Calcif Tissue Int (1999) 64:414–421

Calcified
Tissue
International


# 1,25 Dihydroxyvitamin-$D_3$ Attenuates the Confluence-Dependent Differences in the Osteoblast Characteristic Proteins Alkaline Phosphatase, Procollagen I Peptide, and Osteocalcin

**H. Siggelkow,[1] H. Schulz, S. Kaesler, K. Benzler, M. J. Atkinson, M. Hüfner**

[1]Department of Gastroenterology and Endocrinology, University of Göttingen, Robert-Koch-Str. 40, 37075 Göttingen, Germany
[2]Department of Pathology, GSF München, Neuherberg, Germany

Received: 25 November 1997 / Accepted: 2 September 1998

**Abstract.** In the present study a cell culture model of primary human osteoblasts based on degrees of confluence was investigated by measuring basal and $1,25(OH)_2D_3$-stimulated levels of the osteoblast characteristic proteins alkaline phosphatase (AP), procollagen I-peptide (PICP), and osteocalcin (OC), as well as the corresponding gene expression. Primary osteoblast-like cell cultures from seven donors were treated in the second passage with $1,25(OH)_2D_3$ ($5 \times 10^{-8}$ M for 48 hours) and investigated at four stages of confluence (stage I 50%, stage II 75%, stage III 100%, and stage IV 7 days postconfluence). In untreated cultures passing through the different stages of confluence, we saw a 1.8-fold increase of AP activity, a 2.3-fold increase of OC secretion, but a decrease of PICP levels to 0.36-fold. Gene expression showed only minor variation between the different confluence stages. $1,25(OH)_2D_3$ did not significantly affect PICP production. Alkaline phosphatase protein was stimulated during proliferation until confluence, with no effect thereafter. Surprisingly, OC secretion and mRNA expression were stimulated in all four stages to the same absolute level independent of basal values. We conclude that our results correspond to other studies showing differentiation-stage dependent changes of basal levels of osteoblast-specific proteins. However, $1,25(OH)_2D_3$ stimulation decreased the confluence-dependent difference for AP and abolished it for osteocalcin, thus leading to a more differentiated phenotype of the osteoblast. Therefore, $1,25(OH)_2D_3$ stimulation might improve the reproducibility of results obtained at different confluence stages from cultures of clinical samples.

**Key words:** Human osteoblast — Differentiation — $1,25(OH)_2D_3$ — Osteocalcin — Procollagen I — Alkaline phosphatase.

Osteogenic differentiation has been extensively studied *in vitro* using rat calvarial cells cultured in conditions that facilitate the formation of a mineralized bone matrix [1, 2]. The progressive differentiation of osteoblasts in culture is associated with the expression of alkaline phosphatase, an early marker of the osteoblast phenotype. The subsequent deposition of a collagenous extracellular matrix is accompanied by a decrease of procollagen I mRNA [3] and an increase of osteocalcin expression, a late marker of differentiated osteoblasts [4]. It is not yet clear if human osteoblasts behave in a similar manner *in vivo* and *in vitro*, but initial studies in developing human bone appear to indicate an analogous process [5, 6]. Because differences in hormonal regulation between species have been described [7], characterization and evaluation of human osteoblast systems seem to be essential for the study of human bone biology.

Studies on the differentiation of human bone have been greatly facilitated by the recent development of culture methods for human bone cells [8-14]. Normal adult human cells of the osteoblast lineage derived from various bone compartments are routinely established in short-term cultures. However, over the last years it emerged that all these cultures are a mixture of cells, composed of different subpopulations of bone cells, if not also contaminated with other cell types [10, 15]. The subpopulations seem to be primary osteoblasts in varying differentiation stages, modulated by hormones, depending on the phenotype as well as cell density in culture [16–20] probably accounting for the high variation in results [15, 21–23]. However, the differences in growth and differentiation of these cells in culture is thought to give the most realistic insight into the physiology and possibly pathophysiology of bone differentiation. Therefore, methods were developed to compare early and late stages of differentiation of primary osteoblasts in culture. The effect of hormones was studied on different stages of developing osteoblasts, on different clones of the same tumor cell line [16], on cells treated with dexamethason [24], on osteoblasts derived from spongiosa and marrow [25], and on cells plated at low and high densities [18, 19, 26]. In models of different cell density the influence of cell to cell contacts onto the differentiation was investigated. However, the possible effect of different culture time on a defined stage of confluence was not considered. Lately, we tested the influence of degree of confluence on the growth and differentiation of primary human osteoblasts *in vitro* [27]. In that study, we were able to show an increasing differentiation characterized by the increasing number of alkaline phosphatase-positive cells with decreasing proliferation of cultured cells in four stages of confluence. According to results in the chicken osteogenic system [3], collagen secretion was maximum at early proliferation, decreasing with later confluence stages.

$1,25(OH)_2D_3$ is a hormone known to differentially in-

*Correspondence to:* H. Siggelkow

fluence primary human osteoblast in culture [19] and also to promote differentiation [28–30]. In this study we used the confluence model to investigate the influence of $1,25(OH)_2D_3$ on proteins and gene expression of alkaline phosphatase (AP), osteocalcin (OC), and procollagen I (PICP) in primary human osteoblasts.

## Materials and Methods

All culture media and fetal calf serum (FCS) were purchased from Biochrom (Berlin, Germany). Cell culture disposables were purchased from Nunc (Wiesbaden-Biebrich, Germany) or Greiner (Solingen, Germany), and medium supplements (antibiotics, glutamine) from GIBCO/ BRL (Eggenstein, Germany). Standard laboratory reagents were purchased from Sigma (Deisenhofen, Germany) if not noted otherwise.

### *Cell Culture*

Primary human bone cell cultures were established from bone specimens as previously described [11, 12]. Trabecular bone sections, obtained during hip or knee replacement surgery, were treated identically. The explants were cleaned of adherent tissue and periosteum, cut in 1–3 $mm^3$ pieces, thoroughly rinsed, and maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FCS, glutamine (58.5 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cultures were initiated within 24 hours of bone specimen sampling.

