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Rat growth plate chondrocytes express low levels of 25-hydroxy-1α-hydroxylase☆

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Abstract

Long standing disturbances of Vitamin D-metabolism as well as null-mutant animals for 25-hydroxy-1α-hydroxylase results in disorganised growth plates. Cultured chondrocytes were shown to be target for the hydroxylated Vitamin D-metabolites 1α,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$. Because studies on production of these metabolites were inconclusive in in vitro systems, the expression of the Vitamin D-system was examined in rat growth plate chondrocytes in vitro as well as ex vivo. Gene expression for 25-hydroxy-1α-hydroxylase, 25-hydroxy-24-hydroxylase as well as Vitamin D-receptor and collagen II and X were analysed on mRNA level by RT-PCR and quantitative real-time PCR, on protein level by western blotting and by immunohistochemistry in isolated growth plate chondrocytes or intact growth plates. Compared to UMR or $CaCo_2$ cells and renal homogenates cultured growth plate chondrocytes expressed low levels of 25-hydroxy-1α-hydroxylase mRNA and 25-hydroxy-24-hydroxylase mRNA. The expression of both was modulated by 25$(OH)D_3$, but 1α,25$(OH)_2D_3$ affected only 25-hydroxy-24-hydroxylase. These data were confirmed by Western blotting. Immunohistochemistry demonstrated predominant staining for 25-hydroxy-1α-hydroxylase in chondrocyte nodules and cells embedded in matrix in vitro. Ex vivo, 25-hydroxy-1α-hydroxylase was detected predominantly in late proliferative and hypertrophic zone of the growth plate. In conclusion, growth plate chondrocytes express the key components for a paracrine/autocrine Vitamin D-system.

Keywords: Chondrocytes; 25-Hydroxy-1α-hydroxylase; 25-Hydroxy-24-hydroxylase; Growth plate; Vitamin D

1. Introduction

Growth plate chondrocytes express the Vitamin D receptor (VDR) in vitro as well as in vivo [1]. The hydroxylated Vitamin D metabolites 1α,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ were shown to act on proliferation and specific cell functions of growth plate chondrocytes in in vitro systems [1–3]. The mitochondrial enzyme 24-hydroxy-1α-hydroxylase (1α-OHase) plays an important role in calcium homeostasis by catalysing synthesis of the active form of Vitamin D, 1α,25$(OH)_2D_3$ in the kidney as well as in granulomatous tissue [4]. In vitro production of polar Vitamin D metabolites was described in passaged chondrocytes, but not in primary cultures [1,5]. Furthermore, growth plate chondrocytes in culture tend to dedifferentiate, i.e. loose specific chondrocyte functions, such as VDR or collagen type II expression [3,6]. Therefore, the expression of 1α-OHase and other components of a potential auto/paracrine Vitamin D system was investigated in chondrocytes in vitro as well as ex vivo in the growth plate.

2. Methods

2.1. Cell culture

Chondrocytes were isolated and cultured as described earlier by Benya and Schaffer [6] modified by Klaus et al. [3]. Cells were cultured in flasks or in 60 mm culture dishes. The chondrocytes grew in F12/DMEM 7/5 medium supplemented with 8% FCS, 10 mM HEPES, 2 mM pyruvate, 2 mM L-glutamine, 10 mg/ml penicillin/streptomycin. Ionised calcium, measured by a calcium sensitive electrode, was 1.2 mmol/l. UMR and $CaCo_2$ in DMEM (calcium 0.83 mmol/l) with the same supplements. Chondrocytes became confluent within 6–12 days and were passaged into 60 mm culture dishes or on glass cover slides as described [7,8]. For experiments, cell cycles were synchro-

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nised by starvation in F12-medium (calcium 0.82 mmol/l) and DMEM/MCDB 1/1 (calcium 0.47 mmol/l) for chondrocytes and UMR and $CaCo_2$ cells, respectively [3]. Vitamin D metabolites (gift of Dr. Armbruster, Fa. Imundiagnostik, Bensheim, Germany) were dissolved in ethanol. Maximal final ethanol concentration was 0.05%.

2.2. Preparation of tissue samples

Freshly isolated growth plates free of perichondrium and surrounding connective tissue or rat kidney slices were frozen immediately with liquid nitrogen and pulverised using a mortar. Thereafter the samples were transferred into lysis buffer and sonificated (2×5 s) on ice for RNA or protein isolation. Further development of the samples see sections PCR and Western blotting.

