast numbers and activity were increased in bone of vehicle-treated null animals on a high-calcium intake, consistent with the skeletal anabolic effect of the high circulating concentrations of PTH. Osteoblast activity was reduced by 1,25(OH)2D3 and doxercalciferol as PTH fell, and this likely reflected reduction of excess osteoblast stimulation by the su-
mice on a high-calcium intake. CaSR has been reported to function
versely hyperparathyroid vehicle-treated mutant mice on a
bers and activity at a level significantly lower than in the se-

Figure 5. Active vitamin D compounds, but not cinacalcet, normalize femoral length and BMD. Representative contact radiographs (A), quantitation of femur lengths (B), and BMD of femurs (C) are shown: from wild-type (WT) mice; from \(1\alpha(OH)ase\) null mice [\(1\alpha(OH)ase^{-/-}\)] on a high-calcium intake \((h)\) treated with vehicle, \(1,25(OH)_2D_3\), doxercalciferol, and cinacalcet; from \(1\alpha(OH)ase\) null mice on a rescue diet \((r)\) treated with vehicle, and cinacalcet. Each femur length value is the mean ± SEM. Number of replicates for femur length from left to right are as follows: 6 for WT, 5 for vehicle, 5 for \(1,25(OH)_2D_3\), 7 for doxercalciferol, 6 for cinacalcet, 5 for vehicle, and 5 for cinacalcet. Each BMD value is the mean ± SEM. Number of replicates for BMD from left to right are as follows: 4 for WT, 8 for vehicle, 5 for \(1,25(OH)_2D_3\), 7 for doxercalciferol, 6 for cinacalcet, 6 for vehicle, and 6 for cinacalcet. *\(P < 0.05\) relative to WT mice; **\(P < 0.01\) relative to WT mice; ***\(P < 0.001\) relative to WT mice; **\(P < 0.01\) relative to vehicle-treated \(1\alpha(OH)ase\) null mice on a high-calcium intake; ***\(P < 0.001\) relative to vehicle-treated \(1\alpha(OH)ase\) null mice on a rescue diet; \({}^{10}P < 0.05\) relative to vehicle-treated \(1\alpha(OH)ase\) null mice on a rescue diet.

praphysiologic serum PTH levels. Although cinacalcet cyclically
inhibited PTH in this model, the transient peaks in circulating
PTH produced by cinacalcet were insufficient to maintain osteo-
blast numbers at the elevated levels seen in vehicle-treated null
animals on a high-calcium intake. Furthermore, reductions in
osteoblast numbers were greater with cinacalcet than with the
vitamin D sterols, possibly because of intrinsic bone anabolic
activity of active forms of vitamin D.\(^{18,19,25}\) On a rescue diet, the

A previous study described the effects of therapy with calcii-
mimetics on bone histology in patients treated with hemodial-
ysis\(^{35}\) and noted that a few patients developed adynamic bone,
although the sample size was small and the patients all received
concurrent vitamin D and/or phosphate binder therapy. Overall,
our studies in the current model are not consistent with suppres-
sion of bone turnover by cinacalcet.

We and others have previously noted that in models ex-
pressing the null mutation of either the 1α(OH)ase gene\textsuperscript{18,36} or the VDR gene,\textsuperscript{37} osteoclast activity is either only modestly increased or not increased despite markedly elevated circulating PTH. This observation has been interpreted as evidence that active vitamin D and the VDR are required for optimal PTH stimulated bone resorption, and active vitamin D forms may in fact be more potent osteoclast activators than PTH. The present studies suggest that the mechanism for this effect of vitamin D may lie, in part, in correcting the hypocalemia and thereby enhancing the stimulation of osteoclastic bone resorption via CaSR.

Calcium, phosphorus and 1,25(OH)\textsubscript{2}D have all been implicated in the normal development and mineralization of the growth plate. However, the role of the calcium receptor in growth plate development remains unclear. In the present study, treatment of 1α(OH)ase null mice on a high-calcium intake with active vitamin D compounds improved both the architecture and mineralization of the growth plate and normalized long bone growth. However, our studies in 1α(OH)ase null mice failed to show any positive effect of cinacalcet alone on growth plate architecture, or longitudinal bone growth. There are several potential explanations for this observation. It is possible that calcimimetic-induced modulation of calcium signaling at the CaSR differs in the chondrocyte, as compared with the parathyroid and osseous cells, or that calcium functions independently of the CaSR in the growth plate. Finally, in view of the fact that the growth of vehicle-treated normocalcemic and normophosphatemic mutant mice on the rescue diet was not completely normalized, direct vitamin D action on the growth plate may be required and this requirement was not met in the 1α(OH)ase null mice treated with cinacalcet alone.