The grown cells were released from the surface of the culture dish with 0.25% trypsin after 14–21 days. Cells were divided in two 75 $cm^2$ tissue culture flasks and are referred to as 1st passage cells. After growing near confluence, the cells were released from the culture dish as described above, counted, plated in 75 $cm^2$ flasks at a density of $2 \times 10^5$ cells/dish, and evenly distributed. These cells are referred to as 2nd passage cells. They were analyzed in different growth phases, defined by the extent of cell confluence. We selected cells at a confluence of 50% and 75% as being in the stage of fast proliferation; cells at 100% and 7 days 100% confluence were used to describe the period of diminished proliferation and increasing differentiation. To assess cell density, daily control of all culture dishes was necessary.

Cultures were controlled every day to establish the stage of confluence. At each time point the cells were washed twice with incubation medium (DMEM with 1% BSA) (Sigma), $MgSO_4$ 0.02% and penicillin (100 U/ml), and streptomycin (100 μg/ml) to remove the FCS followed by incubation with $5 \times 10^{-8}$ M $1,25(OH)_2D_3$ or solvent (ethanol <0.01%) for 48 hours at 37°C. After harvesting, cell numbers were determined for each time point by hemocytometer counting. The remaining cells were frozen at −20°C for determination of total protein and AP activity. All cells were regularly tested for mycoplasma contamination by PCR (Mycoplasma PCR Primer Set, Stratagene, Heidelberg, Germany).

### *Biosynthesis of Proteins*

For the determination of total protein and AP, the medium was decanted and the adherent cell monolayer was washed with phosphate-buffered saline (PBS). Cells were lysed in distilled water by repeated freeze-thawing and sonication (Ultrasonics W 185F, 40–60 W, 3 × 30 seconds). The lysate was clarified by centrifugation (10 minutes at 10,000 × *g*) and the soluble protein fraction was quantified using the BioRad protein assay with an albumin standard (BioRad, München, Germany). AP activity was assayed in cell lysates by determining the release of p-nitrophenol from p-nitrophenyl phosphate at 37°C and pH of 10.5. The substrate solution contained 8 mM p-nitrophenyl phosphate in 0.5 M AMP-buffer (2-amino-2-methyl-1-propanol), pH 10.5, supplemented with 0.2 mM $MgCl_2$. After 10 minutes at 37°C the reaction (volume 110 μl) was stopped with 50 μl 1 N sodium hydroxide. AP determinations were done in duplicate and are reported as units per mg protein per minute incubation time (modified after [31]). Secreted osteocalcin (OC) and the carboxyterminal peptide of procollagen I (PICP) were measured in culture supernatants after the incubation with $5 \times 10^{-8}$ M $1,25(OH)_2D_3$ or solvent for 48 hours at 37°C. Osteocalcin was determined in duplicate with an immunoradiometric assay (Nichols, Bad Nauheim, Germany); the detection range of the kit was 0.9–267 ng/ml. Values were corrected for interfering substances present in the culture medium. Collagen type I content was calculated from the c-terminal propeptide concentration [32, 33] measured in duplicate samples by ELISA (Metra Biosystems, Palo Alto, CA, USA). Values were corrected for the presence of background levels of PICP in fresh culture medium. Results are reported as the mean of two supernatants for each time point.

### *RNA Isolation and Northern Blot Analysis*

Total RNA was isolated from cells by the Caesium-chloride method [34]. For Northern blot analysis, 10 μg per lane of total cellular RNA was separated electrophoretically in a 1% agarose gel (2% formaldehyde, 0.3 μg/ml ethidium bromide), blotted to nylon membranes (Zeta Probe, BioRad, München, FRG) by capillary diffusion, and immobilized by UV-crosslinking (Stratalinker, Stratagene, Heidelberg). $^{32}$P-labeled cDNA probes were synthesized by random priming (Stratagene, Heidelberg). Prehybridization and hybridization were each performed in 50% formamide, 4 × SSPE, 5 × Dehnhardt's, 1% SDS, and 500 μg/ml salmon sperm DNA at 42°C for 24 hours. For hybridization, approximately $10^6$ cpm/ml of $^{32}$P-labeled probe was added. Following hybridization, blots were washed for 2 × 30 minutes at room temperature in 2 × SSC/0.1% SDS and 5–30 minutes at 55°C (rat cDNA probes) or 63°C (human cDNA probes) in 0.1 × SSC/0.1% SDS. Autoradiographs were quantified by laser densitometry (Biometra, Göttingen, FRG) and normalized to the ethidiumbromide staining of 18S and 28S ribosomal RNA, using a negative film for densitometric analysis. Values are expressed in relation to the maximal absorbency of each transcript. For each mRNA the maximal absorbency of stimulated and unstimulated values was set to 100.

Values represent the mean of five (OC), seven (PICP), or three (AP) independent experiments. For analysis of GAPDH mRNA expression, seven experiments and two sets of blots were analyzed. Two sets of Northern blots were rehybridized without stripping for each experiment.

### *cDNA Probes*

Procollagen type I expression was measured using the 372 bp fragment of the rat proalpha(I) collagen cDNA clone pHCAL1U [35]. This fragment binds to α1(I) and α2(I) procollagen. The glycero-aldehydephosphatase-dehydrogenase (GAPDH) human cDNA probe was a 1.2 kb insert from clone pHcGAP [36] and the AP human cDNA probe was a 2.5 kb insert of pAT153 [37]. Osteocalcin expression was detected using the human 1.2 kb Sac I fragment of SP 65 [38].

### *Statistical Methods and Mathematics*

Statistical testing was performed with Stat Works statistical package. Student's paired *t*-test, Wilcoxon, and Whitney-U-Test were used where appropriate and as noted. All values are expressed as mean ± SEM. (See Figs. 3 and 4 for significant differences.)

