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Authors

Pereira, Renata C

Salusky, Isidro B

Bowen, Richard E

et al.

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Vitamin D sterols increase FGF23 expression by stimulating osteoblast and osteocyte maturation in CKD bone

Renata C. Pereira, PhD¹, Isidro B. Salusky, MD¹, Richard E. Bowen, MD², Earl G. Freymiller, DDS³, Katherine Wesseling-Perry, MD¹

¹Department of Pediatrics, David Geffen School of Medicine at UCLA

²Department of Orthopedic Surgery, David Geffen School of Medicine at UCLA

³UCLA School of Dentistry

Abstract

Impaired osteoblast and osteocyte maturation contribute to mineralization defects and excess FGF23 expression in CKD bone. Vitamin D sterols decrease osteoid accumulation and increase FGF23 expression; these agents also increase osteoblast maturation *in vitro* but a link between changes in bone cell maturation, bone mineralization, and FGF23 expression in response to vitamin D sterols has not been established. We evaluated unmineralized osteoid accumulation, osteocyte maturity markers (FGF23:early osteocytes; sclerostin: late osteocytes), and osteocyte apoptosis in iliac crest of 11 pediatric dialysis patients before and after 8 months of doxercalciferol therapy. We then evaluated the effect of 1,25(OH)₂vitamin D on *in vitro* maturation and mineralization of primary osteoblasts from dialysis patients. Unmineralized osteoid accumulation decreased while numbers of early (FGF23-expressing) increased in response to doxercalciferol. Osteocyte apoptosis was low but increased with doxercalciferol. Bone FGF23 expression correlated with numbers of early, FGF23-expressing, osteocytes ($r=0.83$, $p<0.001$). *In vitro*, 1,25(OH)₂vitamin D increased expression of the mature osteoblast marker osteocalcin (*BGLAP*) but only very high (100 nM) concentrations affected *in vitro* osteoblast mineralization. High doses (10 and 100 nM) of 1,25(OH)₂vitamin D also increased the ratio of *RANKL/OPG* expression in CKD osteoblasts. Vitamin D sterols directly stimulate osteoblast maturation. They also increase osteocyte turnover and increase osteoblast expression of osteoclast differentiation factors, thus likely modulating osteoblast/osteoclast/osteocyte coupling. By increasing numbers of early osteocytes, vitamin D sterols increase FGF23 expression in CKD bone.

Keywords

osteoblasts; osteocytes; vitamin D; chronic kidney disease

Corresponding Author Katherine Wesseling Perry, A2-383 MDCC, 650 Charles Young Drive, Los Angeles, CA 90095, Phone: 310-206-6987, Fax: 310-825-0442, kwesseling@mednet.ucla.edu.

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INTRODUCTION

Chronic kidney disease (CKD) is associated with changes in mineral ion homeostasis which have implications for both skeletal health and cardiovascular outcomes (1). Secondary hyperparathyroidism causes high turnover bone disease which results in severe bone deformities, bone pain, and fractures (2). Skeletal mineralization defects are also common in children with CKD but the etiology of these defects, which can be identified even in children with normal calcium, phosphorus, and PTH concentrations (3, 4), is incompletely understood. We have recently demonstrated that CKD bone is characterized by impaired osteocyte maturation; primary osteoblasts derived from CKD patients also display a maturation defect *in vitro* (5). This delay associates with defective mineralization of CKD osteoblasts *ex vivo* and may contribute to the skeletal mineralization defects which are widely prevalent in the pediatric CKD population (3, 4).

Active vitamin D sterols are effective at treating secondary hyperparathyroidism; they also directly affect bone cell physiology. 1,25(OH)₂vitamin D stimulates osteoblast maturation *in vitro* (6) and also regulates skeletal hormone expression. Expression of the osteocyte-specific hormone fibroblast growth factor 23 (FGF23), which is markedly increased in CKD bone, increases further in response to 1,25(OH)₂vitamin D (3). We have recently demonstrated that FGF23 is a marker of osteocytes in an early phase of secondary mineralization (7). However, whether 1,25(OH)₂vitamin D increases FGF23 expression by increasing the number of early osteocytes in CKD bone or whether it stimulates per-osteocyte FGF23 expression in CKD bone remains unknown.

In order to investigate the effects of active vitamin D sterols on osteoblast and osteocyte maturation in CKD patients, we evaluated osteoid accumulation, osteocyte maturation markers, and osteocyte apoptosis in bone biopsy specimens from CKD patients before and after 8 months of therapy with the active vitamin D sterol doxercalciferol. We then used primary human osteoblasts from pediatric dialysis patients, which, as we have previously demonstrated (5), maintain intrinsic impairments in maturation and mineralization when removed from the uremic milieu, to evaluate the direct effects of 1,25(OH)₂vitamin D on CKD-mediated osteoblast maturation and mineralization impairments *in vitro*.

MATERIALS AND METHODS

Study Subjects

Full thickness iliac crest bone biopsy samples were obtained after double tetracycline labeling from eleven pediatric dialysis patients (7 male and 4 female/ 9 Hispanic, 1 white, and 1 black). The average age of the patients from whom bone biopsies were obtained was 15.8 ± 0.8 years; these patients had been on dialysis for an average of 1.1 ± 0.4 years. At baseline, patients had evidence of secondary hyperparathyroidism; namely, increased bone turnover and/or bone marrow fibrosis. They were treated for 8 months with doxercalciferol, an active vitamin D analogue, and the phosphate binder sevelamer carbonate, after which time they underwent a second double tetracycline-labeled biopsy. Doxercalciferol and sevelamer were titrated during the treatment period according to a protocol which considered serum PTH, calcium, and phosphate concentrations (3). During the course of

therapy, subjects received an average of 19.3 ± 3.8 mcg of doxercalciferol per week. Circulating values of calcium, phosphorus, and alkaline phosphatase values were assessed at the time of bone biopsy using an Olympus AU5400 analyzer (Olympus America Incorporated, Center Valley, PA). PTH concentrations in EDTA plasma were measured by the 1st generation immunometric assay (Quidel, San Diego, California, normal range: 10-65 pg/ml) and FGF23 levels were determined in EDTA plasma by a 2nd generation C-terminal assay (Quidel). Circulating 1,25(OH)₂vitamin D levels were measured at baseline and in the middle (month 3 to 4) of therapy to monitor medication compliance. As previously reported, calcitriol levels increased by 10.9 ± 5.0 pg/ml and doxercalciferol doses correlated with the change in circulating calcitriol concentration ($r=0.55$, $p=0.08$) (8). The study was approved by the UCLA Human Subject Protection Committee and informed consent was obtained from all patients and/or parents.

Characterization of bone histomorphometry, immunohistochemistry and apoptosis

Histomorphometric analysis was performed in un-decalcified bone from all pediatric CKD patients and healthy controls using the Osteometrics^R system and standard measures of bone turnover, mineralization, and volume were measured and calculated (9). The results were previously described (10). A “mineralization defect” was defined by the presence of excess osteoid volume in combination with a prolonged osteoid maturation rate.

