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Evidence for auto/paracrine actions of vitamin D in bone: 1α-hydroxylase expression and activity in human bone cells

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ABSTRACT **Vitamin D is an important regulator of mineral homeostasis and bone metabolism. 1α-Hydroxylation of 25-(OH)D_3 to form the bioactive vitamin D hormone, 1α,25-$(OH)_2D_3$, is classically considered to take place in the kidney. However, 1α-hydroxylase has been reported at extrarenal sites. Whether bone is a 1α,25-$(OH)_2D_3$ synthesizing tissue is not univocal. The aim of this study was to investigate an autocrine/paracrine function for 1α,25-$(OH)_2D_3$ in bone. We show that 1α-hydroxlase is expressed in human osteoblasts, as well as the vitamin D binding protein receptors megalin and cubilin. Functional analyses demonstrate that after incubation with the 1α-hydoxylase substrate 25-(OH)D_3, the osteoblasts can produce sufficient 1α,25-$(OH)_2D_3$ to modulate osteoblast activity, resulting in induced alkaline phosphatase (ALP) activity, osteocalcin (OC) and CYP24 mRNA expression, and mineralization. The classical renal regulators of 1α-hydroxylase, parathyroid hormone, and ambient calcium do not regulate 1α-hydroxylase in osteoblasts. In contrast, interleukin (IL)-1β strongly induces 1α-hydroxylase. Besides the bone-forming cells, we demonstrate 1α-hydroxylase activity in the bone resorbing cells, the osteoclasts. This is strongly dependent on osteoclast inducer RANKL. This study showing expression, activity, and functionality of 1α-hydoxylase unequivocally demonstrates that vitamin D can act in an auto/paracrine manner in bone.—van Driel, M., Koedam, M., Buurman, C. J., Hewison, M., Chiba, H., Uitterlinden, A. G., Pols, H. A. P., van Leeuwen, J. P. T. M. Evidence for auto/paracrine actions of vitamin D in bone: 1α-hydroxylase expression and activity in human bone cells. *FASEB J.* 20, E000–E000 (2006)**

Key Words: calcium homeostasis · PBMCs · osteoblasts · osteoclasts

VITAMIN D IS ONE OF THE MAJOR factors involved in calcium homeostasis via actions on intestine, kidney, parathyroid gland, and bone (1). The biologically most active vitamin D molecule is 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$). In bone, 1α,25-$(OH)_2D_3$ is important for mineralization, either indirectly via control of calcium absorption in intestine and reabsorption in the kidney, but also via direct action on osteoblasts (2 and unpublished results). 1α,25-$(OH)_2D_3$ is formed from the parental vitamin D molecule by sequential hydroxylations in the liver (25-hydroxylation) and kidney (1α-hydroxylation): 25-hydroxyvitamin D_3 (25-(OH)D_3) formed in the liver is transported to the kidney bound to the vitamin D binding protein (DBP) and is then metabolized to 1α,25-$(OH)_2D_3$ by the renal cytochrome P450 enzyme 25-hydroxyvitamin D_3-1α-hydroxylase (3).

However, 1α-hydroxylase expression and activity are not restricted to the kidney. The first evidence for extrarenal 1α-hydroxylase was based on studies of patients with sarcoidosis in whom conversion from 25-(OH)D_3 to 1α,25-$(OH)_2D_3$ was demonstrated in affected lymph nodes and pulmonary alveolar macrophages (4, 5). More recently it was reported that 1α-hydroxylase expression was increased in pathological parathyroid glands (6, 7) and up-regulated in various other malignant conditions (8–10). Prostate cells also express 1α-hydroxylase, but here the activity of the enzyme is decreased in cancer cells (11–14).

1α-Hydroxylase expression has also been demonstrated in a variety of normal extrarenal tissues like skin, lymph nodes, colon, pancreas, adrenal medulla, dendritic cell, endothelial cells, brain, hypothalamus, and placenta (15–20). Remarkably, ~25 years ago 1α-hydroxylase activity was reported in human bone cells in culture (21–23) but bone has never been considered a vitamin D-synthesizing tissue. The aim of the present study was to settle this issue and comprehensively investigate the expression and activity of 1α-hydroxylase and other components of the vitamin D metabolic pathway in osteoblasts and to define the potential biological activity in these cells. An *in vitro*

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TABLE 1. *Human and mouse genes, the same concentrations used for forward and reverse primers*

Gene	Forward primer	Reverse primer
CYP27B1	GGCAGAGTCTGAATTGCAAAT	CCGGGTCTTGGGTCTAACTG
Osteocalcin	CAGGAGGGCAGCGAGGTA	TGG-GGC-TCC-CAG-CCA
CYP24	CAAACCGTGGAAGGCCTATC	AGTCTTCCCCTTCCAGGATCA
GAPDH	atggggaaggtgaaggtcg	taaaagcagccctggtgacc
CYP27B1[a]	CCCAGCTGCCCCTGTTAAA	GTTTCCTACACGGATGTCTCTGTCT
HPRT[a]	TTATCAGACTGAAGAGCTACGTAATGATC	TTACCAGTGTCAATTATATCTTCAACAATC

[a]Mouse genes.

model of osteoblast differentiation and bone formation was used to examine the impact of osteoblast maturation on the synthesis of $1\alpha,25\text{-(OH)}_2D_3$ and to assess 1α-hydroxylase functionality in bone cell activity.

MATERIALS AND METHODS

Cell culture

Human SV-HFO cells were seeded at a density of 10×10^3 vital cells per cm^2 in phenol-red free α-minimal essential medium (α-MEM Gibco BRL, Paisley, U.K.), pH 7.5, supplemented with 20 mM HEPES (Sigma, St. Louis, MO, USA), 100 IU/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Breda, the Netherlands), 1.8 mM $CaCl_2 \cdot 2H_2O$ (Sigma), and 2% heat-inactivated charcoal-treated FCS at 37°C and 5% CO_2 in a humidified atmosphere. Medium was replaced every 2–3 days supplemented with freshly diluted 10 mM β-glycerophosphate (Sigma) and 1 μM dexamethasone (9α-fluoro-16α-methylprednisolone, Sigma). Cells and culture supernatant from duplicate wells were collected at multiple days (indicated in figures) during culture. Medium was stored at –20°C and cells were scraped in PBS containing 0.1% triton X-100 and stored in −80°C. Before use, cell lysates were sonicated on ice in a sonifier cell disrupter for 2 × 15 s. Human osteoblast-like MG-63 cells were maintained in αMEM medium supplemented with 2 mM L-glutamine, 0.1% glucose (Glc), 100 IU/ml penicillin, 100 IU/ml streptomycin, and 10% FCS at 37°C in 5% CO_2 in a humidified atmosphere. Cells were seeded at 100,000 cells/cm^2 in αMEM containing 10% FCS and cultured for 6 days. The cDNAs from KS483 murine osteoblasts at different time points during culture were generously provided by Dr. M. Karperien (Leiden University Medical Center, the Netherlands).

Human osteoclast cultures

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors and induced to osteoclasts as described earlier (24).