## Results

### *Cell Culture*

The bone used to initiate explant cultures was taken from

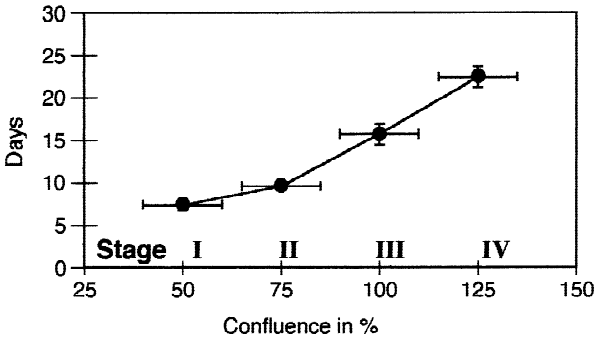


**Fig. 1.** Stages of confluence in relation to time in culture. For reasons of presentation, stage IV is designated as 125% confluence. SEM of days (vertical) and confluence (horizontal) are depicted. Values are mean ± SEM (n = 7).

seven male patients who underwent an arthroplasty (3 hip, 3 knee, 1 talus). The mean age of the patients was 62 ± 4.36 years, range 46–80, median 60 years. The cultures grew predominantly homogeneous and reached the stages of confluence within 7.43 ± 0.63 days (50%, stage I), 9.57 ± 0.49 days (75%, stage II), 15.71 ± 1.27 days (100%, stage III), and 22.43 ± 1.23 days (seven days 100%, stage IV), respectively, showing decreasing proliferation, as demonstrated in Figure 1. The morphological appearance of the four confluence phases is shown in Figure 2. During proliferation, the cells showed a long and narrow fibroblast-like appearance; with further confluence, cells became flattened and widespread. At 100% confluence, cells changed again to a long and extended shape before they started to grow in multilayer formation.

*Protein Synthesis in the Confluence Model*

Cultures showed higher proliferation while passing through stages I and II and growth slowed when reaching stages III and IV (Fig. 1). The secretion of PICP in the supernatant decreased from early proliferation (50%, stage I) to late confluence (stage IV) to 78% of the starting level (factor 0.36, $P < 0.05$, Wilcoxon) (Fig. 3). Alkaline phosphatase (AP) increased from basal level at early proliferation to 1.8-fold values at stage IV (Wilcoxon, $P < 0.05$). In five of the cultures the unstimulated cells were already able to synthesize osteocalcin (OC) during proliferation, with a 2.3-fold increase towards confluence and thereafter (1.27 ng ± 0.4 ng/mg total protein stage I versus 2.91 ± 0.84 stage IV). Due to high culture-dependent variance results did not reach significance levels.

*Gene Expression in the Confluence Model*

To plot the mRNA content in relation to a housekeeping gene we examined GAPDH gene expression in addition to the specific genes. Analyzing seven experiments with a double set of northern blots we found a decrease in GAPDH gene expression from early proliferation to late confluence in unstimulated cultures ($P < 0.05$) (Figs. 4 and 5). Therefore, we adjusted the specific gene expression to the amount of loaded mRNA depicted by the ethidium bromide staining (Figs. 4 and 5). Procollagen I mRNA increased (1.3-fold) significantly in unstimulated cultures from early to late proliferation (stages I and II, $P < 0.05$). There was no further change in stages III and IV (Figs. 4 and 5). Unstimulated OC gene expression was detectable only in two of five cultures at confluence (stage III) with a further 2.2-fold increase at stage IV. During proliferation (stages I and II) no basal osteocalcin expression was detectable (stages I and II, Figs. 4 and 5). Parallel to procollagen I, AP showed an increase to 1.5 levels of basal gene expression from stages I to II and stayed constant thereafter (Figs. 4 and 5).

*Influence of $1,25(OH)_2D_3$ on Specific Proteins*

Alkaline phosphatase/mg protein was increased by $1,25(OH)_2D_3$, the effect being more pronounced in early proliferation; the stimulation was significant only at confluence ($P < 0.05$, Wilcoxon).

Osteocalcin greatly increased after stimulation with $1,25(OH)_2D_3$ independently of the stage of confluence or the increasing basal osteocalcin synthesis, and the maximal level was similar in all stages ($P < 0.05$) (Figs. 3–5).

There were only minor effects on the PICP content in the conditioned medium. PICP/mg protein was inhibited to 0.74-fold by $1,25(OH)_2D_3$ during early proliferation (50% confluence, $P = 0.058$) with no effect thereafter.

*$1,25(OH)_2D_3$ Influence on Gene Expression*

Analyzing seven experiments with a double set of northern blots we found a highly significant inhibition of GAPDH expression by $1,25(OH)_2D_3$ in all stages (to 70% of control). Furthermore, expression decreased from proliferation to late confluence in stimulated as in unstimulated cultures (1.2-fold). Procollagen I gene expression increased 1.2-fold in stimulated cultures from early to late proliferation (stages I to II, $P < 0.05$) with no further change thereafter. $1,25(OH)_2D_3$ slightly stimulated procollagen I gene expression in all stages, the stimulatory effect being significant only at early proliferation and at confluence (1.1-fold, stage III, Wilcoxon, $P < 0.05$) (Figs. 4 and 5). Osteocalcin mRNA was already stimulated by $1,25(OH)_2D_3$ in stages I and II, this increase being 30-fold of the basal level at confluence and 12-fold thereafter (stage IV, $P < 0.01$) (Figs. 4 and 5). The gene expression of AP was detectable in only three cultures. We saw a stimulation of AP gene expression by $1,25(OH)_2D_3$ in all stages which was not significant due to wide scatter and the small number of cases.

*Influence of $1,25(OH)_2D_3$ on Variation of Results*

Overall variation did not show a difference between unstimulated and stimulated cultures, the coefficient of variation for GAPDH gene expression being 6–29% depending on the stage of confluence. The respective values for stimulated OC expression were 27–44% (analysis for unstimulated cultures not done) and 35–58% for procollagen I expression. Variation of basal cell culture analysis was 47–60% for cell number, 32–120% for total protein, 39–106% for AP, 37–121% for osteocalcin, and 38–149% for PICP values.

