

# 1 $\alpha$ ,25-(OH)<sub>2</sub>D<sub>3</sub> Acts in the Early Phase of Osteoblast Differentiation to Enhance Mineralization Via Accelerated Production of Mature Matrix Vesicles

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1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (1,25D<sub>3</sub>) deficiency leads to impaired bone mineralization. We used the human pre-osteoblastic cell line SV-HFO, which forms within 19 days of culture an extracellular matrix that starts to mineralize around day 12, to examine the mechanism by which 1,25D<sub>3</sub> regulates osteoblasts and directly stimulates mineralization. Time phase studies showed that 1,25D<sub>3</sub> treatment prior to the onset of mineralization, rather than during mineralization led to accelerated and enhanced mineralization. This is supported by the observation of unaltered stimulation by 1,25D<sub>3</sub> even when osteoblasts were devitalized just prior to onset of mineralization and after 1,25D<sub>3</sub> treatment. Gene Chip expression profiling identified the pre-mineralization and mineralization phase as two strongly distinctive transcriptional periods with only 0.6% overlap of genes regulated by 1,25D<sub>3</sub>. In neither phase 1,25D<sub>3</sub> significantly altered expression of extracellular matrix genes. 1,25D<sub>3</sub> significantly accelerated the production of mature matrix vesicles (MVs) in the pre-mineralization. Duration rather than timing determined the extent of the 1,25D<sub>3</sub> effect. We propose the concept that besides indirect effects via intestinal calcium uptake 1,25D<sub>3</sub> directly accelerates osteoblast-mediated mineralization via increased production of mature MVs in the period prior to mineralization. The accelerated deposition of mature MVs leads to an earlier onset and higher rate of mineralization. These effects are independent of changes in extracellular matrix protein composition. These data on 1,25D<sub>3</sub>, mineralization, and MV biology add new insights into the role of 1,25D<sub>3</sub> in bone metabolism and emphasize the importance of MVs in bone and maintaining bone health and strength by optimal mineralization status.

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Vitamin D is one of the major factors involved in calcium homeostasis through actions on the intestine, kidney, parathyroid gland, and bone (van Leeuwen et al., 2001). The biologically most active form of vitamin D is 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (1,25D<sub>3</sub>). 1,25D<sub>3</sub> is synthesized from the parental vitamin D molecule by sequential hydroxylations in the liver (25-hydroxylation) and kidney (1 $\alpha$ -hydroxylation) (Christakos et al., 2003). Most of the biological activities of 1,25D<sub>3</sub> are exerted through binding to the vitamin D receptor (VDR) followed by heterodimerization with Retinoid-X-Receptor (RXR; Carlberg et al., 1993). The heterodimer directly binds to the promoter of target genes and controls their expression (Jurutka et al., 2001).

1,25D<sub>3</sub> is involved in bone formation and mineralization and it is used to prevent and treat osteoporosis. These influences on bone can be indirectly established through the control of calcium uptake in the intestine or reabsorption in the kidney. However, direct effects are also likely as the VDR is present in osteoblasts. In addition, 1,25D<sub>3</sub> can directly affect osteoblasts as shown by stimulating *in vitro* mineralization in osteoblast cultures (Miyahara et al., 2002; van Driel et al., 2006b) and by altering gene regulation (Christakos et al., 2003; Shen and Christakos, 2005; van Driel et al., 2006b; Barthel et al., 2007). Moreover, we were able to show that osteoblasts themselves

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can synthesize 1,25D3 (van Driel et al., 2006a), emphasizing the importance of local 1,25D3 for osteoblasts.

Composition of the extracellular matrix (ECM) is an important determinant of mineralization. The ECM contains numerous proteins which can be regulated by 1,25D3 and influence mineralization. Examples are osteocalcin (Ducy et al., 1996; Lian et al., 1989), osteopontin (Reinholt et al., 1990), bone sialoprotein (Gordon et al., 2007), and collagens (Landis, 1999). In addition, we recently demonstrated that follistatin and activin cause major changes in the ECM gene expression profile preceding their stimulatory and inhibitory effect on mineralization, respectively (Eijken et al., 2007).

Another crucial prerequisite for mineralization are matrix vesicles (MVs). MVs are budding from the basolateral side of the osteoblast plasma membrane and are released into the ECM (Anderson, 1995). Various channel proteins (annexin II, V, and VI) allow transportation of calcium and phosphate ions into the vesicles (Kirsch et al., 2000). Furthermore, MVs contain a large amount of alkaline phosphatase (ALP), which is an important enzyme involved in osteoblast mineralization and is generally accepted as a marker for MVs (Ali et al., 1970; Majeska and Wuthier, 1975). Mature MVs, reflected by outgrowth of hydroxyapatite, and high ALP activity are believed to initiate mineralization (Anderson et al., 2005). It is widely believed that following MV maturation, the membrane ruptures and hydroxyapatite (calcium–phosphate) is released into the matrix.

The aim of this study was to identify the processes involved in 1,25D3 stimulation of mineralization. This was assessed by a series of experiments, including *in vitro* timing experiments with differentiating human osteoblasts, transcriptional profiling and isolation and analyses of MV production.