Immunohistochemistry was performed to assess bone FGF23 (225-244) (Qidel), DMP1 (LFMb31(62-513) (Santa Cruz Biotechnology), and sclerostin (R&D Systems) expression in un-decalcified sections of iliac crest. Immunoreactivity for these proteins was quantified using the Ariol scanning system and values were previously reported (11). For the current analysis, numbers of FGF23-expressing osteocytes were also counted. These were normalized to trabecular bone area (11).

Apoptosis was also assessed in non-decalcified bone sections via *in situ* TUNEL reaction in iliac crest samples from 5 healthy controls and 11 pediatric dialysis patients before and after doxercalciferol therapy using Klenow terminal deoxynucleotidyl transferase per manufacturer's instructions (Oncogene Research Products). Positive staining for apoptosis was detected by peroxidase streptavidin conjugated and 3,3'-diaminobenzidine. Sections were counter-stained with methyl green to indentify live (green staining) osteocytes. Sections incubated with vehicle alone served as negative controls and a positive control was generated by treating one of the samples with DNase I.

Primary osteoblast maturation and mineralization potential and gene analysis

Primary osteoblasts were obtained at the time of bone biopsy as previously described (5). Osteoblasts from 5 adolescent CKD patients and from 3 healthy adolescent controls were used for these experiments. The baseline mineralization characteristics of the cells from these patients have previously been reported (7). All CKD patients were treated with maintenance dialysis and end stage kidney disease was due to congenital anomalies of the kidneys and urinary tract ($n=2$) and glomerulonephritis ($n=3$). Bone and cells from healthy adolescent controls were obtained at the time of surgery for idiopathic scoliosis or for maxillofacial surgery requiring grafting. Mineralization potential was assessed in primary osteoblasts plated in 12 well plates at equal density (1×10^4 cells per well). Primary

osteoblasts were grown to confluence in the presence of DMEM, 10% fetal bovine serum, and 100 µg/ml ascorbic acid and then stimulated to mineralize by adding 10 mM β-glycero-phosphate and 10^{-8} M dexamethasone and varying (0 nM, 1 nM, 10 nM, and 100 nM) concentrations of 1,25(OH)₂vitamin D. Mineralization was quantified by staining cultures at weekly intervals with 1% (w/v) solution of Alizarin red S (pH 6.4) (Sigma-Aldrich). Dye was extracted from the cell layer and the supernatant was analyzed at 490 nm. Three technical replicates were evaluated at each timepoint and the coefficient of variation between technical replicates was less than 6%. Cell number was estimated by staining one parallel well at each timepoint with 0.05% Crystal Violet; staining was quantified by extracting the dye from the cell layer by incubation in methanol for 30 min and then analyzing the supernatant at 605 nm.

RNA was isolated from parallel cultures and quantitative real-time PCR amplification was performed using QuantiTect® Probe PCR kit (Qiagen, Hilden, Germany). Taqman assays were used to quantify the expression of osteocalcin (*BGLAP*), Runx2 (*RUNX*), alkaline phosphatase (*ALPL*), receptor activator of nuclear factor kappa-B ligand (*RANKL*), and osteoprotegerin (*OPG*) along with the housekeeping gene *GAPDH*. Relative quantification studies of threshold cycle were performed with Sequence Detector software (Applied Biosystems, Foster City, CA). Samples from each individual patient were assayed in triplicate at each time point; the coefficient of variation between these technical replicates was less than 4% for each gene at each time point.

In vitro evaluation of osteocyte apoptosis

The effects of 1,25(OH)₂vitamin D on osteocyte apoptosis were evaluated in immortalized murine osteocytes (MLO-Y4) which were cultured to 80% confluence. After overnight serum deprivation, cells were cultured in either normal (3 mM phosphate) or phosphate enriched (10 mM phosphate) media in the presence or absence of 1,25(OH)₂vitamin D₃ (100 nM). Apoptosis was assessed visually under fluorescent microscopy (510/540 nm excitation filters) by acridine orange/ethidium bromide with trypan blue exclusion staining at 24 hours. Using this technique, live cells appear uniformly green; early apoptotic cells stain green and contain bright green nuclear dots (indicating chromatin condensation and nuclear fragmentation); late apoptotic cells stain orange and also display bright green nuclear dots. Apoptosis activation was also assessed by caspase-3 activity (Biovision, Milpitas, CA) in cell lysates after 24 hours in culture with phosphate (10 nM), 1,25(OH)₂vitamin D₃ (100 nM), or both (as above).

Statistical analysis

Measurements are reported as median (interquartile range) or mean ± standard error. The Mann-Whitney U and the Wilcoxon Signed Rank tests were used to assess between-group and from-baseline differences, respectively. Spearman correlation coefficients were used to assess relationships between variables. All statistical analyses were performed using SAS software (SAS Institute Inc.) and all tests were two-sided. A probability of type I error less than 5% was considered statistically significant and ordinary *p* values are reported.

RESULTS

Doxercalciferol increases FGF23 expression by increasing numbers of early osteocytes in CKD bone

As previously shown, although circulating calcitriol concentrations correlated with prescribed doxercalciferol dose and increased with therapy (8), PTH levels did not change in response to doxercalciferol (Supplemental Table). By contrast, osteoid volume decreased. Marrow fibrosis area also tended to decrease (from 0.23 (0.11, 0.45) % to 0.07 (0.01, 0.21) %; $p=0.06$ from baseline). These findings confirm previous data suggesting that vitamin D sterols directly affect bone, confounding the relationship between circulating biomarkers and bone histology (3, 12).

We have previously demonstrated that FGF23 protein expression, as determined by ArioI scanning of immunohistochemical detection of FGF23 in trabecular bone, increases in response to doxercalciferol treatment (8). In order to evaluate whether these changes in FGF23 expression are due to increased numbers of FGF23-expressing osteocytes, we counted numbers of FGF23-expressing osteocytes in the trabecular bone of dialysis patients before and after 8 months of doxercalciferol therapy. Circulating FGF23 levels increased in response to doxercalciferol, as did numbers of FGF23-expressing osteocytes (Supplemental Table). As shown in Figure 1, numbers of FGF23-expressing osteocytes correlated closely with bone FGF23 protein expression ($r=0.83$, $p<0.001$). In a subset of 6 patients who had pre/post therapy biopsies adequate for RNA extraction, FGF23 mRNA increased by 226 (124, 320)% ($p<0.05$ from baseline) while numbers of FGF23-expressing osteocytes increased by 226 (83, 440)% in this same subset. The relationship between FGF23 RNA message, FGF23 protein, and numbers of FGF23-expressing osteocytes suggests that much of the doxercalciferol-mediated increase in FGF23 expression is due to increased numbers of FGF23-expressing osteocytes in CKD bone. The previously demonstrated co-localization of FGF23 with e11/gp38 (7) suggests that the increase in numbers of FGF23-expressing osteocytes reflects an increase in early osteocyte numbers.