2.3. Reverse transcriptase-polymerse-chain-reaction (RT-PCR)

2.3.1. RNA preparation in vitro

Total RNA was isolated from first passage monolayer cultures of cells or from developed tissue samples (see Section 2.2). We utilised the high Pure RNA Isolation Kit from Roche. RNA concentration (average 1.06 μg/ μl) was quantitated by measuring the absorbency at 230, 260 and 280 nm. Reverse transkriptase-polymerase-chain-reaction (RT-PCR) of 1α-OHase, 25-hydroxy-24-hydroxylase (24-OHase), VDR, collagen II and X (COLL II, COLL X), and GAPDH. After DNase digestion 2 μg of RNA were transcribed into cDNA using superScript II (invitroGen, Karlsruhe, Germany), PCR Core Kit and Taq-DNA-polymerase (Roche Diagnostics, Mannheim, Germany) for master mix and the cDNA template with the specific primer pairs (Table 1). All primers were deduced from rat sequences. After an initial preincubation step of 10 min, 98 °C the amplification profile consisted of denaturation at 93 °C for 30 s, annealing at 58 °C for 30 s and extension of DNA at 72 °C for 30 s after a 10 min denaturation step at 72 °C. The conditions for RT-PCR using in situ RNA were 30 cycles for all primers except for the 1α-OHase and 24-OHase, for which 52 cycles were run (Table 1). The amplified products were detected by electrophoresis in a 1.5% agarose gel, visualised by ethidium bromide staining and ultraviolet transilumination and compared with a 100 bp-ladder as standard. Bands were detected and evaluated densitometrically by a commercially available computer program (Bio-1 D V.96., Vilber Lourmat, France). The PCR products were verified by sequencing. To exclude any soiling for every sample a parallel control reaction (without cDNA) was tested.

2.4. Quantitative real-time PCR

Quantitative real-time PCR was performed using the PCR Core Kit to prepare Universal Master mix with SYBR Green to detect PCR products at the end of each amplification step. PCR conditions were 10 min 98 °C, followed by 50 cycles of 30 s at 93 °C and 30 s at 58 °C. Relative quantities of RNA levels were determined accounting for amplification efficacy using the software provided with the PCR iCycler iQ-system.

2.5. Antibodies

Polyclonal sheep anti-mouse IgG antibodies against the 25-hydroxyvitamin D_3-1α-hydroxylase (PC290) were purchased from The Binding Site Limited (Birmingham, UK), monoclonal antibody against VDR 9A7y were from Daco (Hamburg, Germany). Polyclonal-rabbit-antibodies against collagen type II were purchased from Biotrend Chemicals GmbH (Cologne, Germany). Monoclonal anti-collagen type X antibody (mouse) was from Quartett (Berlin, Germany).

2.6. Western blot analysis (western immunobloting)

Cells were washed twice with ice-cold PBS and lysed in protein lysis buffer (20 mM Tris–HCl, pH 8, 150 mM NaCl, 1% Triton X) containing a cocktail of proteinase and phosphatase inhibitor (20 mM NaF, 2 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml apro-

Table 1
Primer sequences

	Sequence	Cycles	Loading (ng per lane)
GAPDH (345 bp)	F1: 5′-GCT GGG GCT CAC CTG AAG GG-3′ R1: 5′-GGA TGA CCT TGC CCA CAG CC-3′	30	1.7
VDR (328 bp)	F1: 5′-GCC CAC CAC AAG ACC TAT-3′ R1: 5′-CCT TTT GGA TGC TGT AAC TG-3′	30	4.0
1α-OHase (300 bp)	F1: 5′-TGG GCA AGA GTA CCA GAT CC-3′ R1: 5′-ATG TCA GGG TCC ACC AGT TC-3′	52	12.0
24-OHase (190 bp)	F1: 5′-CCT GCT GGA AGC TCT GTA CC-3′ R1: 5′-CGG GCT TCA TGA GTT TCT TC-3′	52	12.0
COLLl II (361 bp)	F1: 5′-CTC CAG GTG TGA AGG GTG AG-3′ R1: 5′-GAA CCT TGA GCA CCT TCA GG-3′	30	4.0
COLLl X (348 bp)	F1: 5′-TGC CTC TTG TCA GTG CTA AC-3′ R1: 5′-GCG TGC CTG TCT TAT ACA GG-3′	30	4.0