## Material and Methods

### Cell culture

SV-HFO cells (Chiba et al., 1993) were cultured as described previously (Eijken et al., 2007). To induce osteoblast differentiation, medium ( $\alpha$ MEM; Gibco BRL, Invitrogen Ltd., Paisley, UK) was supplemented with freshly added 10 mM  $\beta$ -glycerophosphate (Sigma–Aldrich, St. Louis, MO) and 100 nM dexamethasone (Sigma–Aldrich), with or without 10 nM 1,25D3, and replaced every 2 or 3 days. Hence, one treatment with 1,25D3 is equivalent to a duration of 2–3 days, two treatments with 4–5 days, etc. 1,25D3 was a generous gift from Leo Pharma (Ballerup, Denmark). Cells were harvested at different time points during culture.

### DNA, mineralization, and protein assays

DNA and calcium measurements were performed as described previously (Eijken et al., 2006). Briefly, for DNA measurements cell lysates were incubated with heparin (8 IU/ml in PBS) and Ribonuclease A (50  $\mu$ g/ml in PBS) for 30 min at 37°C. DNA was stained by adding ethidium bromide (25  $\mu$ g/ml in PBS). Analyses were performed by using a Victor2 plate reader (PerkinElmer Life and Analytical Science (Wallac), Waltham, MA) with an extinction filter of 340 nm and an emission filter of 590 nm. For calcium measurements, cell lysates were incubated overnight with 0.24 M HCl at 4°C. Calcium content was colorimetrically determined with a calcium assay kit (Sigma–Aldrich) according to the manufacturer's description. Results were adjusted for DNA content of the cell lysates. For Alizarin Red S staining cell cultures were fixed for 60 min with 70% ethanol on ice. After fixation, cells were washed twice with PBS and stained for 10 min with Alizarin Red S solution (saturated Alizarin Red S in demineralized water adjusted to pH 4.2 using 0.5% ammonium hydroxide). For protein measurement 200  $\mu$ l of working reagent (50 volumes BCA<sup>TM</sup> reagent A, 1 volume BCA<sup>TM</sup> reagent B; Pierce, Rockford, IL) was added to 10  $\mu$ l of sonicated

cell lysate. The mixture was incubated for 30 min at 37°C, cooled down to room temperature and absorbance was measured, using a Victor2 plate reader at 595 nm.

### Affymetrix gene chip-based gene expression

Gene expression arrays were performed as described previously (Eijken et al., 2007). Briefly, cultures, continuously treated with or without 10 nM 1,25D3, were harvested at days 3, 7, 12, and 19 of culture and total RNA was isolated. Quality assessments of arrays were performed by RNA 6000 Nano assay on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). For further procedures several kits were used: cDNA synthesis (Invitrogen), purification, labeling and hybridization (Affymetrix, Santa Clara, CA). As platform we used the Affymetrix "GeneChip Human Genome U133 Plus 2.0 oligonucleotide Genechips." For gene ontology (GO) analysis, selected Affymetrix IDs were analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) 2008 hosted by the National Institute of Allergy and Infectious Diseases (NIAID), NIH (Sherman et al., 2007).

### Quantification of mRNA expression

RNA isolation, cDNA synthesis and PCR reactions were performed as described previously (Bruedigam et al., 2008). Reactions were downscaled to a 384-well-plate system and reactions were performed in 12.5  $\mu$ l volume. Q-PCR was carried out, using an ABI PRISM 7900 HT system (Applied Biosystems, Foster City, CA). Primer pairs, all being either on exon boundaries or spanning at least one exon, were purchased from Sigma–Aldrich. Primers: decorin (DCN): TAACCGAAATCAAAGATGGAGACTT (forward primer; 2.5 pmol/reaction); TCCAGGACTAACTTTGCTAATTTTATTG (reverse primer; 2.5 pmol/reaction); fibulin-1 (FBLN1): GGAGACCGGAGATTTGGATGT (forward primer; 5 pmol/reaction); TCAGATATGGGCTCTTGTTCCCT (reverse primer; 5 pmol/reaction). GAPDH: ATGGGGAAGGTGAAGGTCG (forward primer; 3.75 pmol/reaction); TAAAAGCAGCCCTGGTGACC (reverse primer; 3.75 pmol/reaction); CGCCCAATACGACCAAATCCGTTGAC (FAM probe, 3.75 pmol/reaction).

### Devitalization of osteoblast cultures

Cells were devitalized according to the protocol by Eijken et al. (2007). In short, on day 10 of SV-HFO cultures, medium was removed from the cells and cultures were washed once with PBS (Gibco BRL, Invitrogen Ltd.). Cells were air-dried and stored at  $-20^{\circ}\text{C}$ . After thawing, cells were washed once with PBS. Devitalized cultures were incubated under standard conditions as described above.