Doxercalciferol stimulates osteocyte turnover *in vivo*

We have previously demonstrated that CKD is characterized by stagnant osteoblast and osteocyte maturation, with nearly undetectable osteocytes apoptosis in CKD bone (7). We have also previously shown an increase in expression of the mature osteocyte marker sclerostin in response to doxercalciferol (8). In order to determine whether this increase in mature osteocytes is associated with an overall increase in osteocyte turnover in response to active vitamin D sterols, we performed TUNEL staining on iliac crest bone biopsies before and after 8 months of doxercalciferol therapy. Methyl green staining demonstrated a predominance of live osteocytes with minimal numbers of empty lacunae. Apoptosis was apparent in cortical bone, trabecular bone and bone marrow of normal controls. Cortical and trabecular osteocyte apoptosis and marrow apoptosis were markedly decreased in CKD bone prior to doxercalciferol therapy. Only 27% of dialysis patients had any apoptotic cortical osteocytes and only one dialysis patient (i.e. 9%) displayed any apoptotic trabecular osteocytes at baseline. After doxercalciferol, 100% of patients had evidence of apoptotic cortical osteocytes ($p<0.05$) and 91% had apoptotic trabecular osteocytes ($p<0.05$) (Figure

2); these values were similar to apoptotic osteocytes observed in healthy adolescent bone. Increased numbers of early osteocytes, increased numbers of mature osteocytes, and restoration of osteocyte apoptosis suggest that doxercalciferol increases new osteocyte recruitment and osteocyte turnover in advanced CKD.

Increased numbers of early osteocytes is associated with decreases in osteoid accumulation in response to doxercalciferol.

We have previously shown that FGF23-expressing osteocytes occupy packets of bone that have completed primary mineralization and have entered an early phase of secondary mineralization (7). We have also previously shown that numbers of FGF23-expressing osteocytes correlate inversely with osteoid accumulation in CKD bone (11). To evaluate whether the increase in numbers of early, FGF23-expressing, osteocytes reflects a shift in the mineralization state of peripheral trabecular bone or whether vitamin D sterols stimulate an increase in numbers of early osteocytes, independent of their effect on bone mineralization, we evaluated the relationship between numbers of FGF23-expressing osteocytes and the presence of a mineralization defect (defined by excess osteoid accumulation in combination with a prolonged osteoid maturation time) in iliac crest before and after doxercalciferol therapy. As previously shown (8), osteoid accumulation decreased in response to doxercalciferol (Supplemental Table); however, as many as 36% of patients had a persistent mineralization defect after doxercalciferol therapy. When all biopsies (both before and after doxercalciferol treatment) were considered together, biopsies with normal mineralization parameters had greater numbers of early, FGF23-expressing, osteocytes than those with mineralization defects ($p < 0.05$ between groups), despite similar bone formation rates between groups (13.1 (12.5, 15.7) versus 12.2 (11.5, 19.8) $\mu\text{m}^3/\mu\text{m}^2/\text{day}$ for patients with normal versus abnormal mineralization, respectively) (Table 1). Moreover, the relationship between FGF23-expressing osteocytes and mineralization defects was consistent regardless of whether the measurements were obtained before or after doxercalciferol therapy (Figure 3), suggesting that active vitamin D sterols do not alter the relationship between numbers of FGF23-expressing osteocytes and skeletal mineralization parameters. Rather, increased numbers of FGF23-expressing osteocytes appear to co-occur with increasing amounts of peripheral trabecular bone undergoing secondary mineralization (7).

1,25(OH)₂vitamin D increases the maturation and mineralization of primary CKD osteoblasts *in vitro*

Primary CKD osteoblasts removed from the uremic milieu are highly proliferative, mineralize slowly, and have increased expression of the early osteoblast markers (5). We recently demonstrated that this phenotype is consistent with an impairment in osteoblast maturation due to CKD that persists *ex vivo* (5). We also demonstrated that impaired osteocyte maturation contributes to increased numbers of early, FGF23-expressing osteocytes in CKD bone. Since doxercalciferol decreases osteoid accumulation but increases numbers of early, FGF23-expressing osteocytes in CKD bone, we postulated that active vitamin D sterols might improve, although not completely rescue, the underlying CKD-mediated maturation defect observed in osteoblast lineage cells. We thus evaluated the effects of 1,25(OH)₂vitamin D on CKD osteoblast maturation *in vitro*. As shown in Figure

4a, mineralization, as assessed by alizarin red S staining, occurred more rapidly and completely in healthy control cells than in CKD cells. No dose of 1,25(OH)₂vitamin D altered the mineralization of control cells and high doses (100 nM) decreased cell viability in control cultures (Figure 4b). By contrast, cell numbers were persistently higher and mineralization rates were lower in CKD osteoblast cultures. Although no dose of 1,25(OH)₂vitamin D normalized CKD osteoblast mineralization, the highest concentrations of 1,25(OH)₂vitamin D (100 nM) did decrease excessive cell numbers (Figure 4c) and improve mineralization in CKD cultures. RNA expression analysis revealed that 1,25(OH)₂vitamin D increased expression of the early osteoblast marker *RUNX2* (*RUNX2*) to a minor degree in healthy control, although not in CKD, osteoblasts. The minor increase in *RUNX2* expression in response to 1,25(OH)₂vitamin D was dwarfed by the effect of time on this early osteoblast marker (Figure 5a). By contrast, 1,25(OH)₂vitamin D markedly stimulated expression of the mature osteoblast marker osteocalcin (*BGLAP*) in both CKD and control cultures alike (Figure 5b). 1,25(OH)₂vitamin D had very little effect on alkaline phosphatase (*ALPL*) expression in either control or CKD osteoblasts (Figure 5c). Together, these data suggest that although 1,25(OH)₂vitamin D stimulates RNA expression of mature osteoblast markers, it has very little effect on osteoblast mineralization and does not rescue the mineralization defect intrinsic to CKD osteoblasts.

Bone biopsy data suggest that active vitamin D sterols increase osteocyte maturation and turnover and primary osteoblast cultures suggest that vitamin D directly enhances maturation of cells of the osteoblast lineage. In order to confirm that 1,25(OH)₂vitamin D also directly enhances osteocyte apoptosis, we cultured immortalized murine long bone osteocyte (MLO-Y4) cells with 1,25(OH)₂vitamin D *in vitro*. As shown in Figure 6a, 1,25(OH)₂vitamin D, particularly in the presence of high phosphate, increased the number of dead and apoptotic MLO-Y4 cells at 48 hours in culture. Activity of caspase 3, an enzyme responsible for the execution of apoptosis, simultaneously increased (Figure 6b), suggesting that active vitamin D sterols directly stimulate osteocyte apoptosis, particularly in the context of hyperphosphatemia (13).

Activation of osteoclasts may also indirectly contribute to vitamin D-mediated osteoblast maturation.