tinin, 3 mM benzamidine). Protein extracts were obtained after sonocation of cell lysates 2 × 5 s and centrifugation at 8000 × *g* at +4 °C. Protein was quantified with the Bio-Rad protein colorimetric assay. After addition of sample buffer to the cellular extract and boiling at 96 °C for 5 min 40 μg of total protein lysate were separated on a 10% SDS-polyacrylamide gel. Proteins were transferred onto nitrocellulose membrane. After blocking the membrane blots were incubated overnight at +4 °C with a 1:1000 dilution of primary antibody for 1α-OHase. The secondary HPO-conjugated antibody were diluted at 1:2000 and incubated with the membrane for another 60 min. After chemiluminescence reaction (ECL, Amersham Pharmacia Biotech, Freiburg, Germany), bands were detected after expose to Hyperfilm-MP (Amersham International Plc, Buckinghamshire, UK) and evaluated densitometrically by a commercially available computer program (Bio-1 D V.96., Vilber Lourmat).

2.7. Immunohistochemistry

2.7.1. Tissue preparation

For immunohistochemistry the whole epiphyseal plate was dissected. The isolated tissue was immediately used for frozen sections.

2.7.2. Immunohistological staining

For immunohistology the avidin–biotin technique or the APAAP method was used. Therefore, treated cells on cover slips or frozen sections (4 μm) were fixed in paraformaldehyde (4%). Polyclonal sheep antibodies against 25-hydroxy-1α-hydroxylase PC290 (1:200) as well as a monoclonal rat antibody against VDR 9A7y (1:200) were incubated for 16 h at 4 °C. Further processing was as described earlier [1,9]. Finally, all slides were incubated with DAB or Fast Red for 30 min.

2.7.3. Statistics

Experimental values ± S.D. were expressed as percentage of the control values. Statistical analysis were carried out by ANOVA (Kruskal–Wallis one way analysis of variance on ranks) on SPSS 7.5 software. A *P*-value of <0.05 was considered statistically significant.

3. Results

Subconfluent cultured chondrocytes expressed COLL II, small amounts of COLL X mRNA and VDR mRNA (data not given). Cultures were homogeneously stained for VDR confirming previous studies (Fig. 2F). Using RT-PCR and real-time PCR, low basal expression of 1α-OHase mRNA was found in cultured growth plate chondrocytes. In comparison, rat kidney homogenates, UMR cells or $CaCo_2$ cells expressed 30-, 5- and 2-fold higher levels of 1α-OHase mRNA (Table 2). Furthermore, 24-OHase mRNA was detected in cultured chondrocytes, kidney homogenates as well as in UMR and $CaCo_2$ cells.

Quantitated by real-time PCR, the precursor $25(OH)D_3$ dose dependently [10^{-11}–10^{-7} M] increased 1α-OHase, 24-OHase and VDR mRNA in chondrocytes within 8 h with a further increase after 20 h of incubation. The maximal effect was seen with 10^{-9} M $25(OH)D_3$ (Table 2). In contrast, the polar vitamin D metabolite $1\alpha,25(OH)_2D_3$ did not significantly modulate 1α-OHase mRNA, but VDR mRNA and 24-OHase mRNA was increased, as expected. The stereoisomer $1ß,25(OH)_2D_3$ was without effect (Table 2).

Western blot analysis were carried out to further characterise 1α-OHase expression in cultured chondrocytes, UMR and $CaCo_2$ cells as well as in kidney homogenates. Cultured chondrocytes expressed 1α-OHase protein. In preliminary experiments, approximately 2-fold higher optical density of 1α-OHase was found when chondrocytes were cultured in the presence of low (0.82 mmol/l) ionised calcium in the

Table 2
Effect of $25(OH)D_3$ and $1,25(OH)_2D_3$ on expression of 1α-OHase, 24-OHase and VDR

