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# Exocytotic process as a novel model for mineralisation by osteoblasts *in vitro* and *in vivo* determined by electron microscopic analysis.

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**Abstract**

The process of biomineralisation has been examined during osteoblastic differentiation of bone marrow stroma cells (BMSC) from embryonic chick in culture and in periosteum itself by a number of different techniques including transmission (TEM) and scanning (SEM) electron microscopy. In cell culture of BMSCs at days 20-25 crystals were accumulated extracellularly in the collagen matrix resulting in large plate-like crystallites and non-collagen associated on the culture disk surface. In contrast up to days 10-18 mainly intracellular mineralisation was visible by numerous needle-like crystal structures in the cell cytoplasm and in vacuoles. After 20-30 days the crystal-content of these vacuoles is released -most probably by membrane fusion to the outside of the cells. Energy Dispersive X-ray analysis (EDX), Electron Spectroscopic Imaging (ESI) and Electron Energy Loss Spectroscopy (EELS) demonstrated that Ca, O and P are located in the intra- and extracellular needle-like crystals. From EDX-spectra a Ca/P ratio of 1.3 was estimated for the intracellular structures and a Ca/P ratio of 1.5 for the extracellular material (for comparison the Ca/P ratio in tibiae is 1.6). X-ray diffraction and quantitative IR spectral (IRS) analysis also demonstrated an increase of crystalline bone apatite along the mineralisation process. In addition to the finding *in vitro*, the presence of intracellular needle-like crystals in vacuoles could also be demonstrated *in vivo* in osteoblastic cells of the periosteum in tibia of day 11. The results demonstrated are in favour of a novel model for mineralisation by osteoblasts, in which amorphous Ca-P material is directly secreted via an exocytotic process from vacuoles of the osteoblast and deposited extracellularly and propagated into the collagen fibril matrix and maturated to hydroxyapatite.

## Introduction

The mechanism of mineral formation in bone is best seen at locations, where active new bone is occurring, e. g. in newly forming bone of the periosteum of the embryo, in the growing long bone in the epiphyseal growth plate and in pathological situations in soft tissues. Three patterns of mineral deposition on extracellular matrix have been described: (i) matrix vesicle-mediated mineral initiation [1-7] and (ii) nucleation of mineral crystals on collagen [8-10] , and (iii) ectopic mineralisation in the absence of osteoblasts [11]. To date the accumulation of early calcium phosphate crystals and the transport of early mineral to the extracellular matrix space is uncompletely understood. It is well known, that calcified cartilage and woven bone mineralise via matrix vesicles, membrane-bound bodies, that were generated from plasma membrane and migrate to the loose extracellular matrix space. These matrix vesicles contain often crystals of mineral suggesting that they function as sites of initial calcification. This mechanism has been demonstrated to be involved in the remodeling process in the diaphysis of long bone of embryonic and early postnatal animals [1-7].

In the case of lamellar bone in the periosteum the mineralisation proceeds in association with the hereropolymeric (collagen-noncollagen-protein complex) fibrils themselves. Some of the mineral seems to be associated with the gap regions in the packed fibrils and regions between the collagen fibrils, which are organized in a brick and mortar fashion. However, matrix vesicles are rarely or never seen in lamellar bone and there are some doubt concerning the existence of an association between matrix vesicles and mineralisation in the periosteum and in osteoblastic cells [8-10]. Furthermore, in the case of ectopic mineralisation an osteoblast independent mechanism may also contribute to the mineral formation [11]. A number of problems have hindered the investigation of the calcification process in vivo as the rapid turnover of the developing bone by the presence of osteoclastic activity in young animals. Detailed information about the process of ECM mineralisation has been obtained from *in vitro* cell culture systems, were the kinetic is in a low time frame. The process of ECM mineralisation was found to consist of three distinct time periods, in which each state depend on each other: a proliferative phase with collagen I synthesis, a period of matrix maturation and a period of mineralisation (12). In order to clarify the calcification process in osteoblasts bone marrow derived mesenchymal stem cells (BMSCs) from embryonic chicks have been used as a source of undifferentiated cells. In an effort to standardise culture conditions, in particular to reduce interfering effects of the culture medium towards the mineralisation process, the culture conditions have been defined by replacing FCS with a combination of

physiological regulatory factors such as BMP-2 and Vitamin $D_3$ under serum reduced conditions. Our research focuses on two main goals 1) to investigate at different time points during the calcification process the morphology, chemistry and structure of the CaP mineral phase formed inside the cell and extracellularly in cell culture of BMSCs and 2) to compare the *in vitro* data with the *in vivo* process in the osteoblast of the periosteum in tibia of chick.