## Discussion

In this paper we can show decreasing proliferation and in-

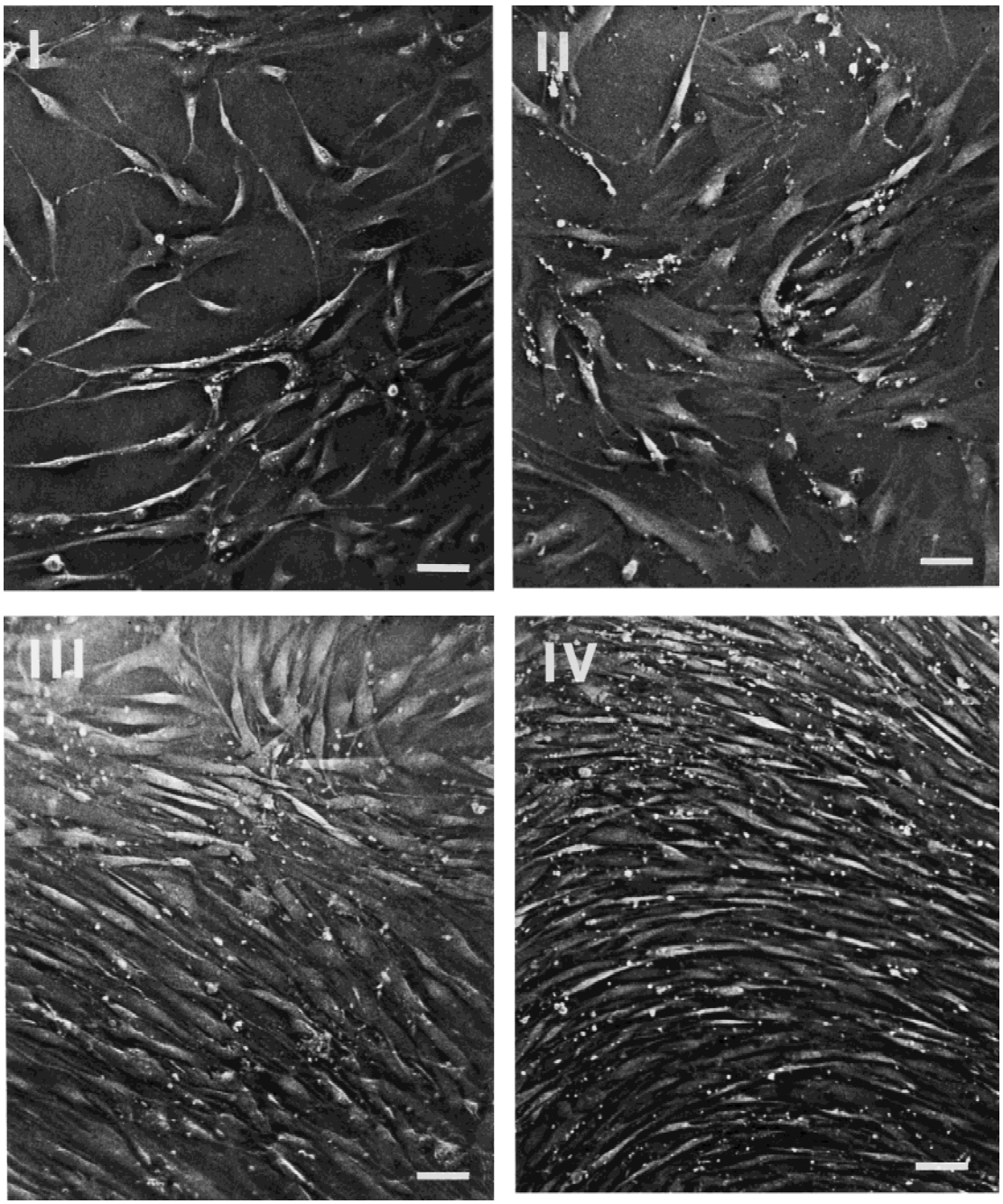


**Fig. 2.** Representative light field microscopy of primary human osteoblast-like cells depicting the four different stages of confluence (single culture at different time points). Stage I depicts 50% confluence, stage II 75%, stage III 100%, and stage IV 7 days 100% confluence. Bar 50 μm.

creasing differentiation in pHOB, characterized by different stages of confluence. The development of a more mature phenotype was shown by an increase in AP activity, decreasing collagen I secretion when cultures became confluent, and increasing OC secretion with further confluence. Finally, OC mRNA expression was detected at a very low level beginning at confluence. So far, these results are in accordance with data from other species and therefore the regulation of synthesis of characteristic proteins seemed to be partly confluence dependent. Concerning the analysis of AP and procollagen I gene expression we did not find any confluence-dependent differences and we conclude that mRNA expression of these proteins does not contribute systematically to the characterization of differentiation stages in pHOB.

Furthermore, we investigated whether the observed stage-dependent differences in protein expression and secretion could be influenced by 1,25$(OH)_2D_3$, a hormone that is known to regulate the differentiation of osteoblasts. We found that confluence-dependent differences were attenuated by 1,25$(OH)_2D_3$, because the hormone caused the proliferating osteoblasts to behave like more differen-