Group	Real-time PCR ($\times10^{-3}$) (product/GAPDH)			RT-PCR (% of control)		
	1α-OHase	24-OHase	VDR	1α-OHase	24-OHase	VDR
Chondrocytes						
Control 4 h	0.48 ± 0.047	0.12 ± 0.033	0.06 ± 0.003	100 ± 7	100 ± 8	100 ± 15
$1,25(OH)_2D_3$ (10^{-12} M)	0.48 ± 0.05	0.06 ± 0.002	0.49 ± 0.05[a]	n.d.	n.d.	n.d.
$25(OH)D_3$ (10^{-9} M)	1.90 ± 0.45[a]	0.09 ± 0.003	0.24 ± 0.04[a]	338 ± 45[a]	106 ± 17	n.d.
Control 20 h	0.44 ± 0.05	0.09 ± 0.01	0.12 ± 0.04	105 ± 15	100 ± 13	110 ± 30
$1,25(OH)_2D_3$ (10^{-12} M)	0.97 ± 0.05	1.22 ± 0.25[a]	1.00 ± 0.09[a]	119 ± 11	204 ± 45[a]	520 ± 100[a]
$25(OH)D_3$ (10^{-11}) 20 h	1.95 ± 0.25[a]	0.19 ± 0.04	n.d.	229 ± 12[a]	n.d.	n.d.
$25(OH)D_3$ (10^{-9}) 20 h	3.17 ± 0.55[a]	0.10 ± 0.02	0.15 ± 0.02	477 ± 81[a]	247 ± 80[a]	309 ± 25[a]
$25(OH)D_3$ (10^{-7} M)	1.31 ± 0.20[a]	0.31 ± 0.02	n.d.	116 ± 12	n.d.	n.d.
$1ß(OH)_2D_3$ (10^{-9} M)	n.d.	n.d.	n.d.	115 ± 12	146 ± 22	n.d.
Growth plate	n.d.	n.d.	n.d.	200 ± 25[b]	268 ± 27[b]	180 ± 19[b]
$CaCo_2$	n.d.	n.d.	n.d.	405 ± 7[b]	224 ± 34	343 ± 20[b]
UMR	n.d.	n.d.	n.d.	482 ± 80	163 ± 27[b]	451 ± 74[b]
Kidney	n.d.	n.d.	n.d.	3356 ± 375[b]	238 ± 74[b]	1156 ± 239

[a] $P < 0.05$ vs. control; [b] $P < 0.05$ vs. chondrocyte control; n.d.: not done; data are given as mean ± S.D.

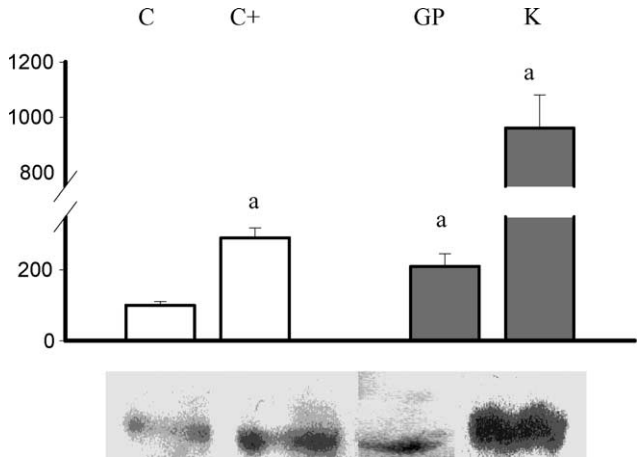

Fig. 1. Western blot analysis 1α-OHase in growth plate chondrocytes in vitro and ex vivo. Western analysis using a 1:200 dilution of the 1α-OHase antibody PCS290 and protein preparations from cultured growth plate chondrocytes (C), chondrocytes with stimulation by 25(OH)D_3 (10^{-9} M) (C+), whole growth plate homogenates (GP) or kidney homogenates. (K) Quantification by arbitrary units. (a) $P < 0.04$ vs. cultured chondrocytes (ANOVA).

medium compared to standard calcium (1.2 mmol/l) (data not given). Therefore, medium with low calcium was used for the following experiments. Homogenates of intact rat tibial growth plate also expressed 1α-OHase, 24-OHase and VDR 2–3-fold compared to cultured chondrocytes (low calcium) (Table 2). Western blotting also confirmed much higher basal levels of 1α-OHase in kidney homogenates compared to cultured chondrocytes (Fig. 1). In the latter, 1α-OHase protein was stimulated by 25(OH)D_3.