With improvements in electron microscopic analysis techniques like Electron Spectroscopic Imaging (ESI) and Electron Energy Loss Spectroscopy (EELS) and EDX-analysis an accurate detection of the elemental distribution of calcium, phosphorus and oxygen in newly deposited crystals at early time points of the mineralisation process in ultrathin sections could be performed [13]. In addition, Fourier transform infrared (FTIR) spectroscopy and X-ray diffraction measurements were used to study the crystallinity of the mineral during the mineralisation process [14].

The applied techniques provided data on the accumulation of CaP needles in early stages of mineralisation first in the cytoplasm of BMSC and then within a vacuole from which the CaP particles are secreted in an exocrine manner extracellularly during the mineralisation process. We have not observed mineralised matrix vesicles extracellulary. At the endpoint of the mineralisation process crystalline hydroxyapatite in association with collagen fibrils could be detected. Analogous findings were obtained *in vivo* in the osteoblast of the periosteum in tibia.

## Materials and Methods

### *Chemicals*

Recombinant human BMP-2 was a kind gift from Genetics Institute (Cambridge, Massachusetts). All tissue culture media was purchased from Flow Laboratories (Meckenheim) and plasticware were obtained from Tecnomara (Fernwald). The growth factors and all chemicals were purchased from Sigma (Deisenhofen).

### *Cell culture*

Fertilized eggs from chickens were obtained from a commercial supplier (Charles River, Sulzfeld, Germany) and incubated in an egg incubator (Ehret, Emmendingen, Germany) with a rotating time of 8 times/ day, at 37°C and 60% humidity. Tibiae and femora were dissected from 17 day old embryonic chicks under sterile conditions and all adherent material removed. The epiphyses were removed and the BMSCs flushed out of the marrow cavity with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin and streptomycin (100 µg/ml) and glutamine (2mM) using a hypodermic syringe. The cells were then transferred to tissue culture bottles and grown in DMEM containing the above supplements plus heat inactivated (56 °C for 30 min) 10 % FCS, the medium was changed every 2 days and the cells were allowed to reach confluency. The cells were sub cultivated into 96 well, 12 well or 6 well plates at a cell density of 10.000 cells/$cm^2$ in DMEM plus 10 % FCS and supplements. After 24 h, the medium was replaced with DMEM containing supplements, 2 % FCS, ascorbic acid (50 µg/ml), ß-glycerophosphate (10 mM) and vitamin $D_3$ ($10^{-9}$M). The cells were then left for another 48 h and challenged with 20 ng/ml BMP-2. The culture without BMP-2 was used as negative control. Culture with 10 % FCS in OI medium was used as positive control. During the course of experiments medium was replaced every two days with fresh medium containing the above supplements together with the inducing agent.

### *Alkaline phosphatase assay*

After the induction period medium was aspirated and then fixed for 2 h with absolute alcohol at room temperature. Alkaline phosphatase (APase) activity was then determined quantitatively by the addition of p-nitrophenylphosphate (1 mg/ml) in AP buffer (20 mM sodium carbonate, 3 mM magnesium chloride, pH 9.5) for 1 h at 20°C. The buffer was aspirated and the liberated p-

nitrophenol determined spectrophotometrically at 405 nm. Alkaline phosphatase was also stained histochemically. In brief, a stock solution of 25 mg napthyl phosphate in 10 ml formamide and 10 ml water was adjusted to pH 8 with 1 M sodium carbonate. The working solution was prepared by adding 1 ml stock solution to 24 ml AP buffer, adding Fast Red TR-salt to a final concentration of 1 mg/ml. The solution was then filtered. The cells were stained for 1 h at 20° C.