RAW 264.7 monocytic cells were cultured in α-MEM containing 10% FCS, with or without 20 ng/ml mRANKL (R&D Systems, Abingdon, UK).

Human femoral head biopsies

Human bone material was obtained from two femoral head biopsies (of ostearthritic bone). These have been collected within a clinical trial, which was approved by the medical ethical commission (MEC No. 204.287). Human biopsy samples were homogenized using a Mikro Dismembrator S (Sartorius, Goettingen, Germany).

Cell culture treatments

$25\text{-(OH)}D_3$ and $1\alpha,25\text{-(OH)}_2D_3$ were generously provided by Dr. L. Binderup, Leo Pharmaceuticals, Ballerup, Denmark. $25\text{-(OH)}D_3$ and $1\alpha,25\text{-(OH)}_2D_3$ were incubated for 24 h, 48 h, or continuously in concentrations indicated in the legends of the figures. Cells were incubated with interleukin-1 (IL-1β) (Peprotech, London, UK), PTH (Bachem, Weil am Rhein, Germany) and different concentrations of $CaCl_2$ (Sigma-Aldrich) for 24 h. $1\alpha,25\text{-(OH)}_2D_3$ production were measured using a gamma-B 1,25-dihydroxy vitamin D RIA according to the manufacturer's protocol (IDS, Boldon, UK); cross-reactivity with $25\text{-(OH)}D_3$ is 0.001%.

RNA isolation

Total RNA was isolated using RNA-Bee solution (Tel-Test, Friendswood, TX, USA) according to the manufacturer's protocol and processed for real-time polymerase chain reaction (PCR) or Affymetrix array-based gene expression. RNA was washed and precipitated overnight at –20°C with equal volumes of 100 mM EDTA and 8 M LiCl. Total RNA was quantified using the Ribogreen assay (Molecular Probes, Eugene, OR, USA). One μg of RNA was reverse transcribed into cDNA, using 0.5 μg oligo$(dT)_{18}$, 0.2 μg random hexamer primers, and Moloney murine leukemia virus according to the protocol of the manufacturer (MBI fermentas, St. Leon-Rot, Germany).

Gene expression (real-time PCR)

Quantitative real-time PCR was carried out using an ABI Prism 7700 sequence detection system (PE Biosystems, Rotkreuz, Switzerland). Reactions were performed in 25 μl volumes using a qPCR™ core kit (Eurogentec, Seraing, Belgium). Reaction mixes contained 2–40 ng cDNA, 5 mM $MgCl_2$, 200 μM dNTPs, and 0.025 U/μl Hot GoldStar enzyme. Primer and probe sets were designed using the Primer Express software (Version 1.5; Applied Biosystems, Foster City, CA, USA) and Biolegio (Malden, the Netherlands). Probes were labeled at the 3′-end with the quencher dye 6-carboxytetramethylrhodamine and at the 5′-end with the reporter dye 6-carboxyfluorescein (Eurogentec, Seraing, Belgium). Primer and probe sequences and concentrations are listed in **Table 1**. Cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Data are presented as relative mRNA levels calculated by the equation $2^{-\Delta Ct}$ (Δ Ct=Ct of target gene minus Ct of GAPDH).

Gene expression (microarrays)

To get a complete overview on the expression of other components of the vitamin D metabolism/endocrine system,

(nM)	Probe (5′-Fam-Tamra-3′)	(nM)
(400)	CTTTGGCCCAGATCCTAACACATTTTGAGG	(200)
(1000)	TGATACAGGTAGCGCCTG`	(200)
(600)	ACTACCGCAAAGAAGGCTACGGGCTG	(300)
(150)	cgcccaatacgaccaaatccgttgacT	(150)
(200)	TGATCAAAGAAGTGTTGAGATTGTACCCTGTGGT	(100)
(600)	TGAGAGATCATCTCCACCAATAACTTTTATGTCCC	(200)

Affymetrix-based microarray studies were performed, according to the manufacturer's protocol.

Western blotting

Cells were scraped in PBS phenylmethylsulfonylfluoride (PMSF) 0.5 mM. Lysates were obtained via three freeze thaw cycles and stored in −80°C. Western blot analysis was performed as described previously (25).

DNA measurement and ALP activity

SV-HFO cell lysates were treated with heparin and RNase A (50 μg/ml in PBS) for 30 min at 37°C. DNA content was measured according to the ethidium bromide method of Karsten and Wollenberger (26). ALP activity was assayed by determining the release of paranitrophenol from paranitrophenylphosphate (20 mM in 1 M diethanolamin buffer supplemented with 1 mM $MgCl_2$ at pH 9.8) in the cell lysates for 10 min at 37°C (27). Adding 0.1 M NaOH stopped the reaction and absorption was measured at 405 nm using a Packard Spectra Count. Results were adjusted for DNA content of the corresponding cell lysates.

Mineralization

SV-HFO cell lysates were incubated overnight with 0.25 M HCl at 4°C. Calcium content was colorimetrically determined after addition of 1M ethanolamine buffer (pH 10.6) 0.35 mM o-cresolphtalein complexone, 19.8 mM 8-hydroxyquinoline, and 0.6 mM hydrochloric acid at 595 nm (Packard Spectra Count). Results were adjusted for DNA content of the corresponding cell lysates.

Statistical analyses

Differences between groups were tested for significance using the Student's t test. Values were considered significantly different at $P < 0.05$.

RESULTS

Figure 1*A* shows the presence of CYP27B1 mRNA in human bone biopsies as well as in cultured human and murine osteoblasts and osteoclasts. Expression of 1α-hydroxylase protein was demonstrated by Western blot analysis of extracts from human osteoblasts (Fig. 1*B*). Besides 1α-hydroxylase, human osteoblasts expressed other components of the 1α,25-$(OH)_2D_3$ endocrine system. These include the two membrane proteins megalin and cubilin and two P450 enzymes, CYP2R1 and CYP3A4, as found by Affymetrix gene expression analyses (Fig. 1*C*).

1α-Hydroxylase activity was shown by the production of ~400 pM 1α,25-$(OH)_2D_3$ after incubations of human osteoblasts (SV-HFO, MG-63) with 1000 nM 25-$(OH)D_3$ for 48 h (**Fig. 2*A***). Time- and dose-dependent incubations with 25-$(OH)D_3$ on SV-HFO cells reveal production of 1α,25-$(OH)_2D_3$ after 1 h and lower concentrations (Km: 370 nM, V_{max}: 33 pmol/h/mg protein) (Fig. 2*B, C*). The 1α-hydroxylase activity and production of 1α,25-$(OH)_2D_3$ by osteoblasts was completely blocked by the P450 inhibitor ketoconazole (Fig. 2*C*).