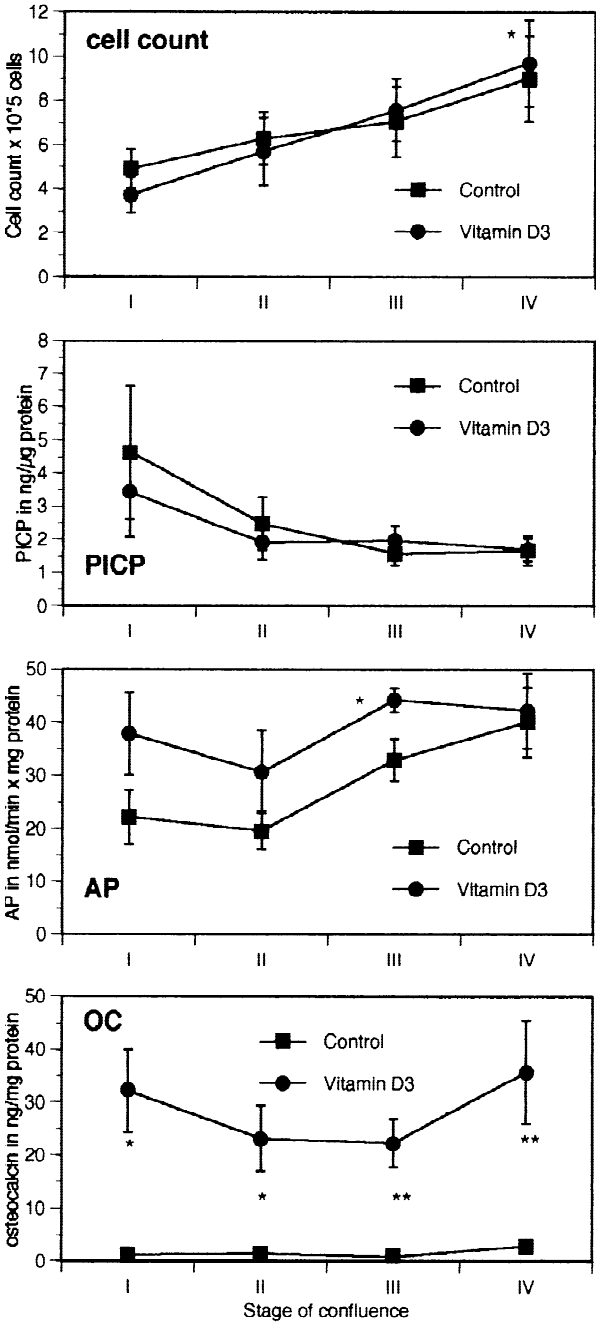


**Fig. 3.** Growth and specific proteins in four different stages of confluence under the influence of $5 \times 10^{-8}$ M $1,25(OH)_2D_3$ in comparison to control values. Stage I means 50% confluence, stage II 75%, stage III 100%, and stage IV 7 days 100% confluence. Procollagen I secretion measured by production of procollagen-I-peptide (PICP) in the supernatant. Alkaline phosphatase (AP), osteocalcin (OC). Significant values: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

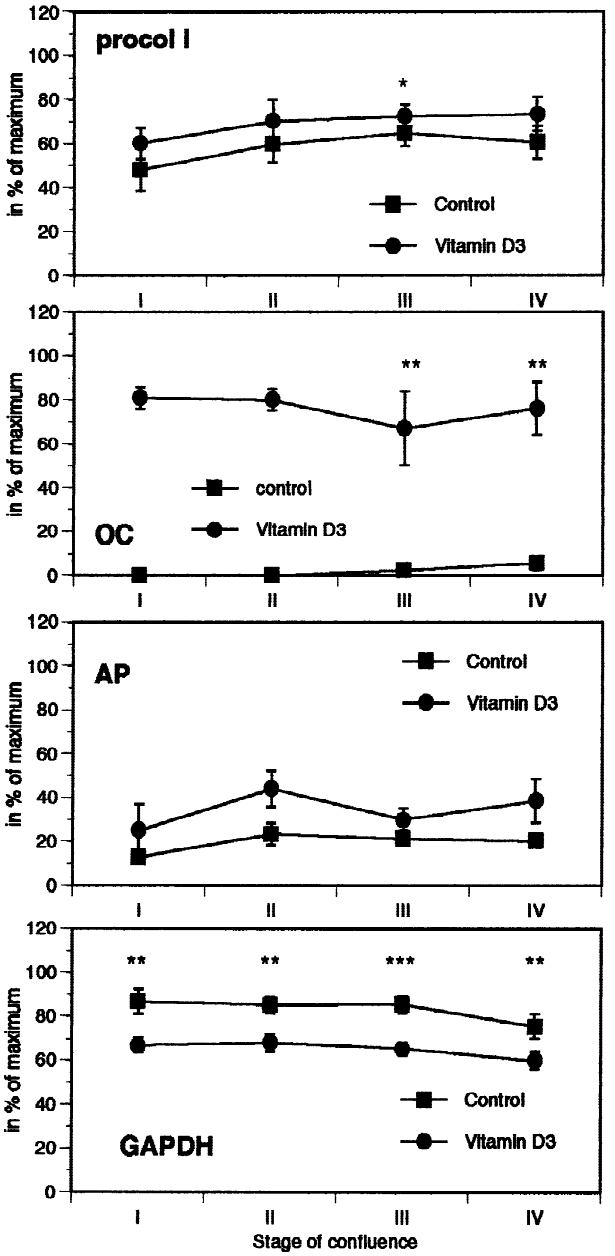


**Fig. 4.** Gene expression of osteoblast specific genes and GAPDH in four different stages of confluence under the influence of $5 \times 10^{-8}$ M $1,25(OH)_2D_3$ compared with control values. Stage I means 50% confluence, stage II 75%, stage III 100%, and stage IV 7 days 100% confluence. Cellular RNA from seven independent experiments was isolated and assayed by northern blot analysis. Expression was quantified by laser densitometry and results are plotted against the maximum expression of each transcript. The panel shows the mean values ± SEM for each specific transcript. α1 and α2 (I) procollagen (procol I, n = 7), akaline phosphatase (AP, n = 4), osteocalcin (OC, n = 7), and glycero-aldehyde phosphate dehydrogenase (GAPDH, n = 14). Significant values: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

tiated cells. In other words, the differentiating influence of $1,25(OH)_2D_3$ was strongest during proliferation whereas only minor effects were seen after cells became more confluent and differentiated. In the following we discuss the different aspects in more detail.