*Calcium determination*

Calcium was measured colorimetrically using arsenazo III calcium reagent. After 10 days in culture, the medium was aspirated and the cells extracted with 0.5 ml 0.1 M HCl for 15 min. 5 µl of the extract was placed in a microtitre plate and 200 µl arsenazo III added. The plates were then read at 595 nm using a plate reader and the calcium concentration calculated according to a standard solution.

*Protein determination*

For protein determination cell layers were lysed with 0,1% Triton X-100 over night and the protein content was determined using a protein assay kit (Pierce) with bovine serum albumin as a standard.

*Physical analysis (X-ray diffraction and FTIR spectroscopy)*

For physical analysis BMSCs were cultured in 6 well plates as above. For wide angle diffraction (WAXD) measurements, a Debye-Scherrer camera with a circumference of 360 mm was used. The detection was carried out with a X-ray sensitive film (Fuji-100) at an exposure time of 20 h. The diameter of each visible ring of interference, measured in mm, is equivalent to four times the diffraction angle Ø. The Debye-Scherrer camera was mounted on a X-ray generator ( Mueller-Mikro III, Philips, Kassel, Germany ) with an accelerator voltage of 46 kV and a current of 20 mA. The indication of the basic cristallographic structures are performed by using a special computer program (Treor 4). FTIR spectra of the culture were

recorded on a Perkin Elmer Fourier transform spectrometer model 1700 and Analect FX 6002, after embedding the dried sample in KBr pellets.

*Transmission electron microscopy and EDX analysis*

Samples were fixed with a fixation solution containing 2% glutaraldehyde and 5% formaldehyde in cacodylate buffer (0.1 M cacodylate, 0.09 M sucrose, 0.01 M $MgCl_2$, 0.01 M $CaCl_2$, pH 6.9) for 1 h on ice. Samples for morphological studies were further fixed and contrasted with 1% aqueous osmiumtetroxide for 1 h at room temperature, washed and dehydrated with a graded series of aceton (10, 30, 50%) for 30 min each step on ice. Another contrasting step was introduced by dehydrating the samples further in 70% acetone containing 0.5% uranyl acetate at 4° C for overnight. Samples were then dehydrated with 90% and 100% acetone on ice. The next 100% acetone step was performed at room temperature. Samples for EDX analysis were not treated with osmiumtetroxide and uranyl acetate to minimize possible line overlapping during EDX analysis. All samples were then embedded in the low viscosity epoxy resin according to Spurr [15]. After heat polymerisation (8 h at 70°C) samples were cut with glass knives (Reichert Ultrotome S, Wien, Austria) and collected onto formvar covered 300 mesh copper grids. Samples for morphological studies were counterstained with 4% aqueous uranyl acetate for 2 min and lead citrate for 1 min. Ultrathin sections (40 nm in thickness) for EELS/ESI analysis were cut with a diamond knife and not counterstained as well as sections for the EDX analysis. Samples were observed in a Zeiss transmission electron microscope EM910 at an acceleration voltage of 80 kV. The EM910 was attached with an Oxford ISIS 300 EDX-analysis system (Oxford, Wiesbaden, Germany) and a digital scanning unit for elemental mapping. Collecting of spectra and processing of data was done according to the manufactors guidelines. ESI and EELS analysis were performed with an Zeiss CEM902 transmission electron microscope with the SIS EFTEM software package (Muenster, Germany) at an accelerating voltage of 80 kV.

*Field emission scanning electron microscopy (FESEM)*

Samples were fixed as described above and dehydrated with a graded series of acetone (10. 30, 50, 70, 90, 100%) on ice for 15 min each step. Samples were brought to room temperature

during the last 100% acetone step and then critical-point dried with liquid $CO_2$. For morphological studies samples were sputter coated with an appr. 10 nm thin gold film. Samples for EDX analysis were carbon coated. To gain access to the underlaying collagen matrix with mineralisation deposits half of the cellular material after critical-point drying was blown away with pressured air and then carbon coated. Samples were examined in a Zeiss field emission scanning electron microscope DSM982 Gemini at an acceleration voltage of 5 kV for morphological studies using the Everhart-Thornley detector and the in-lens detector in a 50:50 ratio. EDX analysis was performed with an acceleration voltage of 15 kV. The microscope was equipped with an Oxford ISIS300 EDX-analysis system (Oxford, Wiesbaden, Germany).