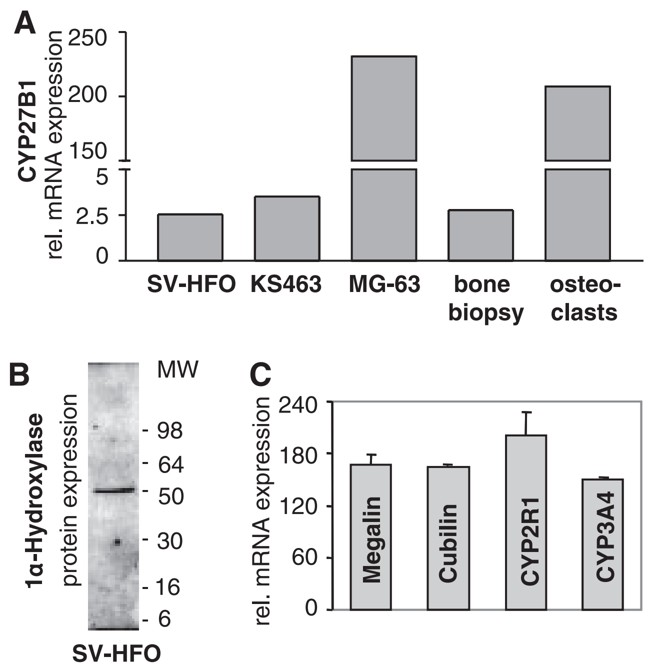

Figure 1. Expression of CYP27B1, 1α-hydroxylase protein, DPB receptors, and vitamin D_3-25-hydroxylases in bone cells. *A)* Real-time PCR analyses of CYP27B1 expression in human (SV-HFO, day 14) and murine (KS463, day 14) osteoblasts, the human osteosarcoma cell line MG-63, a human bone biopsy and human osteoclasts. Values are 2^-delta ct (delta ct=ct of target gene minus ct of GAPDH). *B)* Western blot analyses of 1α-hydroxylase in SV-HFO human osteoblasts at day 14 of culture. *C)* Affymetrix-based gene expression analyses of the DBP receptors megalin and cubilin and the vitamin D_3-25-hydroxylases CYP2R1 and CYP3A4 in human osteoblasts (SV-HFO) at day 14 of culture. Data are presented as means ± SD.

We studied the relationship with differentiation in the differentiating human preosteoblasts (SV-HFO). These cells express maximal ALP activity at day 14 of culture coinciding with the onset of mineralization (**Fig. 3*A, B***). Treatment with 1α,25-$(OH)_2D_3$ during this differentiation sequence caused an increase in ALP activity in the first phase of culture and an earlier maximum calcification. At the different stages of differentiation no changes in the expression of CYP27B1 of both human and murine osteoblasts were observed (data not shown). Despite this, there was a significant increase in 1α-hydroxylase activity during culture and osteoblast differentiation, with a maximum 1α,25-$(OH)_2D_3$ production of 800 fmol/ml at day 16 (**Fig. 4*A***). When 1α,25-$(OH)_2D_3$ production was corrected for cell number (DNA), this translated into a small progressive decrease in production per cell over time (Fig. 4*B*). However, the CYP27B1 mRNA levels and megalin and cubilin mRNA expression did not change throughout the 3 wk culture period (data not shown).

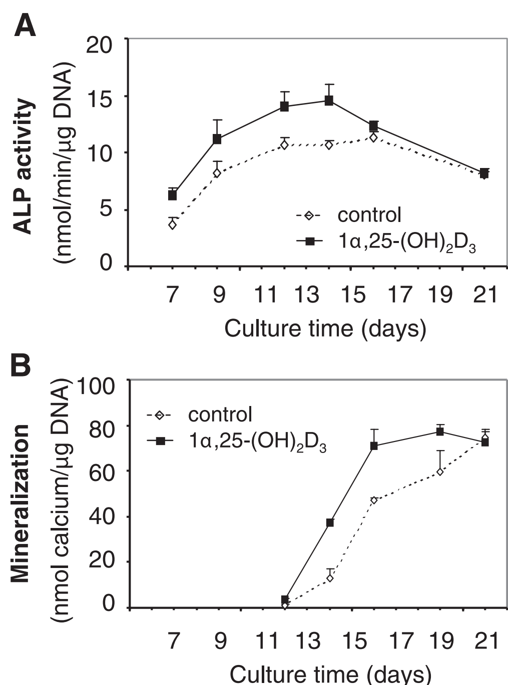

Figure 3. Effect of 1α,25-$(OH)_2D_3$ on human osteoblast activity. SV-HFO human preosteoblasts were induced to differentiate, and at various time points during differentiation ALP activity (*A*) and mineralization (*B*) were measured after incubation with vehicle or 10 nM 1α,25-$(OH)_2D_3$. Data shown are means ± SD.

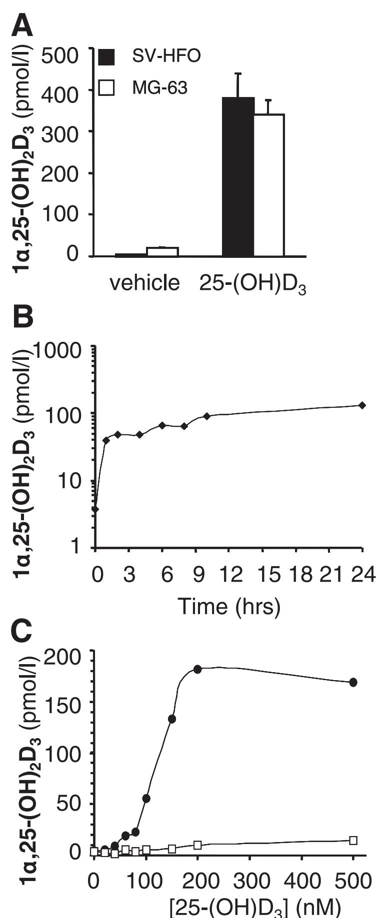

Figure 2. Activity of 1α-hydroxylase in human osteoblasts. *A)* The human osteoblast cell lines SV-HFO and MG-63 were incubated for 48 h with 1000 nM 25-$(OH)D_3$ and next production of 1α,25-$(OH)_2D_3$ was measured. *B)* SV-HFO cells were incubated with 1000 nM 25-$(OH)D_3$ for various periods and *C)* with various concentrations of 25-$(OH)D_3$ for 48 h in the absence (●) or presence (□) of 100 μM ketoconazole, after which 1α,25-$(OH)_2D_3$ production was measured. Data shown are means ± SD.

In contrast to osteoblasts, induction of both murine and human osteoclast differentiation resulted in an increase in 1α-hydroxylase mRNA levels (Fig. 4*C, D*). 1α-Hydroxylase activity (Fig. 4*B*) and CYP27B1 expression (data not shown) were unaffected by 1α,25-$(OH)_2D_3$ pretreatment.

Incubation with increasing doses of 25-$(OH)D_3$ at day 14 of culture for 24 h had no effect on CYP27B1 mRNA expression (**Fig. 5*A***). In addition, PTH (data not shown) and calcium (Fig. 5*B*) did not change 1α-hydroxylase mRNA expression or activity in human osteoblasts. However, production of 1α,25-$(OH)_2D_3$ showed a tendency to increase at higher calcium concentrations. Incubation with IL-1β significantly increased CYP27B1 expression (Fig. 5*C*).