### Isolation of matrix vesicles

MV isolation was performed as described previously (Johnson et al., 1999). Briefly, for isolation of MVs, SV-HFO were cultured with or without 1,25D3 in 175 or 75  $\text{cm}^2$  flasks. For the collection of ECM associated MVs, cells were washed twice with PBS and treated with 1.5 ml (75  $\text{cm}^2$  flasks) of collagenase/dispase (1 mg/ml; Roche, Basel, Switzerland) for 90 min at 37°C. After incubation, 9 ml PBS was added and the suspension was centrifuged for 10 min at 500g, followed by centrifugation at 20,000g for 30 min at 4°C and the resulting supernatant was then subjected to a second centrifugation at 100,000g for 60 min at 4°C. The pellet was dissolved in 100  $\mu$ l PBS. Centrifuges used were the Heraeus Multifuge 1S (DJB Labcare Ltd., Newport Pagnell, UK) and the Ultracentrifuge L-70 (Beckmann Coulter, Fullerton, CA).

### Electron microscopy of MVs

For electron microscopic samples, SV-HFO cells were cultured according to protocol and MVs were harvested on day 14 of culture. To achieve sufficient amounts of MVs, cultures of two flasks (175 cm<sup>2</sup>) were pooled. Isolation of MVs was performed as described; instead of dissolving the pellet in PBS we dissolved it in 200  $\mu$ l fixative (4% formaline, 1% gluteraldehyde; postfixation 1% osmiumtetroxide). Samples were forwarded to the department of Pathology, Erasmus Medical Center, Rotterdam, for further processing and analyses.

### Flow cytometry analyses of MVs

A Becton Dickinson FACS-Canto and DIVA Flow Cytometry System (BD bioscience, San Jose, CA) were used to measure and analyze MVs, respectively. Twenty-five microliter of freshly isolated MVs were incubated for 15 min with 25  $\mu$ l ELF-97 staining solution (0.2 M ELF97 (Invitrogen), 0.11 M acetic acid, 1 mM NaNO<sub>2</sub>, pH 8.0). 250  $\mu$ l PBS was added and stained vesicles were measured in AmCyan-A channel (488 nm). ELF-97 is a phosphatase substrate, which at pH 8 detects alkaline phosphatase. Reference beads (BD bioscience) were added to the samples for size estimation of the isolated vesicles.

### Gene nomenclature

Gene names and symbols were used as provided by the HUGO Gene Nomenclature Committee (Wain et al., 2002).

### Statistics

The data provided are based on multiple independent experiments derived from independent cultures. Experiments were performed at least in triplicate; values are means  $\pm$  SEM. Significance was calculated using the Student's *t*-test and *P*-values <0.05 were considered significant.

### Results

Human osteoblasts continuously treated with 1,25D3 showed enhanced and accelerated mineralization as the onset of mineralization is already observed on day 10 of culture (Fig. 1). Next, we examined the timing of the 1,25D3 effect on mineralization. Treatment prior to the onset of mineralization (i.e., day 12 of control cultures) was crucial and sufficient to observe enhanced mineralization after 19 days of culture (Fig. 2). Therefore, the major effects of 1,25D3 on osteoblasts to stimulate mineralization occur prior to the onset of mineralization.

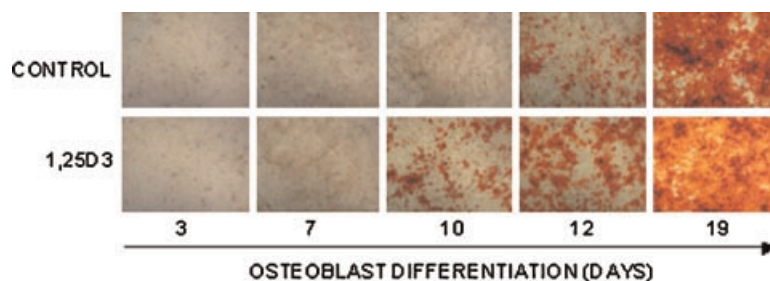
We further examined the timing of the 1,25D3 effect by zooming in on the pre-mineralization period. We reasoned that when 1,25D3 would stimulate a specific step in the differentiation process the precise timing of treatment is very

important. No specific time-point of treatment turned out to be crucial. There was no difference in effect whether treatment started at day 0, 3, or 5 of culture (Supplementary Fig. S1). However, it was obvious that the number of treatments, that is, duration of treatment, determined the magnitude of the 1,25D3 effect on mineralization (Fig. 3). Taken together, the longer the cells were treated with 1,25D3 prior to the onset of mineralization, the stronger the stimulation.

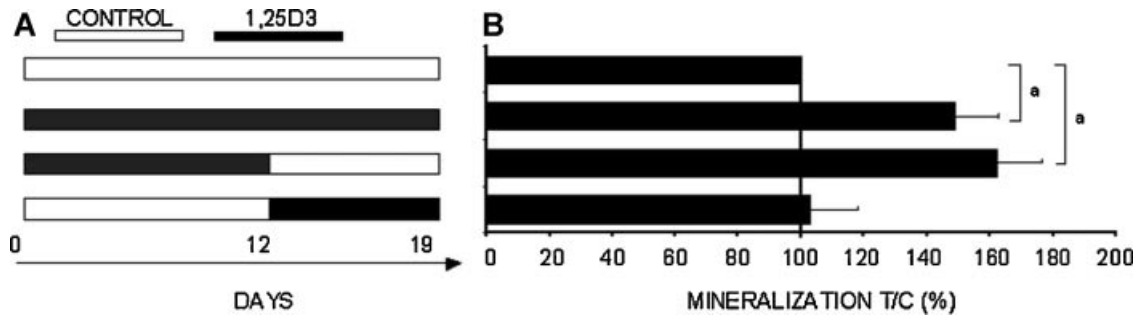
On basis of the findings that 1,25D3 stimulated mineralization by effects in the pre-mineralization period, we performed Gene Chip analyses to identify genes regulated by 1,25D3 at this stage in an unbiased manner. Gene chip profiling was performed at four different time points (days 3, 7, 12, and 19) during the course of osteoblast differentiation, in the presence or absence 1,25D3.