Since osteocyte senescence contributes to osteoclast activation which in turn stimulates osteoblastogenesis *in vivo* (14, 15), we evaluated the effect of doxercalciferol on histomorphometric measures of eroded surface and osteoclast number in CKD patient bone cores before and after doxercalciferol therapy. As previously shown and is reported in the Supplemental Table, number of osteoclasts/tissue area decreased by -0.23 (-1.28, 0.28)/mm² while eroded surface decreased by -1.1 (-4.4, 0.52)% in response to doxercalciferol therapy (NS for both). While osteoclast numbers and eroded surface correlated closely ($r=0.87$, $p<0.001$), there was only a modest, non-significant, correlation between the change in circulating PTH level and the change in eroded surface in response to therapy ($r=0.45$, $p=0.16$).

Given the imperfect correlation between PTH and osteoclast activation, we evaluated whether 1,25(OH)₂vitamin D exerts a direct effect on osteoblast-specific factors known to

regulate osteoclastogenesis. As shown in Figure 7, there was lower expression of receptor activator of nuclear factor kappa-B ligand (*RANKL*) expression in CKD than in control osteoblasts, particularly after one week in pro-mineralizing conditions. *RANKL* expression increased with 10 and 100 nM concentrations of 1,25(OH)₂vitamin D in controls although a more muted and variable increase was observed in CKD osteoblasts. Expression of osteoprotegerin (*OPG*), the decoy receptor for *RANKL*, did not differ between confluent control and CKD osteoblasts prior to exposure to pro-mineralizing conditions. *OPG* expression decreased in CKD and control osteoblasts after one week of culture in standard pro-mineralizing conditions, although expression by two weeks was similar to baseline levels. *OPG* expression increased with the highest doses of 1,25(OH)₂vitamin D in osteoblasts from healthy controls, although no change in *OPG* expression was observed with 1,25(OH)₂vitamin D in CKD cells. These changes in *OPG* and *RANKL* expression translated to an increasing *RANKL/OPG* ratio by one week under pro-mineralizing conditions in control, although not in CKD, osteoblasts. 10 nM and 100 nM doses of 1,25(OH)₂vitamin D, however, increased the ratio of *RANKL/OPG* in CKD osteoblasts. Together, these data suggest that 1,25(OH)₂vitamin D may play an additional role in osteoblast maturation by regulating osteoclast-osteoblast coupling in CKD bone.

DISCUSSION

We have previously demonstrated that CKD is associated with stagnant osteocyte maturation, characterized by an increase number of early osteocytes and a decrease in osteocyte apoptosis and that primary osteoblasts from CKD patients have intrinsic impairments in maturation and mineralization (5). Our current data further these observations, demonstrating an interplay between intrinsic maturation defects in cells of the osteoblast/osteocyte lineage and mineral ion homeostasis in the context of CKD-mediated bone disease. Here we confirm that active vitamin D sterols directly increase the maturation and turnover of cells in the osteoblast/osteocyte lineage. This effect on cell maturation contributes to increased numbers of early osteocytes which constitutively express FGF23 in CKD bone (7). *In vitro* studies confirm that 1,25(OH)₂vitamin D directly increases the maturation of primary osteoblast lineage cells although it fails to rescue the mineralization impairments intrinsic to primary CKD osteoblasts.

In this study, despite clear changes in bone histology and osteocyte-specific protein expression, PTH levels did not change in response to doxercalciferol. This finding is consistent with previous data demonstrating an increase in skeletal PTH-resistance in patients treated with active vitamin D sterols (3). However, treatment with active vitamin D sterols did, as has been previously demonstrated (3, 8), increase circulating levels and bone expression of FGF23. The current data demonstrate that these increases are largely due to increased numbers of FGF23-expressing osteocytes—which are early osteocytes (7)—in CKD bone. Doxercalciferol likewise increased osteocyte apoptosis to levels observed in healthy control subjects, suggesting a vitamin-D mediated increase in osteocyte maturation and turnover which is linked to *in vivo* vitamin-D mediated improvements in skeletal mineralization indices. *In vitro*, 1,25(OH)₂vitamin D stimulated primary osteoblast maturation, increasing expression of the mature osteoblast marker osteocalcin (*BGLAP*) in both healthy control and CKD osteoblasts. 1,25(OH)₂vitamin D also stimulated the

expression of the osteoclast differentiation factor *RANKL*. Particularly in CKD osteoblasts, high doses of 1,25(OH)₂vitamin D increased the ratio of *RANKL* to *OPG*, suggesting that 1,25(OH)₂vitamin D may also indirectly stimulate osteoblast and osteocyte maturation through improved osteoblast/osteoclast/osteocyte coupling. Interestingly, 1,25(OH)₂vitamin D did not induce a corresponding improvement in primary osteoblast mineralization *in vitro*. Mineralization improved only CKD osteoblasts exposed to the highest (100 nM) dose of 1,25(OH)₂vitamin D—a dose which far exceeds the concentrations around 1 nM which are achieved in dialysis patients receiving high doses of calcitriol (16) and which was toxic to the primary osteoblasts evaluated in this study. The discrepancy between maturation markers and primary osteoblast mineralization characteristics may be explained by previous data demonstrating that 1,25(OH)₂vitamin D, while inducing the osteogenic differentiation of osteoblast precursors (17), stimulates pyrophosphate expression, thereby inhibiting *in vitro* mineralization of primary rodent osteoblasts (18).

The discrepancies between the effects of active vitamin D sterols on skeletal mineralization *in vivo* and on isolated osteoblast mineralization *in vitro* may be in part explained by the effects of vitamin D on systemic mineral metabolism and on osteocyte/osteoclast/osteoblast coupling. Vitamin D sterols suppress PTH and increase intestinal calcium and phosphorus absorption. Numbers of osteoblast precursors increase—and their apoptosis decreases—in mice treated with PTH (19). In dialysis patients, the removal of PTH via parathyroidectomy increases bone expression of the mature osteocyte marker sclerostin, although it does not alter numbers of new, FGF23-expressing osteocytes (20). Serum phosphate levels are associated with increased osteoblast/osteocyte maturation and with osteoblast and osteocyte apoptosis (7). Thus, the effects of vitamin D-mediated changes in mineral ion and hormone concentrations, in addition to renal osteodystrophy therapies and intrinsic alterations in osteoblast lineage cell maturation characteristics, likely combine to contribute to abnormal osteocyte-specific hormone expression and skeletal mineralization in the context of chronic kidney disease. This interplay is particularly striking when comparing differences in osteocyte maturation and turnover between uremic mice, in whom preserved circulating 1,25(OH)₂vitamin D levels and hyperphosphatemia co-occur with increase rates of osteocyte apoptosis (21), and dialysis patients, in whom low circulating 1,25(OH)₂vitamin D levels may contribute to low osteocyte apoptosis rates prior to vitamin D sterol therapy (7). In addition, the contribution of differentiation-inducing signals from osteoclasts and osteocytes, which play critical roles in osteoblast maturation (14, 15), are lost in isolated cultures of primary osteoblasts, likely obscuring the true effect of 1,25(OH)₂vitamin D on osteoblast maturation.