Short-term (48 h) incubations with 25-$(OH)D_3$ induced OC (**Fig. 6*A, B***) and CYP24 (Fig. 6*C, D*) mRNA expression and ALP activity (Fig. 6*E*) to a greater extent than when 1α,25-$(OH)_2D_3$ (10 nM) was directly added for 48 h. Induction of OC and CYP24 mRNA expression increased with osteoblast differentiation and was dose dependent. OC protein measurements also demonstrated a stimulation following incubation with 25-$(OH)D_3$ (data not shown). Blocking 1α,25-$(OH)_2D_3$ production by ketoconazole (Fig. 2*C*) almost completely blocked the effect of 25-$(OH)D_3$ on both OC (Fig. 6*B*) and CYP24 (Fig. 6*D*) mRNA expression. ALP activity was induced by incubation with 25-$(OH)D_3$ to a similar extent as when 1α,25-$(OH)_2D_3$ was directly added (Fig. 6*E*). Mineralization was not affected after

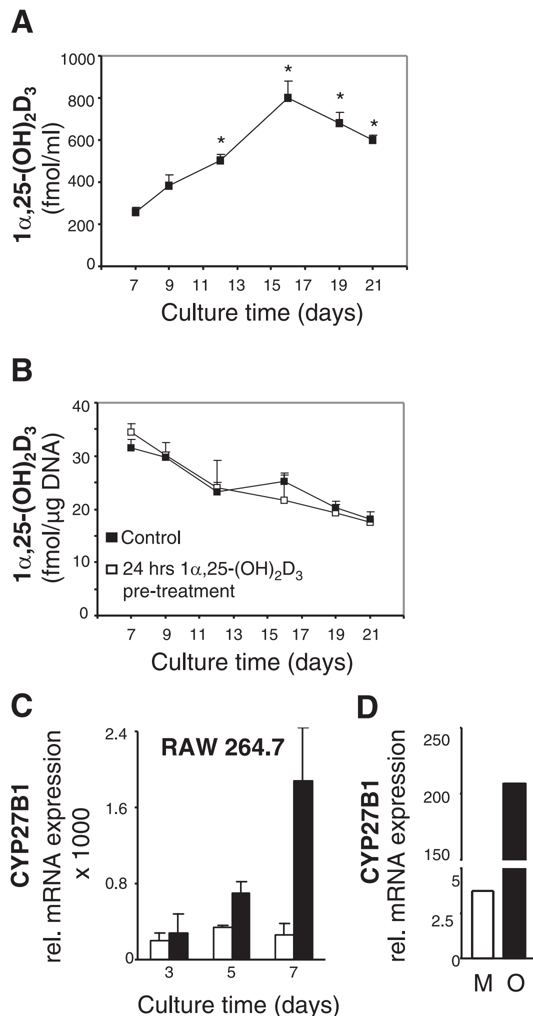

Figure 4. Regulation of 1α-hydroxylase activity. *A)* Total production of 1α,25-$(OH)_2D_3$ at various time points during human osteoblast differentiation, incubated for 48 h with 1000 nM 25-$(OH)D_3$ (*$P<0.01$ *vs.* day 7). *B)* 1α,25-$(OH)_2D_3$ production corrected for total number of cells and effect of 1α,25-$(OH)_2D_3$ preincubation on 1α-hydroxylase activity during human osteoblast differentiation. *C)* Real-time PCR analysis of CYP27B1 expression in murine RAW 264.7 monocytic cells (□) and during osteoclast development (+ RANKL) (■) at days 3, 5, and 7. *D)* Real-time PCR analysis of CYP27B1 expression in human monocytes (M) and osteoclasts (M-CSF/RANKL induced) (O).

short-term incubation of 25-$(OH)D_3$ or 1α,25-$(OH)_2D_3$ (data not shown).

Long-term (continuous) incubations showed no effect on total DNA (**Fig. 7*A***), but treatment with 100 nM 25-$(OH)D_3$ significantly increased ALP activity at days 7 and 14 (Fig. 7*B*) and significantly increased mineralization in the early phase of mineralization at day 14, but not at day 21 (Fig. 7*C*).

DISCUSSION

The current study clearly demonstrates the production of 1α,25-$(OH)_2D_3$ by bone cells. Studies of the autocrine/paracrine actions of 1α-hydroxylase in bone have been relatively limited. To date there are only a few reports from ~25 years ago and a recent paper indicating expression of CYP27B1 in rat femur (21–23, 28). Our current study has taken this further by demonstrating the presence of 1α-hydroxylase mRNA in human bone biopsies as well as in cultured human and murine osteoblasts and osteoclasts. 1α-Hydroxylase protein was detected in human osteoblasts by using the same antibody (Ab) as that shown to detect 1α-hydroxylase expression in human kidney cells (29). A relationship between malignant status and 1α-hydroxylase expression has been described for several extrarenal tissues like colon tumors (higher expression) (30, 31) and prostate cancers (lower expression) (14, 32). In bone cells, the expression of CYP27B1 in the osteoblast-like osteosarcoma cell line MG-63 is highly increased compared with normal osteoblasts.

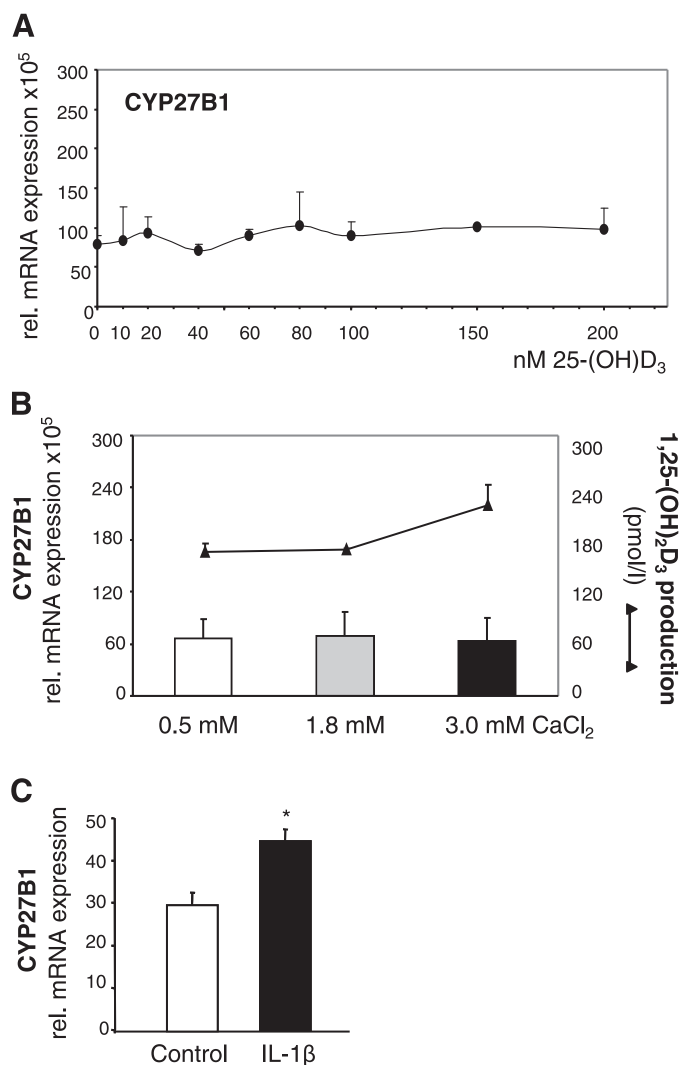

Figure 5. Effects on CYP27B1 mRNA expression. Relative CYP27B1 mRNA expression was measured at day 14 of SV-HFO cell culture after 24 h incubation with *A)* various concentrations of 25-$(OH)D_3$, *B)* various concentrations of $CaCl_2$ (bars), 1α,25-$(OH)_2D_3$ production under the same conditions was also measured (line), and *C)* 10 ng/ml IL-1β. Data are presented as means ± SE. *$P < 0.005$ *vs.* control.