We identified genes which were at least 1.5-fold regulated by 1,25D3 in the pre-mineralization (days 3 and 7) and/or mineralization (days 12 and 19) period. To specifically select for 1,25D3 regulated genes in the mineralizing condition, we excluded genes that were also regulated in the non-mineralizing condition, that is, SV-HFO cultured in the absence of dexamethasone (Eijken et al., 2005). The analyses revealed that 6.4 times more genes are regulated in the mineralization period (470 genes) compared to the pre-mineralization period (74 genes). Amongst them, 13.5% and 18.7% were open reading frames or hypothetical proteins, respectively. Non-annotated transcribed sequences or clone IDs were excluded from further analyses. More genes were down- than up-regulated in both the pre-mineralization and the mineralization period (Table 1). Interestingly, there was an overlap of only 3 genes between the two sets of genes, indicating that the transcriptomes in these two time periods are strongly different and that 1,25D3 has a different role in early compared to late osteoblast differentiation. GO analyses by DAVID 2008 of the mineralization period identified processes of cell maintenance (i.e., RNA splicing (GO:0008380), cell cycle phase (GO:0022403), translation (GO:0006412) and cell death (GO:0008219) to be significantly over-represented (Supplementary Tables S1-S4). The low number of regulated genes precluded significant Gene Ontology analyses of the pre-mineralization period.

We clearly showed that 1,25D3 stimulated mineralization when administered during the pre-mineralization period (ECM formation). We hypothesized that the 1,25D3 effect in the pre-mineralization period is mediated by an effect on ECM genes. To assess this we downloaded a list of genes being annotated with the Gene Ontology (GO) term extracellular matrix (GO:0031012; <http://www.ensembl.org>, Release 49; updated by AmiGO, <http://www.geneontology.org>, Release July 2009) and analyzed their expressions within the pre-mineralization period by merging the list with the



**Fig. 1.** Alizarin Red staining of 1,25D3 enhanced mineralization in SV-HFO cells. Cells were treated with or without 1,25D3. Cells were harvested at days 3, 7, 10, 12, 14, and 19, fixed with 70% EtOH and stained for calcium. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**Fig. 2.** Timing of 1,25D3 effect on mineralization. **A:** Cells were treated with or without 1,25D3 for 19 days. Additionally, the treatment was shortened to only the first 12 days or only the last 7 days of culture. **B:** The cells were harvested at day 19 and the calcium content was measured. Control condition was set to 100%. <sup>a</sup> $P < 0.05$  versus control condition.

Affymetrix Gene Chip expression data. The annotated genes, among others being procollagen I N-proteinase, connective tissue growth factor and matrix-forming genes such as collagens, were up-regulated during osteoblast differentiation (Table 2). However, 1,25D3 had no significant impact on the expression of any of these ECM genes (Table 2). Q-PCR analyses of a subset of these ECM genes confirmed this observation and validated the Gene Chip analyses (Table 2). Osteocalcin appeared not to be part of the GO term extracellular matrix and is therefore not part of Table 2, but as expected 1,25D3 stimulated osteocalcin expression in human osteoblasts (data not shown). However, overall these analyses prove that 1,25D3 stimulates mineralization predominantly independent from changes ECM gene expression.

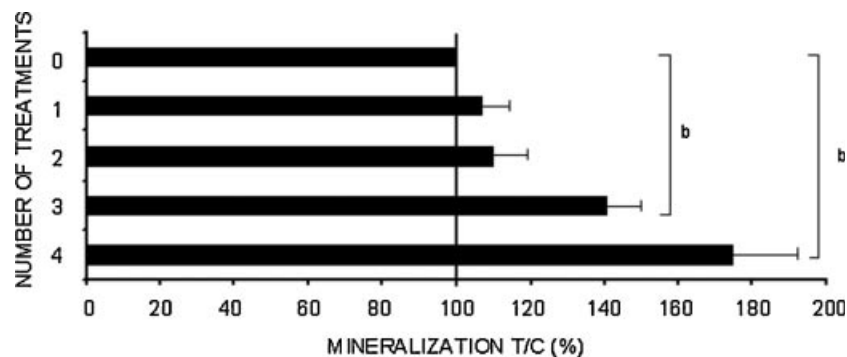
Following the bioinformatic analyses, showing no transcriptional relationship between 1,25D3 and ECM gene expression, we tested whether indeed 1,25D3 does not affect the ECM. We cultured SV-HFO with and without 1,25D3 until the onset of mineralization. Then cells were devitalized and subsequently the remaining ECM that was formed was put into culture again. This allowed for analyses of mineralization on a preformed ECM independent of additional cellular activity. Measurements at 0, 3 and 5 days after devitalization showed that ECM formed by control cultures did not mineralize yet. However, ECM of 1,25D3 treated cultures demonstrated significant mineralization thereby showing an accelerated maturation of the ECM in pre-mineralization phase (Fig. 4). This indicates an earlier onset of mineralization in cells treated with 1,25D3 and underlines the observation that 1,25D3-mediated