In this study, treatment of null mice on a high-calcium intake with doxercalciferol or 1,25(OH)\textsubscript{2}D\textsubscript{3} not only reversed rickets but also, by normalizing ambient calcium and phosphorus,\textsuperscript{18} repaired osseous mineralization. In contrast, cinacalcet only modestly reversed osteomalacia in null mice on a high-calcium intake. Recently, it was reported that mice with osteoblast-specific deletion of CaSR exhibited severely under-mineralized skeletons,\textsuperscript{38} suggesting an important role for the CaSR in osteoblast-mediated bone mineralization, yet in our studies cinacalcet only partially improved bone mineralization. The persistent hypocalemia occurring with cinacalcet treatment may therefore have limited matrix mineralization despite any putative activation of the osseous CaSR. On a rescue diet, mineralization was normalized with cinacalcet treatment despite the fact that transient mild hypocalemia was present. The marked improvement in serum calcium in the null mice on the rescue diet even after cinacalcet treatment, coupled with the transient increase in serum phosphorus in null mice on the rescue diet after cinacalcet treatment, may therefore have improved osseous mineralization.

In summary, our studies show that when 1α(OH)ase null mice are exposed to an environment in which they exhibit persistent hypocalemia, hypophosphatemia, secondary hyperparathyroidism, rickets, and osteomalacia, doxercalciferol or 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment can normalize serum calcium and PTH, parathyroid gland size, and bone parameters, although 1,25(OH)\textsubscript{2}D\textsubscript{3} may cause significant hyperphosphatemia. In contrast, cinacalcet treatment can improve hyperparathyroidism in this model, but does not normalize the accompanying mineral and skeletal abnormalities. When 1α(OH)ase null mice are exposed to an environment in which they become normocalcemic and normophosphatemic, cinacalcet treatment may perturb mineral homeostasis by causing transient hypocalemia and hyperphosphatemia. Furthermore, our data suggest that cinacalcet may exert direct effects on the skeletal calcium receptor that may independently increase bone resorption and modify skeletal homeostasis. Therefore, in the setting of reduced active vitamin D, calcimimetics do not appear optimal for sole treatment of secondary hyperparathyroidism and skeletal abnormalities, especially in the presence of rickets and osteomalacia.

**CONCISE METHODS**

**Mice and Materials**

The derivation of the 1α(OH)ase homozygous null mice [1α(OH)ase\textsuperscript{−/−}], by homologous recombination in embryonic stem cells, was previously described by Panda et al.\textsuperscript{17} Briefly, a neomycin resistance gene replaced exons VI, VII, and VIII of the mouse 1α(OH)ase gene, re-
moving both the ligand-binding and the heme-binding domains. RT-PCR of renal RNA from homozygous 1α(OH)ase−/− mice confirmed the lack of 1α(OH)ase expression. Mice were generated through breeding of heterozygous mice on a C57Bl/6j genetic background and identified by Southern blots and/or PCR with tail genomic DNA as the template as described previously. Wild-type littermates were used as controls in all of the experiments. 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 per gram of vitamin D3 (Ralston Purina Co., St. Louis, MO). At approximately 21 days of age, the animals were weaned and maintained on drinking water containing 1.5% calcium gluconate and either a normal diet of autoclaved chow containing 1% calcium, 0.85% phosphorus, 0% lactose, and 2.2 units per gram of vitamin D3 (Ralston Purina Co.) or a rescue diet of γ-irradiated chow containing 20% lactose, 2% calcium, 1.25% phosphorus, and 2.2 units per gram of vitamin D2 (TD96348; Harlan Teklad, Madison, WI). No significant differences in any parameter determined were observed in wild-type mice on a high-calcium intake or a rescue diet. Consequently, control data are shown for the wild-type mice on the high-calcium intake. Cinacalcet (Amgen, Thousand Oaks, CA) was obtained via the local pharmacy. Doxercalciferol and 1,25(OH)2D3 were provided by Genzyme Corporation, Middleton, WI.

In Vivo Experiments All animal experiments were carried out in compliance with and approval by the Institutional Animal Care and Use Committee. Mice were maintained in a virus- and parasite-free barrier facility and exposed to a 12-hour/12-hour light/dark cycle. From weaning, animals received, every 2 days by subcutaneous injection, either of the following: vehicle (50% propylene glycol, 40% saline, 10% ethanol); doxercalciferol, 150 pg/g; 1,25(OH)2D3, 50 pg/g; or cinacalcet, 30 μg/g daily by oral gavage. The doses of doxercalciferol and 1,25(OH)2D3 used were based on preliminary studies in the null mice on a high-calcium intake; the doses selected for each compound were those that reduced serum PTH to the normal range without causing hypercalcemia. The dose of cinacalcet used was based on previous studies in mice and rats and on preliminary studies in the null mice on a high-calcium intake in which doses of 10, 20, 30, and 50 μg/g were employed; no further PTH suppression was seen at 50 μg/g than at 30 μg/g, so the latter dose was selected. At about 3 and one-half months of age, the animals were killed, and blood and urine samples were collected for analysis. Serum was obtained 2 and 24 hours after the final cinacalcet administration and 24 hours after the final doxercalciferol and 1,25(OH)2D3 injections. Tibias, femurs, and thyroparathyroidal tissue were removed and fixed in 10% buffered formal saline, 0.37 M sodium periodate overnight at 4°C and processed histologically as described previously. The bones were decalcified in EDTA glycerol solution for 14 days at 4°C. Decalcified bones and thyroparathyroidal tissue 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 TRAP activity and alkaline phosphatase (ALP) activity as described below. Alternatively, undecalcified bones were embedded in LR White acrylic resin (London Resin Co., London), 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. Histomorphometric indices were determined as suggested by the ASBMR Histomorphometry Nomenclature Committee.