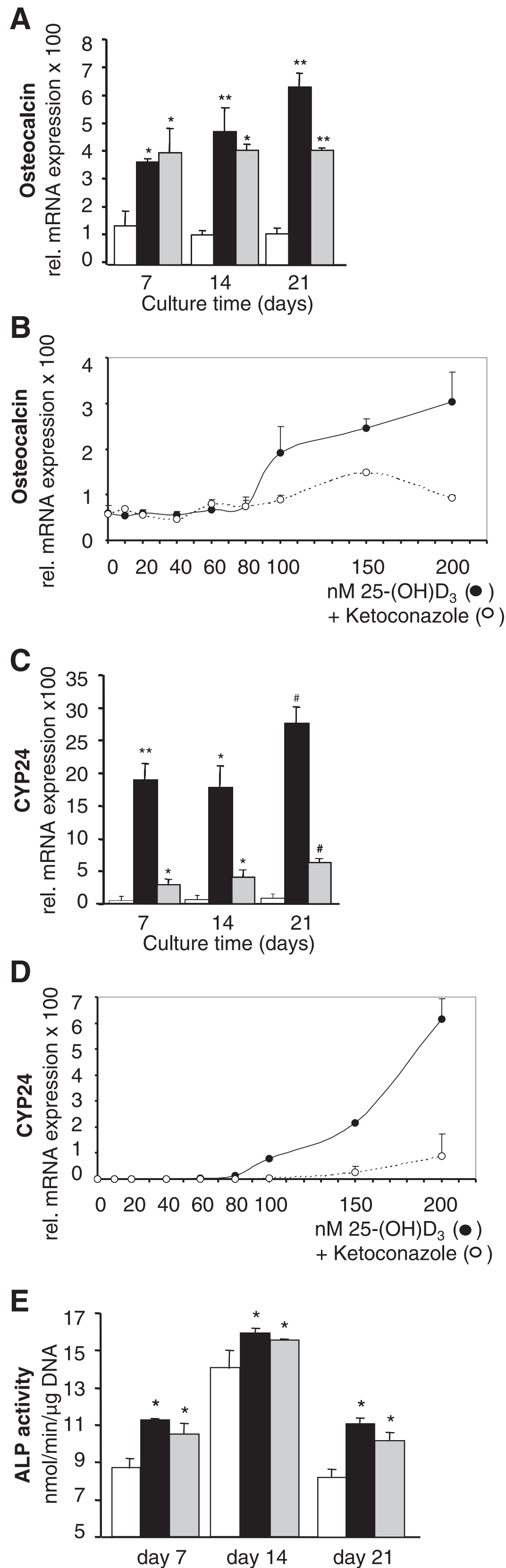

Figure 6. Effect of 25-(OH)D_3 incubation on human osteoblast gene expression and osteoblast function. Human preosteoblasts were induced to differentiate and at various time points during differentiation treated for 48 h with 1000 nM 25-(OH)D_3 (■) or 10 nM 1α,25-$(OH)_2D_3$ (■) (vehicle (□)) and analyzed for effects on *A)* OC mRNA expression and *C)* CYP24 mRNA expression by real-time PCR and calculated as described in Materials and Methods. *B)* OC and *D)* CYP24 mRNA expression was measured after incubation with various concentrations of 25-(OH)D_3 and 100 μM of ketoconazole for 24 h at day 14 of SV-HFO culture, *E)* ALP activity. Data are shown as means ± SD. *$P < 0.05$; **$P < 0.01$, and #$P < 0.005$ *vs.* control at the same day.

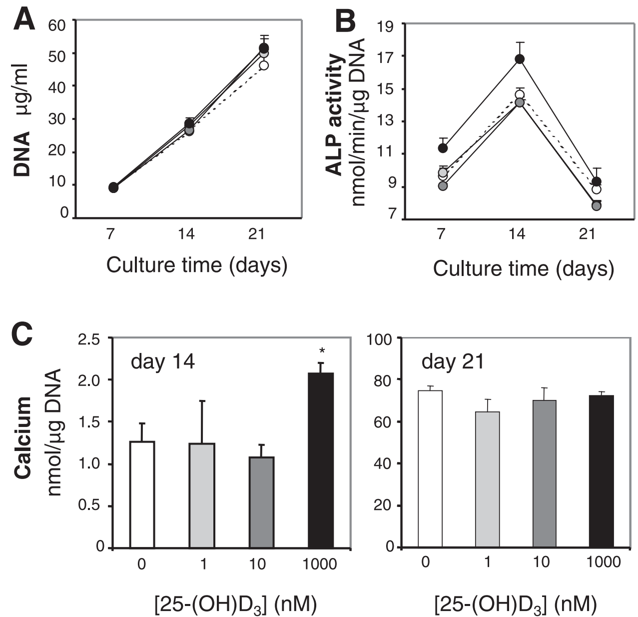

Figure 7. Effect of long-term incubation of 25-(OH)D_3 on human osteoblast differentiation and mineralization. Human preosteoblasts were induced to differentiate and from day 2 on continuously incubated with various concentrations of 25-(OH)D_3: 1 nM, 10 nM, 100 nM *vs.* vehicle (dotted line). Effects were measured on days 7, 14 and 21 on *A)* total DNA, *B)* ALP activity; *$P < 0.05$ *vs.* control of the same day, and *C)* mineralization; $P < 0.025$ *vs.* control of the same day.

Besides 1α-hydroxylase, human osteoblasts expressed other components of the 1α,25-$(OH)_2D_3$ endocrine system. These include the two membrane proteins, megalin and cubilin, which in the kidney are known to be important for cellular uptake of 25-(OH)D_3 associated with DBP (33–35). Two P450 enzymes, CYP2R1 and CYP3A4, identified to have 25-hydroxylase activity and to convert vitamin D_3 into 25-(OH)D_3 (36), were also expressed in human osteoblasts. These data imply that human osteoblasts can function as an almost autonomous 1α,25-$(OH)_2D_3$ producing cell. To date the only tissue that has been shown to be entirely autonomous in synthesizing 1α,25-$(OH)_2D_3$ is human skin, as this can also produce vitamin D_3 from 7-dehydrocholestrol (15, 37). In mouse skin this may be different, as no 1α-hydroxylase promoter activity was found recently in the skin of CYP27B1 null mice (38).