effects are established prior to the onset of mineralization (Fig. 2). Furthermore, despite the absence of an effect on expression of genes coding for ECM proteins (Table 2), the ECM itself is apparently affected by 1,25D3 treatment in the pre-mineralization period.

A potential alternative mechanism to explain the effect of 1,25D3 on mineralization is regulation of MV production and/or MV maturation. MVs attached to the ECM were isolated at different time points after treatment of osteoblasts with vehicle or 1,25D3 in the pre-mineralization period. Electron microscopic and FACS analyses using size reference beads demonstrated that the isolates contained vesicles of the right size (Fig. 5A, and data not shown) (Ali et al., 1970). The TEM picture shows some heterogeneity in MVs which may reflect both ALP-positive and MVs containing no or undetectable ALP (Fig. 5A). With the progress of osteoblast differentiation the number of produced MVs increases in time. The total amount of MVs was not affected by 1,25D3 (Fig. 5A). 1,25D3 significantly increased the number of ALP positive MVs (Fig. 5B) but did not change the ALP signal per MV (Fig. 5C). Quantitative analyses during osteoblast differentiation demonstrated that in the 1,25D3 condition at day 7 already a number of ALP positive MVs was reached that was not yet reached at day 10 in the control condition (Fig. 5B).

### Discussion

In this study, we demonstrate that 1,25D3 accelerates the mineralization process through effects on human osteoblasts in



**Fig. 3.** Impact of duration of 1,25D3 treatment on mineralization. In the pre-mineralization period (days 0–12) SV-HFO cells were treated with 1,25D3 for time periods up to 3, 5, 7, and 10 days representing 1–4 times 1,25D3 treatment, respectively. Cells were harvested at day 19 and calcium content was measured. Control condition was set to 100%. <sup>b</sup> $P < 0.01$  versus no 1,25D3 treatment condition.

TABLE 1. Overview of genes, which are 1.5-fold up- or down-regulated by 1,25D3 during osteoblast differentiation

	Pre-mineralization period	Overlap	Period of mineralization
Total	74	3	470
Up	21	0	154
Down	53	3	316

the period preceding the onset of mineralization. This is the result of an accelerated maturation of the ECM and specifically via effects on MV production but not the ECM protein composition.

During osteoblast differentiation various functional phases can be identified including proliferation, ECM production and maturation and mineralization. In rodent osteoblasts, it was shown that short-term 1,25D3 treatment has different effects on the expression of various osteoblastic marker genes, depending on the period of treatment (proliferation, ECM maturation, or mineralization; Owen et al., 1991). In the human osteoblast model that we used, the proliferation and ECM formation phases are identified as the pre-mineralization phase, which is supported by the gene profiling data showing an increase in expression of a broad range of ECM proteins (Table 2). It is in this pre-mineralization phase that 1,25D3 modulates osteoblast differentiation or function eventually leading to accelerated mineralization. In contrast, 1,25D3 treatment only during ongoing mineralization had no effect on mineralization. The importance of the pre-mineralization period for the 1,25D3 action is further demonstrated by the observation that living cells are not needed during the mineralization process to observe the acceleration by 1,25D3. Thereby our data are in general in line with previous data on rat osteoblasts that the effect of 1,25D3 depends on timing and phase of osteoblast differentiation (Owen et al., 1991).

The current data show the time dependency of the 1,25D3 effect on osteoblast with different effects prior to and during mineralization. This is supported by our findings that there is hardly any overlap (0.6%) between the 1,25D3 transcriptome of

TABLE 2. Regulation of genes annotated to the GO term extracellular matrix during early osteoblast differentiation and compared with 1,25D3 enhanced osteoblast differentiation

	Osteoblast differentiation <sup>a</sup>	1,25D3 enhanced osteoblast differentiation vs. osteoblast differentiation <sup>b</sup>	Ratio verified by real-time PCR
DCN	4.9	0.9	1.1 ± 0.1
COL11A1	4.1	0.7	
COL5A2	3.5	0.8	
COL8A1	3.4	0.8	
NID2	3.3	0.8	0.9 ± 0.1
FBLN1	3.2	1.0	0.9 ± 0.1
COL4A1	2.9	1.0	
FBN2	2.8	1.0	
COL12A1	2.7	1.1	
FN1	2.7	0.9	1.1 ± 0.3
COL3A1	2.3	1.0	
MATN3	2.2	0.8	
CTGF	2.2	1.0	
COL4A2	2.2	1.3	
COL4A5	2.1	0.9	
COL5A1	2.0	1.1	
COL1A1	2.0	1.2	1.3 ± 0.2
COL6A3	1.9	0.9	
COL1A2	1.9	1.0	
FBN1	1.8	0.8	
LUM	1.7	0.7	

Numeric values are fold changes, obtained by mean ratios of (a) differentiating osteoblasts versus non-differentiating osteoblasts and (b) differentiating osteoblasts of 1,25D3 cultures versus differentiating osteoblasts of control cultures.