A stimulation of AP by $1,25(OH)_2D_3$ is seen in rat [16, 39, 40], in human osteosarcoma cell lines [41], and in normal human bone cells [26]. Most of the data concerning the differentiation-dependent AP regulation by $1,25(OH)_2D_3$ have been investigated in rat osteoblast or rat osteosarcoma cells. Early studies in ROS 17/2.8 cells and subclones [16, 39] showed a biphasic regulation for AP by $1,25(OH)_2D_3$,

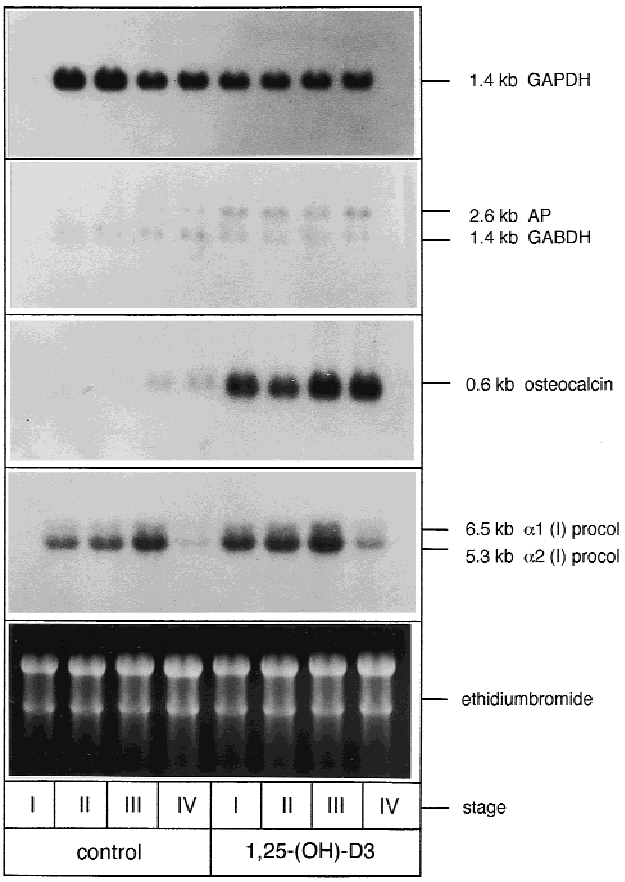


**Fig. 5.** Steady state gene expression during four different stages of confluence under the influence of $5 \times 10^{-8}$ M $1,25(OH)_2D_3$ compared with control values. Stage I means 50% confluence, stage II 75%, stage III 100%, and stage IV 7 days 100% confluence. Cellular RNA from seven independent experiments was isolated and assayed by northern blot analysis. Representative northern blots are depicted to illustrate alterations in mRNA expression. The ethidium bromide staining, which shows equal gel loading, was used as reference. Glycerol-aldehyde phosphate dehydrogenase (GAPDH), alkaline phosphatase (AP), osteocalcin (OC), and procollagen I (procol I) are shown. Due to repeated hybridization, AP has to be shown on a blot together with GAPDH.

dependent on the basal level of enzyme activity. With low basal AP levels, a stimulation of enzyme activity and inhibition of proliferation were seen, an effect that was inverted with high basal AP-enzyme activity. In our confluence model, $1,25(OH)_2D_3$ stimulated AP synthesis from early proliferation to confluence; after confluence, no effect of $1,25(OH)_2D_3$ was seen. The AP mRNA expression in human osteoblasts is known to be increased after $1,25(OH)_2D_3$ stimulation in pHOB [18]. However, we do not have enough good quality data to support these results although we saw an increase in one case. These data on AP protein synthesis support the finding that $1,25(OH)_2D_3$ can positively and negatively regulate expression of osteoblast phenotypic markers as a function of basal levels of expression, which is a reflection of the differentiated state of the osteoblast.

Osteocalcin protein and mRNA synthesis are highly stimulated by $1,25(OH)_2D_3$, as shown in different osteoblast-like cell models [22, 26, 42, 43]. However, in contrast to other studies our data show that the increase was not dependent on confluence, e.g., there were no differences in the stimulated levels between stages [44]. This was also evident for the mRNA expression which was not dependent on basal expression of OC mRNA or the stage of confluence in our model, in contrast to published results in primary rat osteoblasts [44]. Gene expression and protein secretion are similarly stimulated by $1,25(OH)_2D_3$, supporting the hypothesis that $1,25(OH)_2D_3$ controls OC protein synthesis by direct transcriptional mechanisms [45].

Procollagen secretion was measured by a PICP-specific antibody, which binds the intact procollagen I molecule and the free c-terminal propeptide which is split off during collagen I formation. Therefore, we only have information on the release of procollagen from the osteoblasts and the fraction that is transformed to collagen but not on the accumulation of collagen matrix. Gerstenfeld et al. [3] demonstrated in chicken embryo osteoblasts that synthesis of new collagen protein increased during proliferation but decreased thereafter, whereas collagen in the matrix further increased [3]. Our results, showing a decrease in PICP secretion with further confluence, would therefore be in agreement with the decrease of newly synthesized collagen seen in the chicken system. In contrast, the increasing procollagen I mRNA expression until confluence is surprising. Indeed, we have already found a similar expression sequence under basal conditions in cultures from 10 other donors of different ages where the decrease of PICP and increase from procollagen I mRNA was significant from stages I to III [27]. We assumed that the lack of ascorbate, which is a prerequisite for the full expression of collagen protein, was responsible for the expression sequence of procollagen mRNA and protein secretion. This is supported by the finding that in pHOB, ascorbate stimulates PICP secretion up to 5.8-fold values after confluence, probably by stimulating hydroxylation and secretion of accumulated procollagen I peptides [46, 47]. In rat and chicken calvarial cells the total collagen accumulation was increased as a function of ascorbic acid concentration, with no collagen in the matrix without the addition of ascorbate [3, 4]. Alternatively, the cultures might not have been analyzed long enough after confluence to see the decrease in procollagen I mRNA.

There was only a minor inhibitory effect of $1,25(OH)_2D_3$ on procollagen I-peptide secretion in the early proliferative phase but there was no effect thereafter. We found a small but confluence-independent stimulation of the procollagen mRNA message by this hormone. This supports findings in the human osteosarcoma cell line MG 63 that the effect of $1,25(OH)_2D_3$ on collagen production is only partly regulated by mRNA, but mainly at a post transcriptional level [28]. In rat calvarial cells, collagen I mRNA was inhibited by $1,25(OH)_2D_3$ at its peak level during proliferation and stimulated at its lowest basal level during the mineralization period, demonstrating that the effect of $1,25(OH)_2D_3$ is dependent on the proliferative and differentiated phenotype of the osteoblast [44]. In pHOB cells only marginal influence [48] or an increase independent of the seeded cell density on procollagen I synthesis after $1,25(OH)_2D_3$ stimulation [26] was seen. In addition, procollagen I mRNA was stimulated at high cell density [18]. In human bone cells cultivated from periosteum, $10^{-8}$ M $1,25(OH)_2D_3$ stimulated collagen I synthesis and mRNA expression to the same extent after confluence [49]. In immortalized human bone marrow cells, $1,25(OH)_2D_3$ stimulated collagen mRNA and protein secretion into the same direction as proliferating and differenti-

ating cells [50]. Regarding the differences in results between the rat and the human system we suggest that differences in the expression sequence of collagen I might be due to species differences.