*Statistical analysis*

Statistics were performed with the Microsoft Excel program. Values are given as mean ± SEM. The *P* values $*P < 0.05$ and $***P < 0.001$ indicate statistical significance.

## Results

*Synchronized cellular osteogenic differentiation by osteogenic compounds using bone marrow derived mesenchymal stem cells from embryonic chick*

In order to clarify the calcification process by osteoblasts *in vitro* BMSCs from embryonic chick were used. The choice of culture conditions is obviously of critical importance for the kinetics of the osteogenic process. Therefore, we reinvestigated the culture conditions to determine optimal conditions for induction the mineralisation process. To achieve this, firstly the induction of ALP activity was used to measure the inductive role of compounds in osteogenesis. In a basal medium (DMEM in the presence of ß-GP and ascorbic acid in 2% FCS) no ALP activity was detectable. Vitamin $D_3$ at a concentration of $10^{-7}$ M, however, slightly increased ALP activity, whereas dexamethason had no effect. Of the growth factors only BMP-2 was found to be positive with maximum effect at 20 ng/ml, whereas TGF-ß1, IGF-II, bFGF, EGF had no significant effect on ALP induction. Furthermore, the induction of osteocalcin as a late marker of osteogenesis was found to be induced by BMP-2 was higher compared to $VitD_3$ at day 10. Finally, in regards to their effect on cell proliferation$VitD_3$ was found to cause an inhibition, whereas BMP-2, slightly induced cell proliferation. We found that both ascorbate ( 50 µg/ml) and ß-GP (10 mM) are required for mineralisation, however dexamethasone showed no effect. From this data combinations of 1,25-$(OH)_2$ $D_3$ ($10^{-9}$ M) and FCS (2%) in the presence of ß-GP and ascorbic acid with 20 ng/ml BMP-2 were used. As seen in Fig. 1 over the culture period of 30 days the ALP expression was found to be transient and mineralisation increased from day 15 in the BMP-2 treated culture continuously. In situ staining for ALP revealed nearly homogenous ALP positive cells in the BMP-2 treated culture at a confluent state. ALP activity and mineralisation was not seen in the control culture without BMP-2. Furthermore, no mineral particles has been seen in the absence of cells even at incubation for 30 days of the medium. These data suggest that the expression of mineralisation is strictly dependent on the presence of an osteoblastic phenotype.

Gene expression profiling data for osteogenic markers like BMP-2 and the BMP-receptors IA / ALK-3 BMPR-II and BMPR IB/ ALK-6 and osteopontin and osteocalcin were assessed. The expression data confirmed the expression of an osteogenic phenotype during the culture period.. Furthermore, the absence of collagen II expression ( Fig. 1) and the negative results in staining with alcian blue and the inhibition of sulfate incorporation as an indicator for chondroitinsulfate suggests that under these conditions there is no evidence for the presence

of a significant number of chondrocytes in the culture (data not shown). Furthermore, morphologically no significant number of adipocytes have been detected. In situ staining for ALP, revealed homogenously ALP positive cells in the BMP-2 treated culture and not in untreated cells. In summary, the data suggest that mainly the osteogenic lineage is expressed. From this data it can be suggested that the osteogenic process, characterized by ALP expression, enhanced collagen I synthesis and increases in mineralisation can be precisely controlled by osteogenic modulators *in vitro*. In vivo the mineralisation in embryonic chick bones occurs very rapidly whereas in cell culture the kinetic of the mineralisation process is slowed down allowing dissection of the complex process of mineralisation.

*Analysis of the mineralisation process at the end of the culture period by TEM* .