While common in children (4, 10), skeletal mineralization defects have more variably been reported in the adult population, in part due to variation in how different investigators define mineralization defects (22, 23). Despite these discrepancies, the prevalence of mineralization defects, defined by the presence of increased osteoid accumulation in combination with prolonged mineralization time, appears to be substantially more prevalent in children than in adults with CKD (4, 10). The reason behind this difference is not entirely clear but may speak to differences in biology between young people who are both forming and remodeling bone and adults who are maintaining and remodeling it. Thus, it is possible that skeletal mineralization defects resulting from CKD-mediated osteoblast and osteocyte maturation

failure may be a uniquely pediatric problem. Further studies are warranted to determine whether agents that increase bone cell maturation and stimulate osteoclast recruitment, such as active vitamin D sterols, may have different degrees of benefit for bone health in children as compared to adults.

Active vitamin D sterols have previously been shown to increase circulating FGF23 in dialysis patients (3) and circulating FGF23 levels correlate closely with number of FGF23-expressing osteocytes in CKD patients (11). Circulating level of FGF23 have been linked to infection (24), cardiovascular disease (25), and early mortality (26) in dialysis patients. Thus, by improving bone mineralization and overall osteocyte turnover, active vitamin D sterols may contribute to off-target co-morbidities of FGF23. It is important to note, however, that pharmacologic manipulation of FGF23 have not been shown to alter mortality; thus, the systemic benefits of active vitamin D sterol treatment—and its potential adverse effect on FGF23 excess in the CKD population—remain an open question

We acknowledge certain limitations in the current study. Namely, while valuable in its use of a rare library of human bone samples, this study is, by its rarity, limited in sample size. These samples were obtained from children and young adults; generalizability to the adult population thus remains unknown. However, FGF23 expression is increased and skeletal mineralization defects have also been identified in adult CKD bone biopsy studies (22), consistent with data from the pediatric population (3, 4). In addition, the skeletal site of primary osteoblast origin may affect osteocalcin and alkaline phosphatase expression (27) and, while 1,25(OH)₂vitamin D has previously been shown to affect maturation and mineralization of osteoblasts, its effects may depend on the stage of maturation of the cells in this lineage (6). Furthermore, the current data, in five select patients with low (n=2) and high (n=3) bone turnover show lower baseline *ALPL* and *RUNX* expression than was previously reported in a cohort of 24 primary osteoblast samples from CKD patients (5). Two of the CKD “lines” used for the current analysis were from the original cohort; three of the others (obtained in the same manner) were obtained subsequently. While there has been variability in the *ALPL* and *RUNX* expression amongst cells, lower osteocalcin expression has been consistently low in the CKD osteoblasts, consistent with delayed in maturation in CKD osteoblasts in general (5, 7). Finally, it should be noted that while doxercalciferol (1 α -(OH)₂vitamin D₂)—a prohormone that is converted by the liver to 1,25(OH)₂vitamin D₂—was used for the *in vivo* portion of this study while calcitriol (1,25(OH)₂vitamin D₃) was used *in vitro*. While it is possible that the two hormones exert different effects on osteoblast and osteocyte maturation, previous *in vivo* data suggest that doxercalciferol and calcitriol have similar effects on PTH, bone turnover, bone mineralization, and circulating FGF23 levels (3).

In summary, this study demonstrates that 1,25(OH)₂vitamin D directly increases maturation of osteoblast lineage cells. By stimulating osteocyte apoptosis and increasing *RANKL/OPG* expression, 1,25(OH)₂vitamin D may also play a role in modulating osteoblast/osteoclast/osteocyte coupling. The overall effects of active vitamin D sterols are to increase the number of osteocytes in a relatively early phase of maturation which constitutively express FGF23 and to also increase in osteocyte turnover. RNA data further demonstrates that CKD osteoblasts, as compared to healthy control osteoblasts, maintain biological differences

which are not due to vitamin D deficiency and which may contribute to the persistence of skeletal disease in the CKD population, despite optimal treatment for renal osteodystrophy. Further studies are needed to define the mechanism by which this maturation failure occurs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

CKD	chronic kidney disease
PTH	parathyroid hormone
FGF23	fibroblast growth factor 23
RUNX2	runt related transcription factor 2
BGLAP	bone gamma-carboxyglutamate protein; osteocalcin
ALPL	tissue non-specific alkaline phosphatase
MLO-Y4	murine osteocyte-like cell line-Y4
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

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Highlights

1. The overall effects of active vitamin D sterols on CKD bone are to increase the number of osteocytes in a relatively early phase of maturation, to increase numbers of late osteocytes, and to increase osteocyte turnover.
2. 1,25(OH)₂vitamin D directly increases maturation of osteoblast lineage cells.
3. By stimulating osteocyte apoptosis and increasing *RANKL/OPG* expression, 1,25(OH)₂vitamin D may play a role in modulating osteoblast/osteoclast/osteocyte coupling.
4. CKD osteoblasts, as compared to healthy control osteoblasts, maintain biological differences which are not due to vitamin D deficiency and which may contribute to the persistence of skeletal disease in the CKD population, despite optimal treatment for renal osteodystrophy.
5. Active vitamin D sterols increase FGF23 expression in bone by increasing the number of early osteocytes which constitutively express this hormone.