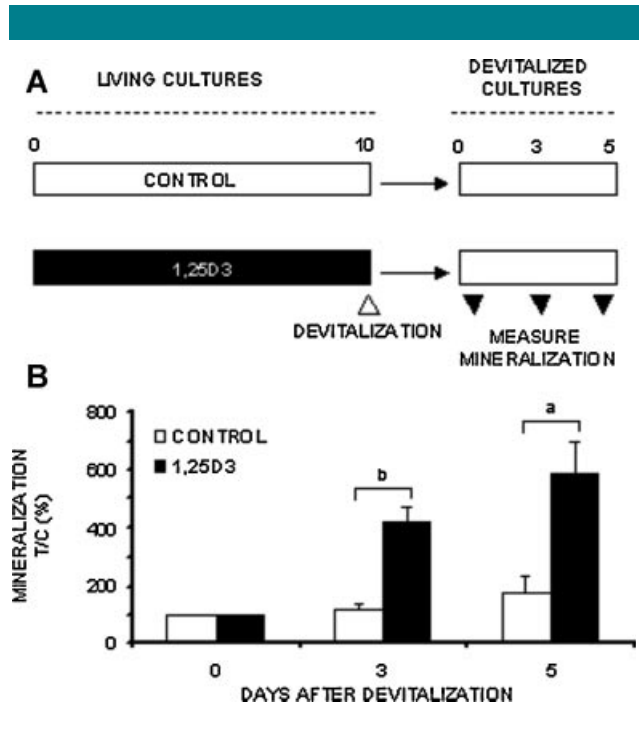
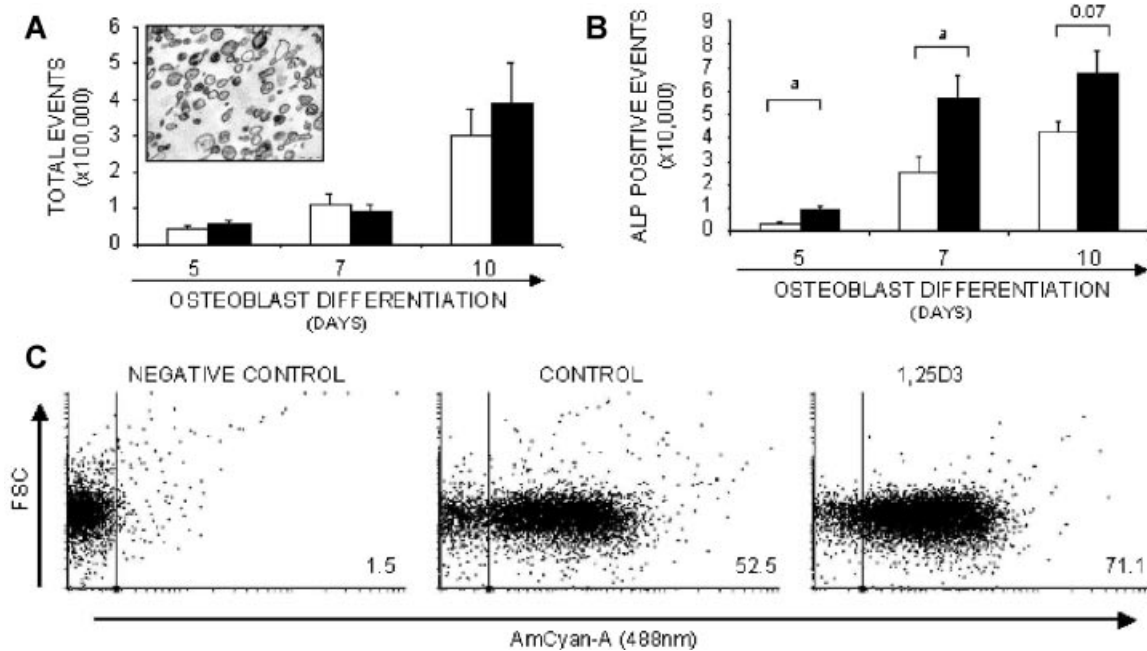


Fig. 4. Effect of 1,25D3 pre-treatment on subsequent mineralization of formed ECM. A: Culture and treatment scheme of SV-HFO cells. Cells were treated continuously with or without 1,25D3 and after 10 days of culture the cells were devitalized according to the protocol described in the Materials and Methods Section. B: Mineralization of ECM in devitalized cultures. Cultures were harvested at day 0, 3, and 5 and analyzed for calcium content. <sup>a</sup>P < 0.05; <sup>b</sup>P < 0.01 versus control condition.