1α-Hydroxylase activity was shown by a time- and dose-dependent production of 1α,25-$(OH)_2D_3$ after incubations of human osteoblasts with 25-(OH)D_3. Kinetics were on the same order of magnitude as that reported earlier for cultured human renal proximal

tubule cells (25). The 1α-hydroxylase activity and production of 1α,25-$(OH)_2D_3$ by osteoblasts was completely blocked by the P450 inhibitor ketoconazole, which is in line with data in kidney cells (25).

In keratinocytes there has been found an inverse relation between 1α,25-$(OH)_2D_3$ formation and differentiation (15, 16), but no differences in CYP27B1 expression at different stages of differentiation of either human or murine osteoblasts were observed. However, a small progressive decrease was observed in 1α,25-$(OH)_2D_3$ production per cell over time, suggesting an inverse relationship between 1α-hydroxylase activity and osteoblast differentiation. However, the CYP27B1 mRNA levels did not change throughout the 3 wk culture period, suggesting an additional differentiation-dependent regulatory step. The current data do not suggest a decrease in 1α-hydroxylase activity and 1α,25-$(OH)_2D_3$ formation due to decreased DPB binding as both megalin and cubilin mRNA expression did not change during osteoblast differentiation. In contrast to osteoblasts, induction of both murine and human osteoclast differentiation, which is dependent on the critical osteoclast inducer RANKL that acts via NF-κB, resulted in an increase in 1α-hydroxylase mRNA levels. This follows a similar pattern to that observed for other monocyte-derived cells such as macrophages and dendritic cells (19, 39).

Regulation by 1α,25-$(OH)_2D_3$ itself provides an elegant feedback mechanism to control 1α-hydroxylase activity (40–43). However, autocrine/paracrine 1α-hydroxylase activity in extrarenal tissues is modulated differently and is less sensitive to autoregulation by 1α,25-$(OH)_2D_3$ (44). Our data support this notion, as in human osteoblasts 1α-hydroxylase activity and CYP27B1 expression were unaffected by 1α,25-$(OH)_2D_3$. This is in line with the observation that CYP27B1 expression in rat femur is independent of circulating 1α,25-$(OH)_2D_3$ levels (28) and resembles data obtained in the skin (37).

CYP27B1 mRNA expression and activity were not affected by incubation with increasing doses of 25-$(OH)D_3$ or by two major regulators of renal 1α-hydroxylase activity: PTH and calcium. These data suggest alternative regulatory mechanisms in target tissues. We found that a potent regulator of CYP27B1 mRNA in human osteoblasts appeared to be the NF-κB activator IL-1β. This further substantiates the significance of immune modulatory cytokines and the 1α,25-$(OH)_2D_3$ endocrine system (17, 19). Studies have reported both a positive (19) and negative (45) effect of NF-κB on 1α-hydroxylase expression. However, the current IL-1β data together with the effects of RANKL incubations implicate a role for NF-κB in regulation of 1α-hydroxylase in bone cells. All in all, it indicates the existence of a local (autocrine/paracrine) regulation of 1α-hydroxylase by factors (growth factors, cytokines) derived from bone cells and/or cells in the bone marrow.

The functional biological significance of CYP27B1 expression and 1α-hydroxylase activity in osteoblasts is shown by the positive short-term effects of 25-$(OH)D_3$ on OC and CYP24 mRNA expression and ALP activity and by the positive long-term effects on ALP activity and mineralization. It is remarkable that the biological effects are much stronger when derived from the locally produced 1α,25-$(OH)_2D_3$ after 25-$(OH)D_3$ treatment than with directly added exogenous 1α,25-$(OH)_2D_3$. This is all the more interesting because the self-produced amounts of 1α,25-$(OH)_2D_3$ (~400–800 pM, as shown in the different 1α,25-$(OH)_2D_3$ production experiments) are much lower than the 10 nM of 1α,25-$(OH)_2D_3$ that is directly added. An explanation for this may include aspects related to the balance between synthesis and catabolism of 1α,25-$(OH)_2D_3$ when continuous production of 1α,25-$(OH)_2D_3$ takes place in the presence of 25-$(OH)D_3$ and direct availability at the site of action (binding to the vitamin D receptor in the osteoblasts). These data strongly support a two-step model similar to that which has already been demonstrated in skin cells (37) and in prostate cancer cells, where incubation with 25-$(OH)D_3$ also leads to regulation of cellular activity (46).

The absence of long-term effects on mineralization at day 21 is in line with that observed after direct 1α,25-$(OH)_2D_3$ incubations and can be attributed to a limitation of the culture system, *i.e.*, full mineralization has been reached in the culture well at ~day 21.

The significance of 1α-hydroxylase activity for the eventual biological response of 25-$(OH)D_3$ is shown by studies using ketoconazole. Blocking 1α,25-$(OH)_2D_3$ production by the P450 inhibitor ketoconazole almost completely blocked the effect of 25-$(OH)D_3$. This indicates that the enzyme that is blocked is 1α-hydroxylase, because the only substrate added is 25-$(OH)D_3$. However, direct effects of 25-$(OH)D_3$ itself may contribute to the overall effect because 25-$(OH)D_3$ can bind to the vitamin D receptor, though with a 50- to 600-fold lower affinity (47), depending on cell origin.

In conclusion, this study provides the unequivocal answer that human osteoblasts contain all the components necessary to fulfill an autocrine/paracrine action of vitamin D in bone. Specifically, we have demonstrated the expression of 1α-hydroxylase mRNA and protein, enzyme activity, and the induction of 1α,25-$(OH)_2D_3$-mediated responses in osteoblasts incubated with the inactive precursor molecule 25-$(OH)D_3$. It has been postulated that vitamin D plays a role in tissues that have a barrier function that is either immunological or truly physical (39). It is tempting to speculate that vitamin D in osteoblasts serves a similar role and regulates the mineralization process mediated by osteoblasts that cover the bone surface as a barrier. The current data provide a solid basis for further *in vivo* analyses to address these issues and clearly warrant development of osteoblast- as well as osteoclast-specific CYP27B1 knockout mice. These findings also hold a possible clue to explain the association studies between serum 25-$(OH)D_3$ levels and bone parameters like bone mineral density and fractures (48). In a general perspective, this study contributes to the currently emerging concept of steroid hormone production

within its target tissues. This implicates a transition from a hormone acting at a distant site of synthesis to a local factor acting in an auto/paracrine manner. FJ