We analysed by TEM the mineralisation process intracellularly and extracellularly at different time points. At the end of the culture period a dense calcified connective matrix had been synthesized and ultrastructural analysis revealed after 21 –25 days structural features similar to young osteoid *in vivo*, including cells arranged in a mono/bilayer fashion and well developed, orthogonally arranged collagen fibrils. Analysis of mineral indicated two fractions. An accumulation of small mineralised particles outside the cells could be seen without collagen (Fig. 2, a) and mineralised sheets were found to be embedded in a collagen matrix (Fig. 2, b). In addition, non induced BMSCs formed also a collagen matrix which did not exhibit any mineralisation (Fig. 2, d). A typical organizational feature for the osteoid was observed in the collagen matrix organisation, specifically that layers of collagen fibres run perpendicular to each other. In Fig. 2, c the numbers indicate the different orientation of the collagen fibres in the extracellular matrix; 1. shows a cross section, 2. a longitudinal section through the fibres and 3 again depicts a cross-section of collagen. This organisation was also detectable in non-induced BMSCs (Fig. 2, d). In all differently orientated collagen layers we were able to detect mineralisation plate like aggregates in the BMP2-induced cells. However, we did not detect any mineralisation deposits in small matrix vesicles. In addition, we were unable to enrich matrix vesicles by centrifugation of BMSCs in contrast to the control tissue of epiphysis confirming our ultrastructural analysis.

*Formation of non collagen and collagen associated mineralisation as seen by high resolution FESEM*

The mineralisation process of the extracellular matrix was followed by electron microscopic methods demonstrating the high resolution power, especially when applying modern analysis

methods like high resolution FESEM, Electron Spectroscopic Imaging (ESI) and Electron Energy Loss Spectroscopy (EELS) as well as energy-dispersive x-ray analysis (EDX). When applying high resolution FESEM to our in vitro mineralisation system we observed at higher magnifications small island-like deposits on the culture disk surface after 18 days which were formed out of a multitude of smaller mineralisation deposits (Fig. 3, a-c). At day 18-20 sphere-like material was deposited on the top of these mineralisation islands (Fig. 3, d and e) and with time larger aggregates have been formed (Fig. 3, f-h). These aggregates on the culture disc surface were not embedded in collagen fibrils. The collagen fibrils were first observed in the following layer of mineralisation aggregates (see also Fig. 5). EDX- analysis revealed that even in the small island-like deposits as well as in the larger aggregates Ca, P and O could be detected (Fig. 4, a). The signals for all three elements were more pronounced in the large aggregates suggesting more deposition of these elements in the large mineralisation areas. Analysis of the plain culture disk surface gave no signal for Ca and P (data not shown). We then applied EDX elemental mapping analysis to detect colocalisation of Ca, P and O. Results of the mapping analysis revealed that all three elements are colocalised in the mineralisation deposits (Fig. 4, b-e). After 25 days of BMP-2 induction a very pronounced formation of an extracellular matrix comprised of collagen fibres was detectable. Within this matrix we could detect different sized mineralisation aggregates (Fig. 5, a). Even on the collagen fibres we were able to detect some small mineralisation deposits (Fig. 5, b-d).

*Visualisation of intracellular mineralisation needles by ultrathin section analysis*

At this time no information was available on the distribution of mineral particles in the early mineralisation state inside as well as outside the osteoblast. Therefore, we analysed the presence of mineral particles in ultrathin sections over a time period covering the entire mineralisation process. At days 15-18 the process started with the formation of mineralisation needles in a distinct area of the cytoplasm (Fig. 6, a arrow head). Mineralisation needles then formed non membrane-bound aggregates (Fig. 6, a, arrow and b). At higher magnifications the mineralisation needles exhibited a very characteristic morphology, namely consisting of two different phases, one phase being a dark part representing most probably the calcification phase of the mineralisation needles (Fig. 6, c, circles and d, arrow heads). The other phase of a greyish to pale structure could be distinguished exactly positioned underneath the dark area. From the morphological appearance in the ultrathin section this luminescent part of the

structure represents a proteinaceous structure (Fig. 6, d, arrows) and does not consist of collagen because the ring-like structures, typical for collagen, are not visible.

Starting between day 21-25 cytoplasmically formed mineralisation needle aggregates were engulfed by a membrane (Fig. 7, a). We found several of these membrane-bound mineralisation compartments in a single BMSC cell. These compartments have the ability to fuse with each other to form larger compartments (Fig, 7, b, arrow and c) containing more and more mineralisation needle aggregates. After 25 days we observed that the content of the mineralisation compartments can be released to the outside of the osteoblast most probably by exocytosis (Fig. 7, d and e).