Histochemical Staining for TRAP Enzyme histochemistry for TRAP staining was performed as described previously. De-waxed sections were preincubated 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-Aldrich, St. Louis) in dimethylformamide as substrate and 0.5 mg/ml fast garnet GBC (Sigma-Aldrich) as a color indicator for the reaction product. After the product was washed with distilled water, the sections were counterstained with methyl green and mounted in Kaiser’s glycerol. ALP staining was performed on methyl methacrylate (MMA)-embedded undecalcified bone samples using a vector red alkaline phosphatase substrate kit (Vector Laboratories).

Double Calcein Labeling Double calcein labeling was performed by intraperitoneal injection of mice with 10 μg of calcein per gram of body weight (C-0875; Sigma-Aldrich) at 10 and 3 days before the mice were killed. Bones were harvested and embedded in LR White acrylic resin as described above. Serial sections were cut, and the freshly cut surface of each section was viewed and imaged using fluorescence microscopy. The double calcein interlabel width was measured using Northern Eclipse image analysis software version 6.0 (Empix Imaging Inc., Mississauga, ON, Canada), and the mineral apposition rate (MAR; MAR = interlabel width/labeling period) was calculated. The bone formation rate was calculated according to the following formula: BFR = MAR X (MS/BS), where MS is the mineralizing surface and BS is the bone surface. Calculations were made on a minimum of duplicate specimens from replicate mice of each group.

Histomorphometry After H&E staining or immunohistochemical staining, histomorphometric measurements were performed as described previously in the measurement of serum osteocalcin levels according to the manufacturer’s specifications. A mouse TRAP5b assay (IDS, Fountain Hills, AZ) was used according to the manufacturer’s specifications to determine osteoclast-derived TRAP5b in mouse serum samples.
secondary spongiosa in the metaphyseal area (0.5 mm below the growth plate) at the distal end of femur. The parameters obtained for bone formation were the osteoblast surface per bone surface (Ob.S/BS; percentage), and the MAR (micrometers per day) and the BFR/BS(μm²/μm² per day). The parameter measured for bone resorption was the number of osteoclasts per bone perimeter [N.Oc./bone perimeter (mm)]. After H&E staining or histochemical staining of sections from replicate mice of each group, images of fields were photographed with a digital camera. Images of micrographs from single sections were digitally recorded using a rectangular template, and recordings were processed and analyzed using Northern Eclipse image analysis software as described.44–46

BMD Analysis
Densitometry was performed by PIXImus on the right femur under anesthesia as described previously.46 In brief, mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (0.1 mg/kg) in PBS and placed prone on the platform of a PIXImus densitometer (software version 1.46.007; Lunar Corp., Madison, WI) for BMD measurements according to the manufacturer’s instruction. In some experiments, the variability in measurements was examined by repeating scans after repositioning the animals. Percent coefficient of variation of BMD for the repeated scans was 1 to 3%.

Skeletal Radiography
After removal of soft tissue, radiographs were taken using a Faxitron model 805 radiographic inspection system (Faxitron Contact, Faxitron, 22-kV voltage, and 4-minute exposure time). X-Omat TL film (Eastman Kodak, Rochester, NY) was used and processed routinely.

Microcomputed Tomography
Femurs obtained from mice were dissected free of soft tissue, fixed overnight in 70% ethanol, and analyzed by microcomputed tomography with a SkyScan 1072 scanner and associated analysis software (SkyScan, Antwerp, Belgium) as described.46 Scans were performed on the distal part of the femur. Image acquisition was performed at 100 kV and 98 μA with a 0.9° rotation between frames. Thresholding was applied to the images to segment the bone from the background. Three-dimensional renderings of the distal 3.5 mm of the femora was reconstructed using two-dimensional data from scanned slices with the 3D Creator software supplied with the instrument. The trabecular bone region of interest was drawn to include all cancellous bone in the 3D Creator software supplied with the instrument. The trabecular bone region of interest was drawn to include all cancellous bone in the 3D Creator software supplied with the instrument. The trabecular bone region of interest was drawn to include all cancellous bone in the 3D Creator software supplied with the instrument. The trabecular bone region of interest was drawn to include all cancellous bone in the 3D Creator software supplied with the instrument.

Computer-Assisted Image Analysis
After H&E staining or histochemical staining of sections from replicate mice of each group, 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 and analyzed using Northern Eclipse image analysis software.46 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.

Statistical Analysis
Statistical comparisons, using Graph-Pad Prism (GraphPad Software Inc., San Diego) analysis software were made using t test or ANOVA, followed by a Bonferroni adjustment or a Neuman-Keuls multiple comparison test. P < 0.05 was considered significant.

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DISCLOSURES
D.G. has served as a consultant for Amgen, Genzyme, Eli Lilly, Merck, Novartis, Proctor and Gamble/Aventis, and Servier. S.S. is an employee of Genzyme.

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