Immunohistochemistry demonstrated ubiquitous nuclear staining of VDR in cultured chondrocytes (Fig. 2F) as well as in intact growth plate (Fig. 2D). In contrast, 1α-OHase was visible in chondrocytes arranged in nodules or chondrocytes surrounded by extensive matrix in vitro indicating its expression in differentiated chondrocytes (Fig. 2E). The intracellular distribution was cytosolic. With respect to chondrocyte phenotype, the same pattern of 1α-OHase distribution was found in the intact growth plate. Whereas no constant staining was observed in the reserve zone, highest levels of 1α-OHase were expressed in late proliferating and in early hypertrophic zone (Fig. 2E), the former being characterised by presence of COLL II, the latter by presence of COLL X (Fig. 2A and B).

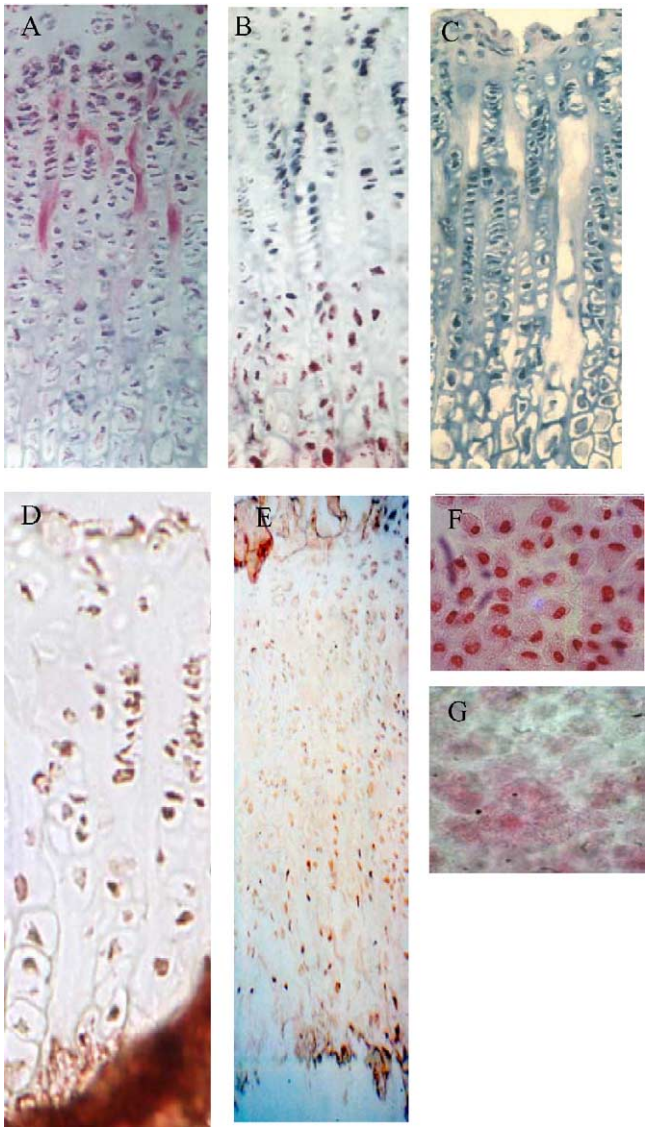

Fig. 2. Immunolocalisation of collagen II, collagen X, VDR, and 1α-OHase in cultured chondrocytes and in the intact growth plate. Immunolocalisation in growth plate (A–E) and cultured cells (F and G). Collagen II was predominately seen in proliferative zone of the growth plate (A), collagen X in hypertrophic zone (B). VDR was stained within the whole growth plate (D) and homogeneously in cultured chondrocytes (F); 1α-OHase staining was found in late proliferating (E1) and hypertrophic chondrocytes (E2) and in cultured chondrocytes embedded in matrix (G).

4. Discussion

To date, analysis of the expression of 1α-OHase in growth cartilage cells has relied upon studies on enzyme activity using radiolabelled 25(OH)D_3 as a substrate [1,4,5,10]. The present study demonstrates for the first time the expression of 1α-OHase in growth plate chondrocytes in vitro and ex vivo, extending the distribution of extrarenal 1α-OHase. This was shown on mRNA level, on protein level and by immunohistochemical analysis. The latter demonstrated that 1α-OHase expression is mainly restricted to mature chondrocytes. Furthermore, other components of a paracrine/autocrine Vitamin D system, i.e. VDR and 24-OHase, were confirmed in rat growth cartilage cells.