In conclusion, when regarding the basal levels of the osteoblast specific proteins in correlation to the stage of confluence we show an increase of AP and OC and a decrease of procollagen I secretion, representing the development from proliferation to differentiation. These data support the current view on osteoblast differentiation in culture. Stimulation with $1,25(OH)_2D_3$ decreased the difference between confluence stages by causing the proliferating cultures to behave like more differentiated cells, whereas there was little effect except on osteocalcin after confluence. In other words, the functional status of differently differentiated cell populations became more alike under the influence of $1,25(OH)_2D_3$. Therefore $1,25(OH)_2D_3$ might improve the reproducibility of results obtained from clinical samples.

*Acknowledgments.* We thank Hoffmann-La Roche for providing the $1,25(OH)_2D_3$ and Prof. Willert of the Orthopaedic Department for making the bone specimens available to us.

## References

1. Nefussi J-R, Boy-Lefevre ML, Boulekbach H, Forest N (1985) Mineralization in vitro of matrix formed by osteoblasts isolated by collagenase digestion. Differentiation 29:160–168
2. Bellows CG, Aubin JE, Heersche NM, Antosz ME (1986) Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations. Calcif Tissue Int 38: 143–154
3. Gerstenfeld LC, Chipman SD, Kelly CM, Hodgnes KJ, Lee DD, Landis WJ (1988) Collagen expression, ultrastructural assembly and mineralization in cultures of chicken embryo osteoblasts. J Cell Biol 106:979–989
4. Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, Kennedy MB, Pockwinse S, Lian JB, Stein GS (1990) Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J Cell Physiol 143:420–430
5. Dodds RA, Merry K, Littlewood A, Gowen M (1994) Expression of mRNA for IL1β, IL6 and TGFβ1 in developing human bone and cartilage. J Histochem Cytochem 42:733–744
6. Mundlos S (1994) Expression patterns of matrix genes during human skeletal development. Prog Histochem Cytochem 28: 1–47
7. Chen TL, Cone CM, Feldman F (1983) Effects of 1,25-dihydroxyvitamin D3 and glucocorticoids on the growth of rat and mouse osteoblast-like bone cells. Calcif Tissue Int 35: 806–811
8. Mills BG, Singer FR, Weiner LP, Holst PA (1979) Long-term culture of cells from bone affected by Paget's disease. Calcif Tissue Int 29:79–87
9. Aubin JE, Heersche JNM, Jerrilees MJ, Sodek K (1982) Isolation of bone cell clones with differences in growth, hormone response and extracellular matrix production. J Cell Biol 92: 452–461
10. Wergedal JE, Baylink DJ (1984) Characterization of cells isolated and cultured from human bone. Proc Soc Exp Biol Med 176:60–69
11. Beresford JN, Gallagher JA, Poser JW, Russell RGG (1984) Production of osteocalcin by human bone cells in vitro. Effects of $1,25(OH)_2D_3$, $24,25-(OH)_2D_3$, parathyroid hormone, and glucocorticoids. Metab Bone Dis Rel Res 5:229–234
12. Auf'm Kolk B, Hauschka PV, Schwartz ER (1985) Characterization of human bone cells in culture. Calcif Tissue Int 37:228–235
13. Gehron Robey P, Termine JD (1985) Human bone cells in vitro. Calcif Tissue Int 37:453–460
14. Marie PJ, Lomri A, Sabbagh A, Basle M (1989) Culture and behaviour of osteoblastic cells isolated from normal trabecular bone surfaces. In Vitro Cell Biol Dev 25:373–380
15. Manduca P, Sanguineti C, Pistone M, Boccignone E, Sanguineti F, Santolini F, Federici A (1993) Differential expression of alkaline phosphatase in clones of human osteoblast-like cells. J Bone Miner Res 8:291–300
16. Spiess YH, Price PA, Deftos JL, Manolagos SC (1986) Phenotype-associated changes in the effects of 1,25-dihydroxyvitamin D3 on alkaline phosphatase and bone GLA-protein of rat osteoblastic cells. Endocrinology 118:1340–1346
17. Thavarajah M, Evans DB, Kanis JA (1993) Differentiation of heterogeneous phenotypes in human osteoblast cultures in response to 1,25-dihydroxyvitamin D3. Bone 14:763–767
18. Wergedal JE, Matsuyama T, Strong DD (1992) Differentiation of normal human bone cells by transforming growth factor beta and 1,25(OH) vitamin D3. Metabolism 41:42–48
19. Evans DB, Thavarajyh M, Binderup L, Kanis JA (1991) Actions of calcipotriol (MC 903), a novel vitamin D3 analog, on human bone-derived cells: comparison with 1,25-dihydroxyvitamin D3. J Bone Miner Res 6:1307–1315
20. Rickard DJ, Gowen M, Macdonald BR (1993) Proliferative responses to estradiol, Il-1α and TGFβ by cells expressing alkaline phosphatase in human osteoblast-like cell cultures. Calcif Tissue Int 52:227–233
21. Evans CE, Galasko CSB, Ward C (1990) Effect of donor age on the growth in vitro of cells obtained from human trabecular bone. J Orthop Res 8:234–237
22. Keeting PE, Scott RE, Colvard DS, Anderson MA, Oursler MJ, Spelsberg TC, Riggs BL (1992) Development and characterization of a rapidly proliferating, well-differentiated cell line derived from normal adult human osteoblast-like cells transfected with SV large T antigen. J Bone Miner Res 7:127–136
23. Chavassieux PM, Chenu C, Valentin-Opran A, Merle B, Delmas PD, Hartmann DJ, Saez S, Meunier PJ (1990) Influence of experimental conditions on osteoblast activity in human primary bone cell cultures. J Bone Miner Res 5:337–343
24. Rao LG, Wylie JN, Sutherland MSK, Murray TM (1994) 17β-estradiol and parathyroid hormone potentiates each other's stimulatory effects on alkaline phosphatase activity in AsOS-2 cells in a differentiation-dependent manner. Endocrinology 134:614–620
25. Kassem M, Mosekilde L, Eriksen EF (1994) Effects of fluoride on human bone cells in vitro: differences in responsiveness between stromal osteoblast precursors and mature osteoblasts. Eur J Endocrinol 130:381–386
26. Beresford JN, Gallagher JA, Russel RGG (1986) 1,25-dihydroxyvitamin D3 and human bone-derived cells in vitro: effects on alkaline phosphatase, type I collagen and proliferation. Endocrinology 119:1776–1785
27. Siggelkow H, Benzler K, Atkinson MJ, Hüfner M (1998) Phenotypic stability of primary human osteoblast–like cells at different cell densities and passage numbers. Exp Clin Endocrinol Diabetes 106:217–225
28. Franceschi RT, Romano PR, Park K-Y (1988) Regulation of type I collagen synthesis by 1,25-dihydroxyvitamin D3 in human osteosarcoma cells. J Biol Chem 263:18938–18945
29. Franceschi RT, James WM, Zerlauth G (1985) 1-alpha,25-dihydroxyvitamin D3 specific regulation of growth, morphology and fibronectin in a human osteosarcoma cell line. J Cell Physiol 123:410–409
30. Fedarko NS, Bianco P, Vetter U, Gehron Robey P (1990) Human bone cell enzyme expression and cellular heterogeneity: correlation of alkaline phosphatase enzyme activity with cell cycle. J Cell Physiol 144:115–121
31. Boyan BD, Schwartz Z, Bonewald LF, Swain LD (1989) Localization of 1,25-(OH)2D3-responsive alkaline phosphatase