the pre-mineralization and mineralization period. The impact of 1,25D3 on gene transcription is about sixfold stronger in the mineralization than in the pre-mineralization period with 470 and 74 genes being regulated, respectively. However, the current data show that the 74 genes are important for the 1,25D3 effect on mineralization as the acceleration is still observed in absence of living cells (and thus the 470 genes) during the mineralization period. Although not important for acceleration of mineralization it is interesting to understand the function of these 470 genes in osteoblast biology and bone metabolism. It is tempting to speculate that these genes and the processes they are driving are involved in the control of extent of bone formation by controlling cell survival, additional gene expression and potentially also in interaction (cross-talk) of osteoblasts with other cells in the bone micro-environment. GO analyses provided terms like cell cycle control and processes involved in transcription/translation but analyses to assess in detail these processes in osteoblast function and bone metabolism were beyond the scope of this study.

The importance of the pre-mineralization period for the regulation of mineralization is not unique for 1,25D3 as it has also been shown for activin A (Eijken et al., 2007). Moreover, it has been shown that treatment with FGF-8 within the first three days of murine bone marrow cultures stimulates mineralization 14 days later (Valta et al., 2006). In that study, treatment with FGF-8 during the entire pre-mineralization period did not lead to increased bone nodule formation, whereas continuous treatment even inhibited bone nodule formation. These observations pinpoint to the fact that timing of treatment is important for the process of osteoblast differentiation and that treatment at different time points during culture affects mineralization.



**Fig. 5.** Effect of 1,25D3 on MVs. SV-HFO cells were treated with or without 1,25-D3. At days 5, 7, and 10 of differentiation, MV isolation and analyses were performed as described in the Materials and Methods Section. Open bars represent control and solid bars represent 1,25D3 condition. **A:** Total number of MVs during the pre-mineralization period and an exemplified TEM picture of an MV isolate of control culture, day 5. Magnification: 44,000 $\times$ . **B:** Amount of ALP positive MVs during the pre-mineralization period. Results were achieved by determining Elf97 positive events in FACS analyses. **C:** An example of MVs FACS analyses of negative control (unstained MVs), control and 1,25D3 treated cultures, isolated on day 7 of culture. Numbers in the right corners of the pictures represent the percentage of ALP positive MVs. <sup>a</sup>P-value < 0.05.

In contrast to FGF-8, the effect of 1,25D3 was not dependent on a specific time-point during the pre-mineralization. It was the duration of treatment that determined the magnitude of accelerated mineralization rather than the time-point of treatment. This observation implicates that it is unlikely that 1,25D3 is stimulating a specific step in the differentiation of osteoblasts. If this would be the mechanism by which 1,25D3 accelerates mineralization, the precise timing of the effect would have been crucial. The current data about duration rather than timing suggests that 1,25D3 enhances one or more structural processes involved in the mineralization process by osteoblasts.

Two candidate processes to explain the 1,25D3 accelerated mineralization are the expression of ECM genes and/or the production of MVs. For the pre-mineralization effect of activin A on mineralization we have shown that it specifically regulates the expression of a broad range of ECM proteins (Eijken et al., 2007). However, 1,25D3 did not have any significant effects on the expression of a large list of ECM genes in the pre-mineralization period, thereby excluding a major involvement of ECM composition leading to accelerated mineralization.

Data on the effect of 1,25D3 on collagen type I are variable (van Driel et al., 2004). For example, we could not detect any effect of 1,25D3 on collagen type I expression in differentiating human osteoblasts while Maehata et al. (2006) have shown in MG63 osteosarcoma cells a stimulation by 1,25D3. Whether this is related to the osteosarcoma origin of MG63 is unclear but the basal collagen type I expression in these cells is already high suggesting an altered genomic promoter organization potentially making it also responsive to 1,25D3. Irrespective of this difference, the current study demonstrates that independent of changes in collagen type I expression 1,25D3 accelerates mineralization. An interesting observation by

Maehata et al. was that via the collagen-mediated signaling ECM affects 1,25D3 responsiveness, which may be part of the long-term 1,25D3 effects in our osteoblast model.

Another possible explanation is the regulation of MV production by osteoblasts. The current concept is that these vesicles originate from the plasma membrane and bind to the ECM and initiate mineralization (Anderson et al., 2005). The biological importance of MVs is demonstrated in a broad range of pathological calcifications, that is, pulmonary alveolar microlithiasis (Bab et al., 1981) and atherosclerosis (Tanimura et al., 1983). Furthermore, patients with hypophosphatasia, a disease resulting in rickets and osteomalacia, lack the tissue non-specific isoenzyme of ALP (TNSALP) in MVs (Anderson et al., 1997). MVs contain a large amount of ALP (Ali et al., 1970; Majeska and Wuthier, 1975) and we have previously shown that in the pre-mineralization period 1,25D3 enhances the expression of ALP in osteoblastic cultures (van Driel et al., 2006a). MVs can be affected in two ways, firstly, the production as assessed by the number of ALP-positive MVs, and/or secondly, the maturation as assessed by the amount of ALP per MV can be regulated. Our data show that the first way is the mechanism by which 1,25D3 acts. We demonstrated that in the pre-mineralization period 1,25D3 increases the number of ALP-positive MVs. This is in line with effects of 1,25D3 on MVs from the MG63 osteosarcoma cell line (Bonewald et al., 1992) and on chondrocytic MVs (Schwartz et al., 1988, 2002; Dean et al., 1996; Schmitz et al., 1996; Boyan and Schwartz, 2008).