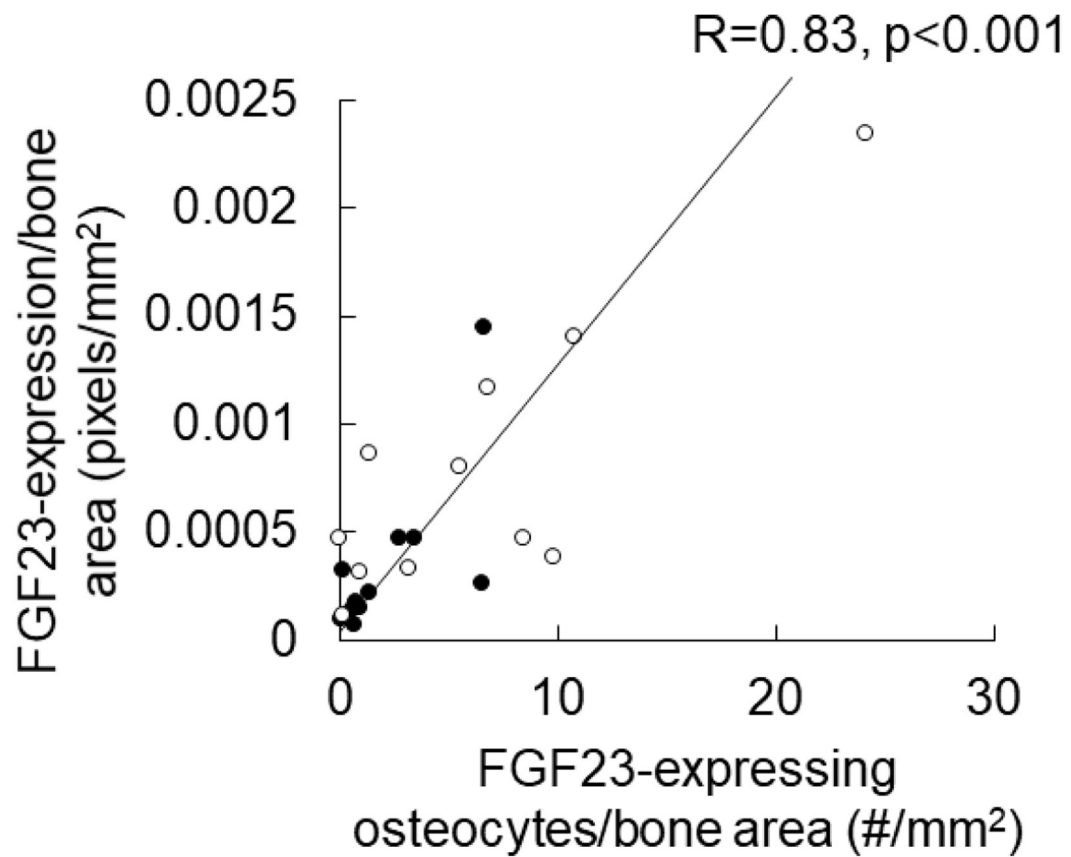


Figure 1: Numbers of FGF23-expressing osteocytes increase in response to active vitamin D sterols.

Scatterplot demonstrating the tight ($r=0.83$; $p<0.001$) relationship between numbers of FGF23-expressing osteocytes (X-axis) and quantification of FGF23 immunostaining in bone. Closed circles represent values in bone biopsies prior to doxercalciferol therapy and open circles represent values in samples from the same patients treated with doxercalciferol.

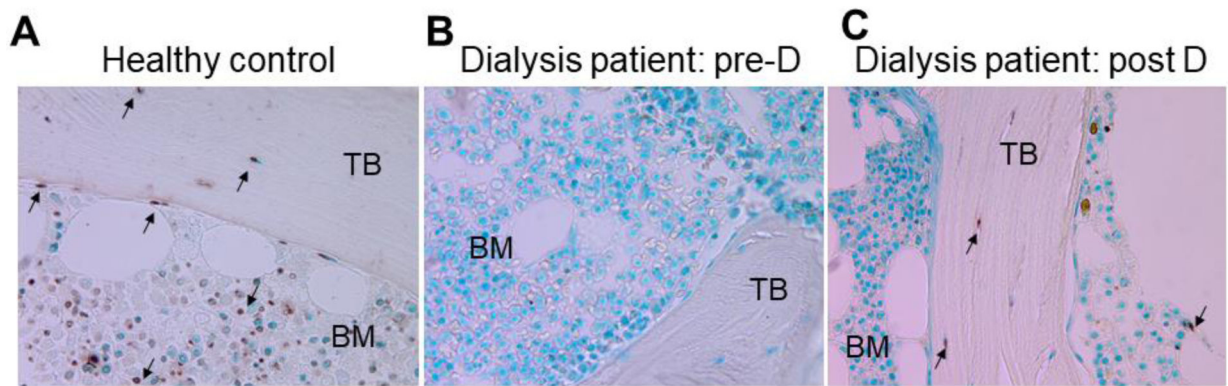


Figure 2: Osteocyte apoptosis is decreased in advanced CKD but increases in response to active vitamin D sterols.

TUNEL staining of iliac crest in a normal control (A) and in a dialysis patient before (B) and after (C) 8 months of therapy with doxercalciferol ("D") and phosphate binders. Arrows point to apoptotic cells.

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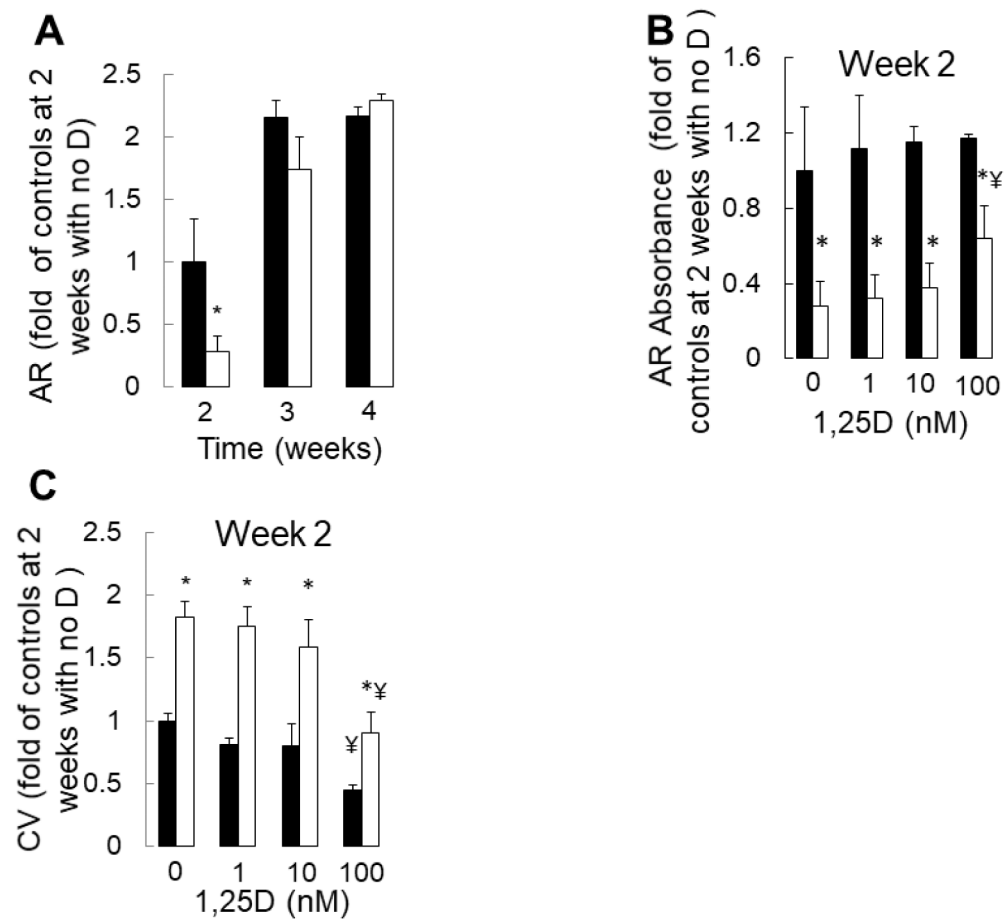


Figure 4: Mineralization of primary CKD osteoblasts is delayed but increases in response to high doses of 1,25(OH)₂vitamin D.