Extrarenal distribution of the 1α-OHase in normal and diseased tissue was based on studies of granulomatous tissue, which showed ectopic 1,25$(OH)_2D_3$ production [4,10]. In recent studies, the tissue distribution of 1α-OHase was further characterised by complementary RNA probes and polyclonal antisera. These studies demonstrated 1α-OHase in

the proximal and distal nephron, in skin (keratinocytes, hair follicles), granulomatous lymph nodes, colonic epithelial cells, endocrine organs (pancreas islets, adrenal medulla), placenta and in macrophages [11]. This widespread distribution of 1α-OHase suggests diverse (local) functions of $1,25(OH)_2D_3$ in peripheral tissues.

In primary cultures of tibial growth plate chondrocytes expressing a mainly proliferative phenotype, no $25(OH)D_3$ conversion to $1,25(OH)_2D_3$ could be measured in earlier experiments [1], but third passage costal chondrocytes did express 1α-OHase activity [5]. This difference might be due to different differentiation stages of the chondrocytes used in the two models. In VDR null mutant mice as well as in 1α-OHase null mutant mice, stunting of growth with widened growth plates were described [12,13]. In VDR null mutant mice, most histological changes could be reversed by normalising ambient calcium and phosphate [12]. All chondrocyte layers within the growth plate expressed the VDR, indicating local actions of the active Vitamin D metabolite $1,25(OH)_2D_3$. In view of these conflicting results it is still debated on, what function $1,25(OH)_2D_3$ exerts within the growth plate. The detection of 1α-OHase in addition to VDR and 24-OHase in growth plate chondrocytes points to a paracrine/autocrine role of the Vitamin D system. It is noteworthy, that in contrast to VDR 1α-OHase was not homogeneously distributed within the growth plate nor in cultured cells. The 1α-OHase expression was most intense in cell nodules or cells surrounded by extensive matrix in culture, in which we could demonstrate differentiated chondrocyte phenotype in earlier studies using the same culture system [7,8]. In the intact three-dimensional organisation of the growth plate, 1α-OHase expression was most intense in the early hypertrophic zone, corresponding to findings in the 1α-OHase null mutant mouse model, in which the hypertrophic zone was most widened and disorganised [12].

Messenger RNA for 1α-OHase is similar in renal and extrarenal tissues [14]. However, modulation of 1α-OHase activity is different in renal and extrarenal tissues. Whereas production of $1,25(OH)_2D_3$ is tightly regulated in proximal tubule cells, it is mainly substrate-dependent in granulomatous tissues [4]. Furthermore, $1,25(OH)_2D_3$ suppressed 1α-OHase expression in renal cells, but not in growth plate chondrocytes, in parallel to findings in macrophages or keratinocytes [4,15]. In contrast, low calcium stimulated 1α-OHase in the proximal renal tubule cells [16] as well as in our chondrocyte model.

In summary, this study demonstrates that growth plate chondrocytes express the key components of the Vitamin D system in vitro and in vivo indicating a auto/paracrine action of Vitamin D within the growth plate.

References

[1] G. Klaus, J. Merke, H. Eing, U. Hügel, P. Milde, H. Reichel, E. Ritz, O. Mehls, $1,25(OH)_2D_3$ receptor regulation and $1,25(OH)_2D_3$ effects in primary cultures of growth cartilage cells of the rat, Calcif. Tissue Int. 49 (1991) 340–348.

[2] Z. Schwartz, R.M. Gilley, V.L. Sylvia, D.D. Dean, B.D. Boyan, Prostaglandins mediate the effects of $1,25\text{-}(OH)_2D_3$ and $24,25\text{-}(OH)_2D_3$ on growth plate chondrocytes in a metabolite-specific and cell maturation-dependent manner, Bone 24 (1999) 475–484.

[3] G. Klaus, B. v. Eichel, T. May, U. Hügel, H. Mayer, E. Ritz, O. Mehls, Synergistic effects of parathyroid hormone and 1,25-dihydroxyvitamin D_3 on proliferation and vitamin D receptor expression of rat growth cartilage cells, Endocrinology 135 (1994) 1307–1315.