In the ECM we observed an accumulation in time of both ALP positive MVs and MVs containing low or non-detectable ALP. Apparently osteoblasts produce both types of MVs and this heterogeneity in MVs can be seen from the TEM picture in Figure 5A. The function of these ALP negative MVs is yet elusive but from our study it is clear that 1,25D3 shift the ratio towards

ALP-positive MVs and accelerates the increase in ALP-positive MVs.

The main mechanism of initiating mineralization is supposed to constitute the formation of hydroxyapatite, arising from crystallization of inorganic phosphate (Pi) groups, which are provided by ALP (Whyte, 1994), and calcium ions (Anderson, 1995). There are several indications that ALP is present in the ECM (Salomon, 1974; Bonucci et al., 1992; Groeneveld et al., 1996). Therefore, we postulate that MVs translocate ALP to the ECM where it is incorporated. We hypothesize that within the ECM a certain quantity threshold of incorporated ALP has to be achieved to initiate mineralization, which is schematically depicted in Figure 6. Treatment with 1,25D3 reaches this threshold earlier through accelerated synthesis of ALP positive MVs (Fig. 6A). At the onset of mineralization, 1,25D3-treated cultures contain more ALP within the ECM leading to a higher conversion of calcium ions and Pi into hydroxyapatite, eventually resulting in accelerated mineralization. Intermittent treatments of 1,25D3 accelerate mineralization to a lesser degree (Fig. 6B;  $a = 7$  days). This hypothesis is supported by the fact that a higher ratio of Pi over pyrophosphates (PPi), which is thought to be determined by ALP levels (Thouverey et al., 2009), leads to increased mineralization. This new insight

into MV functionality may shed light on the complex mechanism behind MV function.

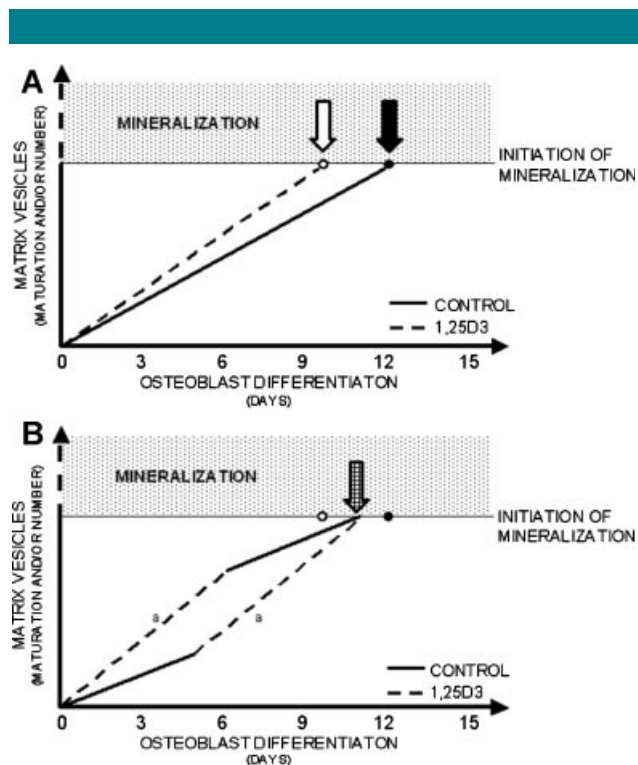
In conclusion, we propose the concept that besides indirect effects via intestinal calcium uptake, 1,25D3 can directly accelerate osteoblast-mediated mineralization by stimulating the production of ALP-positive MVs in the period prior to mineralization. More MVs lead to an earlier onset and higher rate of mineralization. These effects are independent of changes in extracellular matrix protein composition. These data on 1,25D3, mineralization, and MV biology add new insights into the role of 1,25D3 in bone metabolism and emphasize the importance of MVs in bone as well as maintaining bone health and strength by optimal mineralization status.

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**Fig. 6.** Diagram depicting the conclusion that length of treatment rather than starting point of treatment determines the MV-driven acceleration of mineralization by 1,25D3. **A:** Continuous treatment with 1,25D3 (dashed line) leads to an earlier onset of mineralization than in the control cultures (solid line). The time of mineralization in the 1,25D3 condition is indicated by the open arrow and open circle (○) and mineralization in the control condition by the solid arrow and solid circle (●). **B:** Impact of two equally long ( $a = 7$  days) intermittent treatments with 1,25D3 but starting at different days (day 0 and day 5, respectively) during the pre-mineralization period. The dashed lines indicate 1,25D3 treatment and the solid lines the control treatment. These treatments will result in the same acceleration of initiation of mineralization (indicated by patterned arrow) compared to the control condition (indicated by the solid circle (●), see also part A) but later than in the continuous treatment with 1,25D3 (indicated by the open circle (○); see also part A).

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