(A) Absorbance of extracted alizarin red S dye at 405 nm, in healthy control osteoblasts (closed bars) and in CKD osteoblasts (open bars) cultured for 4 weeks under pro-mineralizing conditions in the absence of 1,25(OH)₂vitamin D ("D"). (B) Absorbance of extracted alizarin red S dye at 405 nm, in healthy control osteoblasts and in CKD osteoblasts after 2 weeks of growth under pro-mineralizing conditions with varying concentrations of 1,25(OH)₂vitamin D. (C) Relative live cell numbers over time, as assessed by the absorbance of extracted crystal violet (CV) staining at 605 nm, in healthy control (closed bars) and CKD (open bars) primary osteoblasts cultured under standard pro-mineralizing conditions. The asterisk (*) indicates a difference ($p < 0.05$) between healthy control cells and CKD cells at each timepoint or dosage. The ¥ indicates a difference ($p < 0.05$) from control (no added 1,25(OH)₂vitamin D) conditions. 1,25D: 1,25(OH)₂vitamin D; CV: crystal violet.

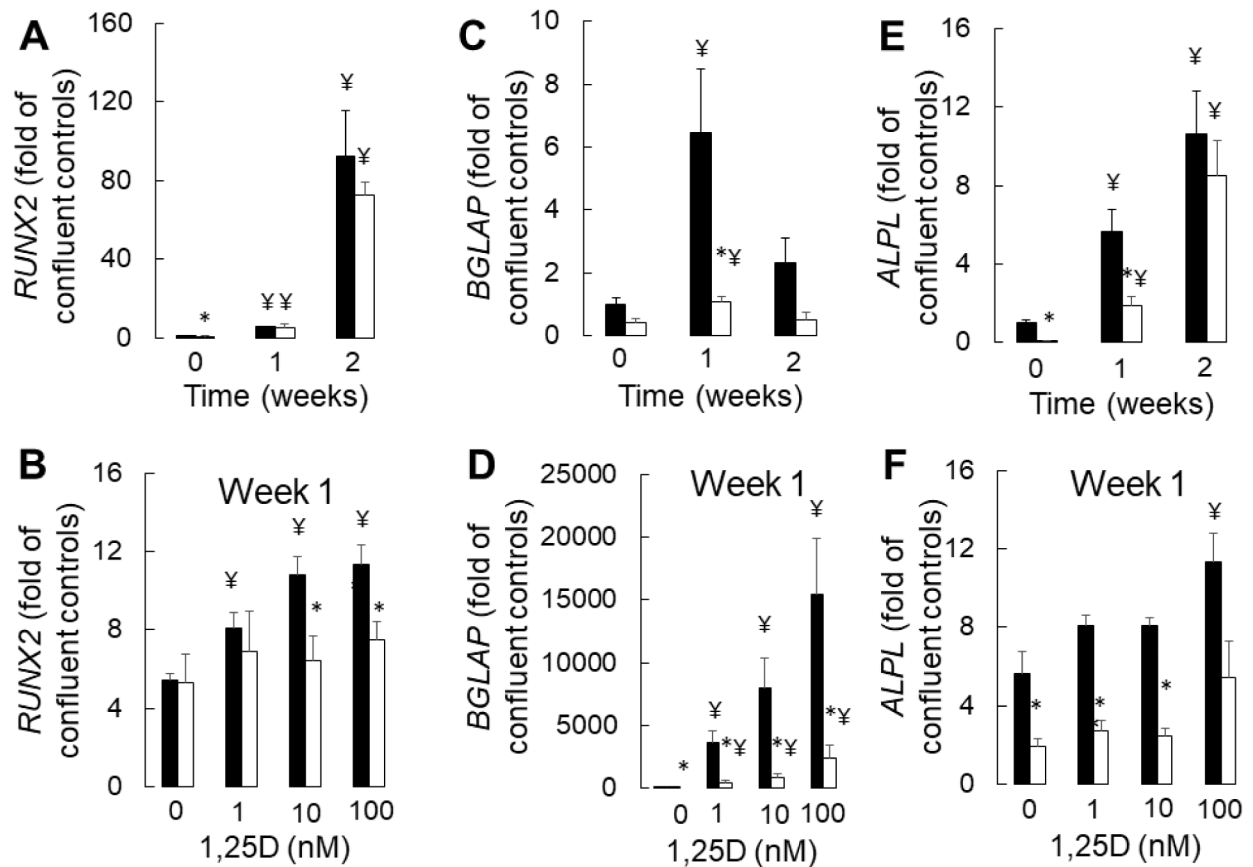


Figure 5: 1,25(OH)₂vitamin D stimulates expression of the mature osteoblast marker, osteocalcin (BGLAP).

(A) *RUNX2* expression over time under standard pro-mineralizing conditions (A) over time and (B) with increasing concentrations 1,25(OH)₂vitamin D. (C) *BGLAP* expression over time under standard pro-mineralizing conditions and (D) with increasing concentrations of 1,25(OH)₂vitamin D (E) *ALPL* expression over time under standard pro-mineralizing conditions and (D) with increasing concentrations of 1,25(OH)₂vitamin D. Expression is displayed as the multiple (fold) of expression identified in confluent healthy control osteoblasts which have not been stimulated to mineralize and have not been exposed to vitamin D (“confluent controls”). The asterisk indicates a difference (p<0.05) between CKD and control osteoblasts; the ¥ indicates a difference from the control (time 0 for A, C, and E and from the no added 1,25(OH)₂vitamin D condition for B, D, and F). Healthy control osteoblasts are depicted by closed bars and CKD osteoblasts by open bars. 1,25D: 1,25(OH)₂vitamin D.

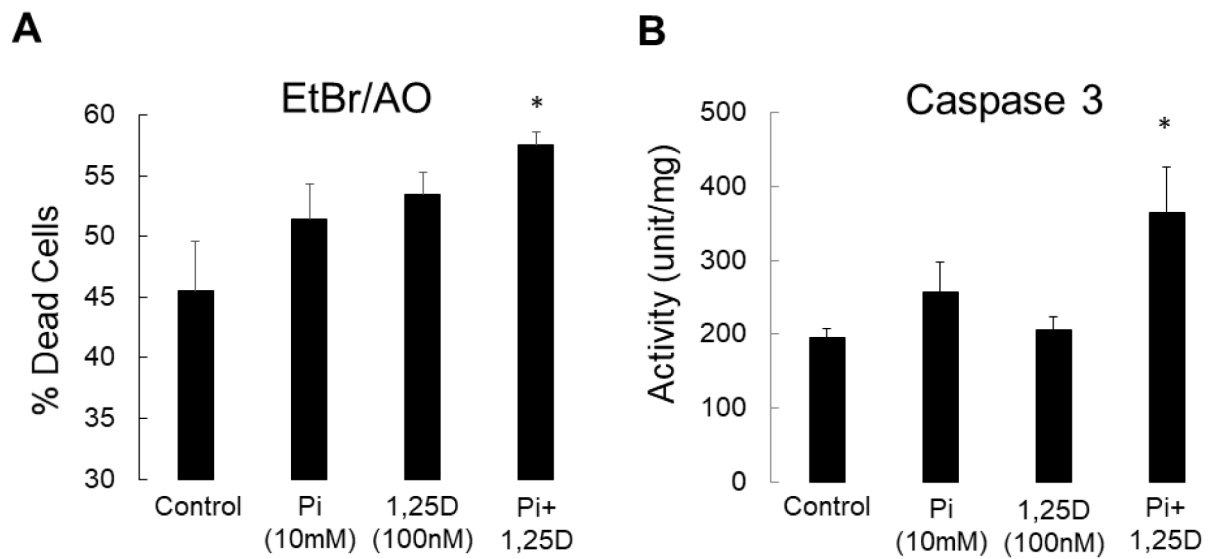


Figure 6: 1,25(OH)₂vitamin D and phosphate directly increases osteocyte apoptosis *in vitro*.