in osteoblast-like cells (ROS 17/2.8, MG 63, and MC3T3) and growth cartilage cells in culture. J Biol Chem 264:11879–11886
32. Melkko J, Niemi S, Risteli L, Risteli J (1990) Radioimmunoassay of the carboxyterminal propeptide of human type I procollagen. Clin Chem 36:1328–1332
33. Jukkola A, Risteli J, Risteli L (1991) Effect of dextran on synthesis, secretion and deposition of type III procollagen in cultured human fibroblasts. Biochem J 279:49–54
34. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294–5299
35. Vuorio T, Mäkelä TJ, Kähäri V-M, Vuorio E (1987) Coordinated regulation of type I and type III collagen production and mRNA levels of pro a1(I) and pro a2(I) collagen in cultured morphea fibroblasts. Arch Dermatol Res 279:154–160
36. Tso JY, Sun XH, Kao TH, Reece KS, Wu R (1985) Isolation and characterisation of rat and human glyceraldehyde 3′-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. Nucleic Acids Res 13:2485–2502
37. Weiss MJ, Henthorn PS, Lafferty MA, Slaughter C, Raducha M, Harris H (1986) Isolation and characterization of a cDNA encoding a human liver/bone/kidney-type alkaline phosphatase. Proc Natl Acad Sci USA 83:7182–7186
38. Celeste AJ, Rosen V, Buecker JL, Kriz R, Wang EA, Wozney JM (1986) Isolation of the human gene for bone gla protein utilizing mouse and rat cDNA clones. EMBO J 5:1885–1890
39. Majeska RJ, Rodan GA (1982) The effect of 1,25(OH)2D3 on alkaline phosphatase in osteoblastic osteosarcoma cells. J Biol Chem 257:3362–3365
40. Fraser JD, Otawara Y, Price PA (1988) 1,25-dihydroxyvitamin D3 stimulates the synthesis of matrix g-carboxyflutamic acid protein by osteosarcoma cells. J Biol Chem 263: 911–916
41. Nyombi EK, Lau KHW, Baylink DJ, Strong DD (1989) Stimulation of cellular alkaline phosphatase activity and its messenger RNA level in a human osteosarcoma cell line. Arch Biochem Biophys 275:363–370
42. Fournier B, Price PA (1991) Characterization of a new human osteosarcoma cell line OHS-4. J Cell Biol 114:577–583
43. Spelsberg TC, Harris SA, Riggs BL (1995) Immortalized osteoblast-cell systems (new human fetal osteoblast systems). Calcif Tissue Int 56:S18–S21
44. Owen TA, Aronow MS, Barone LM, Bettencourt B, Stein GS, Lian JB (1991) Pleiotropic effects of vitamin D on osteoblast gene expression are related to the proliferative and differentiated state of the bone cell phenotype: dependency upon basal levels of gene expression, duration of exposure, and bone matrix competency in normal rat osteoblast cultures. Endocrinology 128:1496–1504
45. Lian JB, Stein GS (1992) Transcriptional control of vitamin D-regulated proteins. J Cell Biochem 49:37–45
46. Siggelkow H, Rebenstorff K, Kurre W, Niedhart C, Schulz H, Hüfner M (1997) Gene expression and protein synthesis in primary human osteoblast-like cells in vitro compared to an osteosarcoma cell line. Bone 20:P231
47. Franceschi RT, Iyer BS, Cui Y (1994) Effects of ascorbic acid on collagen matrix formation and osteoblast differentiation in murine MC3T3-E1 cells. J Bone Miner Res 9:843–854
48. Ingram HT, Bonde SK, Riggs BL, Fitzpatrick LA (1994) Effects of transforming growth factor beta (TGFβ) and 1,25-dihydroxyvitamin $D_3$ on the function, cytochemistry and morphology of normal human osteoblast-like cells. Differentiation 55:153–163
49. Tasaki Y, Takamori R, Koshihara Y (1991) Prostaglandin D2 metabolite stimulates collagen synthesis by human osteoblasts during calcification. Prostaglandins 41:303–313
50. Hicok KC, Thomas T, Gori F, Rickard DJ, Spelsberg TC, Riggs BL (1998) Development and characterization of conditionally immortalized osteoblast precursor cell lines from human bone marrow stroma. J Bone Miner Res 13:205–217