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REFERENCES

1. Van Leeuwen, J. P., van Driel, M., van den Bemd, G. J., and Pols, H. A. (2001) Vitamin D control of osteoblast function and bone extracellular matrix mineralization. *Crit. Rev. Eukaryot. Gene Exp.* **11,** 199–226
2. Miyahara, T., Simoura, T., Osahune, N., Uchida, Y., Sakuma, T., Nemoto, N., Kozakai, A., Takamura, T., Yamazaki, R., Higuchi, S., *et al.* (2002) A highly potent 26,27-Hexafluoro-1a,25-dihydroxyvitamin D3 on calcification in SV40-transformed human fetal osteoblastic cells. *Calcif. Tissue Int.* **70,** 488–495
3. Christakos, S., Dhawan, P., Liu, Y., Peng, X., and Porta, A. (2003) New insights into the mechanisms of vitamin D action. *J. Cell. Biochem.* **88,** 695–705
4. Barbour, G. L., Coburn, J. W., Slatopolsky, E., Norman, A. W., and Horst, R. L. (1981) Hypercalcemia in an anephric patient with sarcoidosis: evidence for extrarenal generation of 1,25-dihydroxyvitamin D. *N. Engl. J. Med.* **305,** 440–443
5. Adams, J. S., and Gacad, M. A. (1985) Characterization of 1 alpha-hydroxylation of vitamin D3 sterols by cultured alveolar macrophages from patients with sarcoidosis. *J. Exp. Med.* **161,** 755–765
6. Segersten, U., Correa, P., Hewison, M., Hellman, P., Dralle, H., Carling, T., Akerstrom, G., and Westin, G. (2002) 25-hydroxyvitamin D_3-1alpha-hydroxylase expression in normal and pathological parathyroid glands. *J. Clin. Endocrinol. Metab.* **87,** 2967–2972
7. Correa, P., Segersten, U., Hellman, P., Akerstrom, G., and Westin, G. (2002) Increased 25-hydroxyvitamin D3 1alpha-hydroxylase and reduced 25-hydroxyvitamin D3 24-hydroxylase expression in parathyroid tumors–new prospects for treatment of hyperparathyroidism with vitamin D. *J. Clin. Endocrinol. Metab.* **87,** 5826–5829
8. Friedrich, M., Rafi, L., Mitschele, T., Tilgen, W., Schmidt, W., and Reichrath, J. (2003) Analysis of the vitamin D system in cervical carcinomas, breast cancer and ovarian cancer. *Recent Results Cancer Res.* **164,** 239–246
9. Evans, K. N., Taylor, H., Zehnder, D., Kilby, M. D., Bulmer, J. N., Shah, F., Adams, J. S., and Hewison, M. (2004) Increased expression of 25-hydroxyvitamin D-1alpha-hydroxylase in dysgerminomas: a novel form of humoral hypercalcemia of malignancy. *Am. J. Pathol.* **165,** 807–813
10. Jones, G., Ramshaw, H., Zhang, A., Cook, R., Byford, V., White, J., and Petkovich, M. (1999) Expression and activity of vitamin D-metabolizing cytochrome P450s (CYP1alpha and CYP24) in human nonsmall cell lung carcinomas. *Endocrinology* **140,** 3303–3310
11. Schwartz, G. G., Whitlatch, L. W., Chen, T. C., Lokeshwar, B. L., and Holick, M. F. (1998) Human prostate cells synthesize 1,25-dihydroxyvitamin D3 from 25-hydroxyvitamin D3. *Cancer Epidemiol. Biomarkers Prev.* **7,** 391–395
12. Chen, T. C., Wang, L., Whitlatch, L. W., Flanagan, J. N., and Holick, M. F. (2003) Prostatic 25-hydroxyvitamin D-1alpha-hydroxylase and its implication in prostate cancer. *J. Cell. Biochem.* **88,** 315–322
13. Ma, J. F., Nonn, L., Campbell, M. J., Hewison, M., Feldman, D., and Peehl, D. M. (2004) Mechanisms of decreased vitamin D 1alpha-hydroxylase activity in prostate cancer cells. *Mol. Cell. Endocrinol.* **221,** 67–74
14. Whitlatch, L. W., Young, M. V., Schwartz, G. G., Flanagan, J. N., Burnstein, K. L., Lokeshwar, B. L., Rich, E. S., Holick, M. F., and Chen, T. C. (2002) 25-Hydroxyvitamin D-1alpha-hydroxylase activity is diminished in human prostate cancer cells and is enhanced by gene transfer. *J. Steroid Biochem. Mol. Biol.* **81,** 135–140
15. Van Leeuwen, J. P., Pols, H. A., van den Bemd, G. C., Kempenaar, J., Thio, H. B., Birkenhager, J. C., and Ponec, M. (1994) Role of extracellular calcium in the regulation of 1,25-dihydroxyvitamin D3 formation in cultured human keratinocytes. *Biochim. Biophys. Acta* **1221,** 167–170
16. Zehnder, D., Bland, R., Williams, M. C., McNinch, R. W., Howie, A. J., Stewart, P. M., and Hewison, M. (2001) Extrarenal expression of 25-hydroxyvitamin D_3-1 alpha-hydroxylase. *J. Clin. Endocrinol. Metab.* **86,** 888–894
17. Zehnder, D., Bland, R., Chana, R. S., Wheeler, D. C., Howie, A. J., Williams, M. C., Stewart, P. M., and Hewison, M. (2002) Synthesis of 1,25-dihydroxyvitamin D_3 by human endothelial cells is regulated by inflammatory cytokines: a novel autocrine determinant of vascular cell adhesion. *J. Am. Soc. Nephrol.* **13,** 621–629
18. Bland, R., Markovic, D., Hills, C. E., Hughes, S. V., Chan, S. L., Squires, P. E., and Hewison, M. (2004) Expression of 25-hydroxyvitamin D3–1alpha-hydroxylase in pancreatic islets. *J. Steroid Biochem. Mol. Biol.* **89–90,** 121–125
19. Hewison, M., Freeman, L., Hughes, S. V., Evans, K. N., Bland, R., Eliopoulos, A. G., Kilby, M. D., Moss, P. A., and Chakraverty, R. (2003) Differential regulation of vitamin D receptor and its ligand in human monocyte-derived dendritic cells. *J. Immunol.* **170,** 5382–5390
20. Zehnder, D., Evans, K. N., Kilby, M. D., Bulmer, J. N., Innes, B. A., Stewart, P. M., and Hewison, M. (2002) The ontogeny of 25-hydroxyvitamin D_3 1alpha-hydroxylase expression in human placenta and decidua. *Am. J. Pathol.* **161,** 105–114
21. Turner, R. T., Puzas, J. E., Forte, M. D., Lester, G. E., Gray, T. K., Howard, G. A., and Baylink, D. J. (1980) In vitro synthesis of 1 alpha,25-dihydroxycholecalciferol and 24,25-dihydroxycholecalciferol by isolated calvarial cells. *Proc. Natl. Acad. Sci. U. S. A.* **77,** 5720–5724
22. Howard, G. A., Turner, R. T., Sherrard, D. J., and Baylink, D. J. (1981) Human bone cells in culture metabolize 25-hydroxyvitamin D3 to 1,25-dihydroxyvitamin D3 and 24,25-dihydroxyvitamin D3. *J. Biol. Chem.* **256,** 7738–7740
23. Pols, H. A., Schilte, H. P., Nijweide, P. J., Visser, T. J., and Birkenhager, J. C. (1984) The influence of albumin on vitamin D metabolism in fetal chick osteoblast-like cells. *Biochem. Biophys. Res. Commun.* **125,** 265–272
24. Van der Eerden, B. C., Hoenderop, J. G., de Vries, T. J., Schoenmaker, T., Buurman, C. J., Uitterlinden, A. G., Pols, H. A., Bindels, R. J., and van Leeuwen, J. P. (2005) The epithelial Ca^{2+} channel TRPV5 is essential for proper osteoclastic bone resorption. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 17507–17512
25. Bland, R., Walker, E. A., Hughes, S. V., Stewart, P. M., and Hewison, M. (1999) Constitutive expression of 25-hydroxyvitamin D3–1alpha-hydroxylase in a transformed human proximal tubule cell line: evidence for direct regulation of vitamin D metabolism by calcium. *Endocrinology* **140,** 2027–2034
26. Karsten, U., and Wollenberger, A. (1977) Improvements in the ethidium bromide method for direct fluorometric estimation of DNA and RNA in cell and tissue homogenates. *Anal. Biochem.* **77,** 464–470
27. Lowry, O. H., Roberts, N. R., Wu, M. L., Hixon, W. S., and Crawford, E. J. (1954) The quantitative histochemistry of brain. II. Enzyme measurements. *J. Biol. Chem.* **207,** 19–37
28. Anderson, P. H., O'Loughlin, P. D., May, B. K., and Morris, H. A. (2005) Modulation of CYP27B1 and CYP24 mRNA expression in bone is independent of circulating $1,25(OH)_2D3$ levels. *Bone* **36,** 654–662
29. Zehnder, D., Bland, R., Walker, E. A., Bradwell, A. R., Howie, A. J., Hewison, M., and Stewart, P. M. (1999) Expression of 25-hydroxyvitamin D3–1alpha-hydroxylase in the human kidney. *J. Am. Soc. Nephrol.* **10,** 2465–2473
30. Cross, H. S., Bareis, P., Hofer, H., Bischof, M. G., Bajna, E., Kriwanek, S., Bonner, E., and Peterlik, M. (2001) 25-Hydroxyvitamin D_3-1alpha-hydroxylase and vitamin D receptor gene expression in human colonic mucosa is elevated during early cancerogenesis. *Steroids* **66,** 287–292
31. Bareis, P., Bises, G., Bischof, M. G., Cross, H. S., and Peterlik, M. (2001) 25-hydroxy-vitamin D metabolism in human colon can-