A) The percentage of dead cells, as defined by the percentage of cells staining with ethidium bromide (Et.Br) relative to the total number of live cells staining with acridine orange (Ac.O), after 48 hours in culture. Values represent the average $\pm$ standard error for n=8 replicates. B) Caspase 3 activity in MLO-Y4 cells exposed to phosphate and 1,25(OH)₂vitamin D. The asterisk indicates a difference ($p < 0.01$) from cells treated under control (3mM Pi with no added 1,25(OH)₂vitamin D) conditions. 1,25D: 1,25(OH)₂vitamin D; Pi: phosphate.

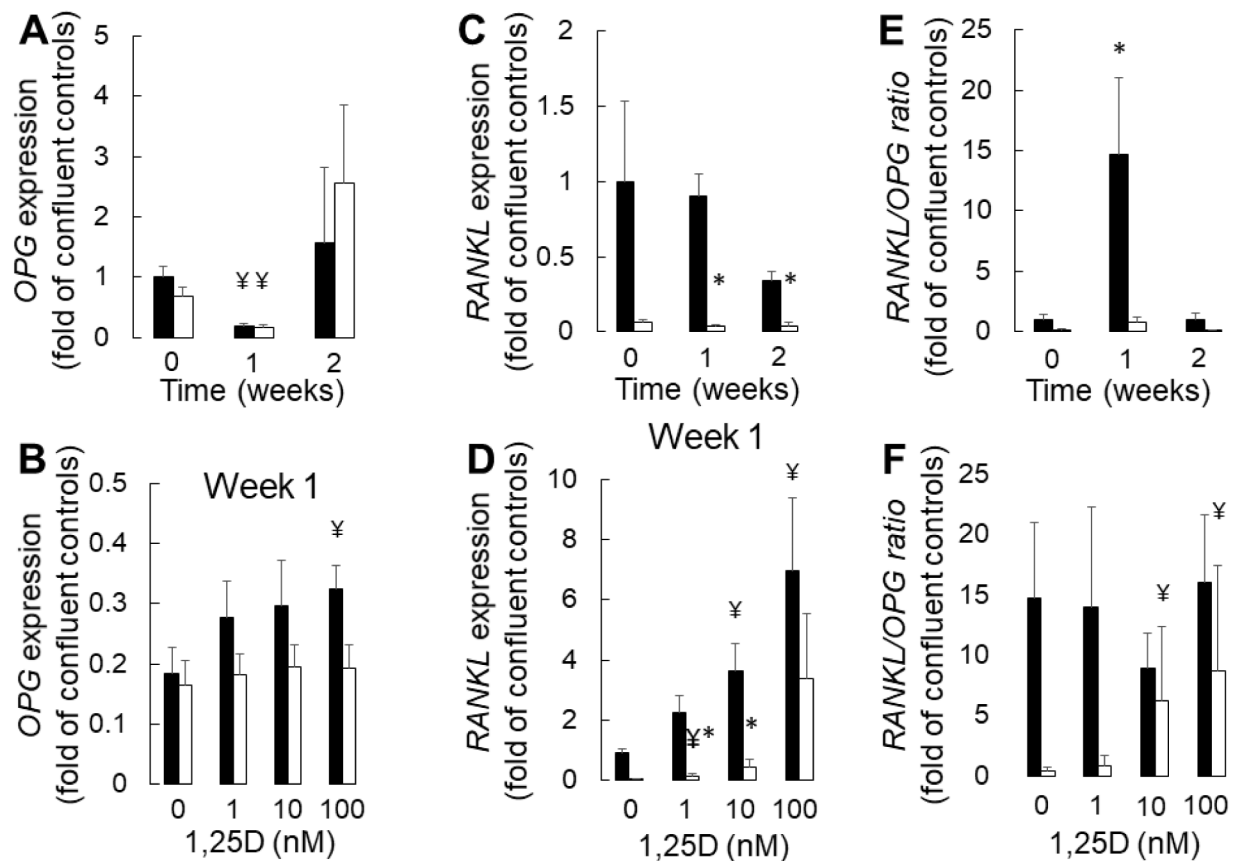


Figure 7: 1,25(OH)₂vitamin D stimulates osteoclastogenesis.

(A) *OPG* expression over time under standard pro-mineralizing conditions (A) over time and (B) with increasing concentrations 1,25(OH)₂vitamin D. (C) *RANKL* expression over time under standard pro-mineralizing conditions and (D) with increasing concentrations of 1,25(OH)₂vitamin D (E) *RANKL/OPG* expression over time under standard pro-mineralizing conditions and (D) with increasing concentrations of 1,25(OH)₂vitamin D. Expression is displayed as the multiple (fold) of expression identified in confluent healthy control osteoblasts which have not been stimulated to mineralize and have not been exposed to vitamin D (“confluent controls”). The asterisk indicates a difference ($p < 0.05$) between CKD and control osteoblasts; the ¥ indicates a difference from the control (time 0 for A, C, and E and from the no added 1,25(OH)₂vitamin D condition for B, D, and F). Healthy control osteoblasts are depicted by closed bars and CKD osteoblasts by open bars. 1,25D: 1,25(OH)₂vitamin D.

Table 1:

Biochemical variables and numbers of FGF23-expressing osteocytes in patients with normal mineralization versus mineralization defects.

	Normal mineralization (n=12; 3 initial biopsies)	Mineralization defects (n=10; 8 initial biopsies)
Biochemical values		
Calcium (mg/dL)	8.8 (8.6, 9.3)	8.7 (8.1, 9.1)
Phosphorus (mg/dL)	5.8 (5.3, 6.8)	6.8 (5.2, 7.4)
PTH (pg/mL)	458 (377, 660)	772 (535, 1068)
Alkaline phosphatase (IU/L)	165 (123, 245)	381 (287, 543) *
FGF23 (RU/mL)	1826 (683, 4069)	522 (348, 793) *
25(OH)vitamin D (ng/mL)	23.9 (19.0, 29.8)	25.9 (19.4, 30.1)
Numbers of FGF23-expressing osteocytes		
FGF23-expressing osteocytes/bone area (FGF23/B.Ar) (#/mm ²)	6.11 (0.95, 8.84)	0.33 (1.06, 2.45) *

*
p<0.05 between groups