[4] S.E. Papapoulos, T.L. Clemens, L.J. Fraher, I.G. Lewin, L.M. Sandler, J.L. O'Riordan, 1,25-Dihydroxycholecalciferol in the pathogenesis of the hypercalcaemia of sarcoidosis, Lancet 1 (8117) (1979) 627–630.

[5] Z. Schwartz, B. Brooks, L. Swain, F. Del Toro, A. Norman, B. Boyan, Production of 1,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 by growth zone and resting zone chondrocytes is dependent on cell maturation and is regulated by hormones and growth factors, Endocrinology 130 (5) (1992) 2495–2504.

[6] P.D. Benya, J.D. Shaffer, Dedifferentiated chondrocytes re-express the dedifferentiated collagen phenotype when cultured in agarose gels, Cell 30 (1982) 215–224.

[7] N. Balmain, B. von Eichel, R. Toury, F. Belquasmi, M. Hauchecorne, G. Klaus, O. Mehls, E. Ritz, Calbindin-D28K and -D9K and $1,25(OH)_2$ vitamin D_3 receptor immunolocalization and mineralization induction in long-term primary cultures of rat epiphyseal chondrocytes, Bone 17 (1995) 37–45.

[8] K. Krohn, D. Haffner, U. Hügel, R. Himmele, G. Klaus, O. Mehls, F. Schaefer, $1,25(OH)_2D_3$ and dihydrotestosterone interact to regulate proliferation and differentiation of epiphyseal chondrocytes, Calcif. Tissue Int. 73 (2003) 400–410.

[9] C. Jux, K. Leiber, U. Hugel, W. Blum, C. Ohlsson, G. Klaus, O. Mehls, Dexamethasone impairs growth hormone (GH)-stimulated growth by suppression of local insulin-like growth factor (IGF)-I production and expression of GH- and IGF-I-receptor in cultured rat chondrocytes, Endocrinology 139 (1998) 3296–3305.

[10] J.S. Adams, O.P. Sharma, M.A. Gacad, F.R. Singer, Metabolism of 25-hydroxyvitamin D_3 by cultured pulmonary alveolar macrophages in sarcoidosis, J. Clin. Invest. 72 (5) (1983) 1856–1860.

[11] D. Zehnder, R. Bland, M.C. Williams, R.W. McNinch, A.J. Howie, P.M. Stewart, M. Hewison, Extrarenal expression of 25-hydroxyvitamin $D(3)^{-1}$ alpha-hydroxylase, J. Clin. Endocrinol. Metab. 86 (2) (2001) 888–894.

[12] Y.C. Li, M. Amling, A.E. Pirro, M. Priemel, J. Meuse, R. Baron, G. Delling, M.B. Demay, Normalization of mineral ion homeostasis by dietary means prevents hyperparathyroidism, rickets, and osteomalacia, but not alopecia in vitamin D receptor-ablated mice, Endocrinology 139 (10) (1998) 4391–4396.

[13] D.K. Panda, D. Miao, M.L. Tremblay, J. Sirois, R. Farookhi, G.N. Hendy, D. Goltzman, Targeted ablation of the 25-hydroxyvitamin D 1alpha-hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction, Proc. Natl. Acad. Sci. U.S.A. 98 (13) (2001) 7498–7503.

[14] S.J. Smith, A.K. Rucka, J.L. Berry, M. Davies, S. Mylchreest, C.R. Paterson, D.A. Heath, M. Tassabehji, A.P. Read, A.P. Mee, E.B. Mawer, Novel mutations in the 1alpha-hydroxylase (P450c1) gene in three families with pseudovitamin D-deficiency rickets resulting in loss of functional enzyme activity in blood-derived macrophages, J. Bone Miner. Res. 14 (5) (1999) 730–739.

[15] D.D. Bikle, M.K. Nemanic, E. Gee, P. Elias, 1,25-Dihydroxyvitamin D_3 production by human keratinocytes. Kinetics and regulation, J. Clin. Invest. 78 (2) (1986) 557–566.

[16] R. Bland, E.A. Walker, S.V. Hughes, P.M. Stewart, M. Hewison, Constitutive expression of 25-hydroxyvitamin D_3-1alpha-hydroxylase in a transformed human proximal tubule cell line: evidence for direct regulation of vitamin D metabolism by calcium, Endocrinology 140 (5) (1999) 2027–2034.