cer cells during tumor progression. *Biochem. Biophys. Res. Commun.* **285,** 1012–1017
32. Hsu, J. Y., Feldman, D., McNeal, J. E., and Peehl, D. M. (2001) Reduced 1alpha-hydroxylase activity in human prostate cancer cells correlates with decreased susceptibility to 25-hydroxyvitamin D3-induced growth inhibition. *Cancer Res.* **61,** 2852–2856
33. Nykjaer, A., Fyfe, J. C., Kozyraki, R., Leheste, J. R., Jacobsen, C., Nielsen, M. S., Verroust, P. J., Aminoff, M., de la, C. A., Moestrup, S. K., Ray, R., *et al.* (2001) Cubilin dysfunction causes abnormal metabolism of the steroid hormone 25(OH) vitamin D_3. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 13895–13900
34. Hilpert, J., Wogensen, L., Thykjaer, T., Wellner, M., Schlichting, U., Orntoft, T. F., Bachmann, S., Nykjaer, A., and Willnow, T. E. (2002) Expression profiling confirms the role of endocytic receptor megalin in renal vitamin D3 metabolism. *Kidney Int.* **62,** 1672–1681
35. Willnow, T. E., and Nykjaer, A. (2002) Pathways for kidney-specific uptake of the steroid hormone 25-hydroxyvitamin D3. *Curr. Opin. Lipidol.* **13,** 255–260
36. Prosser, D. E., and Jones, G. (2004) Enzymes involved in the activation and inactivation of vitamin D. *Trends Biochem. Sci.* **29,** 664–673
37. Schuessler, M., Astecker, N., Herzig, G., Vorisek, G., and Schuster, I. (2001) Skin is an autonomous organ in synthesis, two-step activation and degradation of vitamin D_3: CYP27 in epidermis completes the set of essential vitamin D_3-hydroxylases. *Steroids* **66,** 399–408
38. Vanhooke, J. L., Prahl, J. M., Kimmel-Jehan, C., Mendelsohn, M., Danielson, E. W., Healy, K. D., and DeLuca, H. F. (2006) CYP27B1 null mice with LacZreporter gene display no 25-hydroxyvitamin D3–1{alpha}-hydroxylase promoter activity in the skin. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 75–80
39. Hewison, M., Zehnder, D., Chakraverty, R., and Adams, J. S. (2004) Vitamin D and barrier function: a novel role for extra-renal 1 alpha-hydroxylase. *Mol. Cell. Endocrinol.* **215,** 31–38
40. Takeyama, K., Kitanaka, S., Sato, T., Kobori, M., Yanagisawa, J., and Kato, S. (1997) 25-Hydroxyvitamin D3 1alpha-hydroxylase and vitamin D synthesis. *Science* **277,** 1827–1830
41. Bland, R., Zehnder, D., Hughes, S. V., Ronco, P. M., Stewart, P. M., and Hewison, M. (2001) Regulation of vitamin D-1alpha-hydroxylase in a human cortical collecting duct cell line. *Kidney Int.* **60,** 1277–1286
42. Bikle, D. D., Nemanic, M. K., Gee, E., and Elias, P. (1986) 1,25-Dihydroxyvitamin D3 production by human keratinocytes. Kinetics and regulation. *J. Clin. Invest.* **78,** 557–566
43. Henry, H. L., Dutta, C., Cunningham, N., Blanchard, R., Penny, R., Tang, C., Marchetto, G., and Chou, S. Y. (1992) The cellular and molecular regulation of $1,25(OH)_2D3$ production. *J. Steroid Biochem. Mol. Biol.* **41,** 401–407
44. Hewison, M., Zehnder, D., Bland, R., and Stewart, P. M. (2000) 1alpha-Hydroxylase and the action of vitamin D. *J. Mol. Endocrinol.* **25,** 141–148
45. Ebert, R., Jovanovic, M., Ulmer, M., Schneider, D., Meissner-Weigl, J., Adamski, J., and Jakob, F. (2004) Down-regulation by nuclear factor kappaB of human 25-hydroxyvitamin D3 1alpha-hydroxylase promoter. *Mol. Endocrinol.* **18,** 2440–2450
46. Schwartz, G. G., Eads, D., Rao, A., Cramer, S. D., Willingham, M. C., Chen, T. C., Jamieson, D. P., Wang, L., Burnstein, K. L., Holick, M. F., and Koumenis, C. (2004) Pancreatic cancer cells express 25-hydroxyvitamin D-1 alpha-hydroxylase and their proliferation is inhibited by the prohormone 25-hydroxyvitamin D3. *Carcinogenesis* **25,** 1015–1026
47. Bouillon, R., Okamura, W. H., and Norman, A. W. (1995) Structure-function relationships in the vitamin D endocrine system. *Endocr. Rev.* **16,** 200–257
48. Lips, P. (2001) Vitamin D deficiency and secondary hyperparathyroidism in the elderly: consequences for bone loss and fractures and therapeutic implications. *Endocr. Rev.* **22,** 477–501

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