The presence of calcium in the cytoplasmic mineralisation needles and mineralisation needle aggregates was verified using EDX-analysis. The EDX spectra recorded from the free, intracellular mineralisation needles formed in the cytoplasm and the aggregates revealed the presence of Ca, P and O (Fig. 8, a, spectrum 2) whereas the surrounding ribosome rich cytoplasm showed no Ca signal, a weak P signal and an O signal (Fig. 8, a, spectrum 1). The EDX spectra displayed in Fig. 8, b demonstrate the presence of Ca, P and O in the intra- and extracellular mineralisation aggregates. From the height of the signals it is obvious that the extracellular mineralisation deposits exhibit a higher Ca signal when compared to the intracellular Ca signal (compare Fig. 8, b spectrum 1 with spectrum 2) resulting in a different Ca/P ratio (see Tab.1). EDX elemental mapping also revealed the presence of all three elements in the intra- as well as extracellular mineralisation deposits (data not shown).

*Electron Spectroscopic Imaging (ESI) and Electron Energy Loss Spectroscopy (EELS) of intracellular mineralisation needle aggregates and extracellular mineralisation deposits*

ESI and EELS have proven to give reliable results especially for the detection of Ca in the mineralisation process. We have performed EELS analysis on intracellular mineralisation aggregates in vacuoles as well as on extracellular deposits. The energy loss spectra represent the intensity distribution of the scattered electrons as a function of the energy loss. As is evident from Fig. 9, a calcium, phosphorus and oxygen were detectable in the same mineralisation aggregates represented by the increase of intensity after the elemental characteristic lines; thus, again demonstrating colocalisation of the three elements. ESI analysis was then performed applying the two-window method. In Fig. 9, b an image at the carbon edge at 250 eV is depicted showing the morphology of the investigated extracellular mineralisation deposits. Another image was taken before the Ca-edge at 341 eV and a second

image was taken behind the Ca–edge at 358 eV. From these two images the net Ca distribution was calculated and is depicted as net Ca. Thus, ESI demonstrated the presence of Ca and EELS revealed the colocalisation of Ca, P and O in the intra- and extracellular mineralisation deposits as well as the EDX analysis.

The results presented here demonstrate formation of small mineralisation needles intracellularly in the osteoblast cytoplasm and with prolonged induction time formation of mineralisation needle aggregates which are subsequently surrounded by a membrane. These membrane-surrounded vacuoles have the ability to fuse with the osteoblast membrane. Subsequent release of the mineral needle aggregates and embeddment of the deposits in the extracellular collagen fibril matrix, supports an exocytotic pathway as a novel mechanism for mineralisation by the osteoblast.

*Analysis of intracellular and extracellular mineralisation in vivo in osteoblasts of the periostal regions in tibia*

The osteoblast of the periostal region of chick tibia was used for comparing our *in vitro* findings with the *in vivo* situation in the chick bone. Morphological analysis of ultrathin sections revealed that under *in vivo* conditions mineralisation aggregates which are located in membrane-surrounded vacuoles are also formed (Fig. 10 a-c). Furthermore, mineralisation deposits are also located in the extracellular space in near vicinity to the calcified bone (Fig. 10, a and b, arrow heads). One striking feature observed was the identical morphological appearance of the early mineralisation needles as seen in cell culture (compare Fig. 6, d with Fig. 10, d). When EDX analysis of the mineralisation aggregates was performed, again colocalisation of Ca, P and O was obvious (Fig. 10, e). Therefore, our results give strong evidence that mineralisation *in vitro* using BMSCs exactly mimics the *in vivo* situation of bone mineralisation in the periosteum.

*Physical characterisation of mineralisation during osteogenesis*

Quantification of EDX analysis signals in 25 days BMP-2 induced BMSCs revealed a Ca/P ratio of 1,21 for intracellular mineralisation needle aggregates and a Ca/P ratio of 1,38 for extracellular mineralisation deposits. After 30 days of induction the Ca/P ratio for the extracellular deposits was 1. 45. This value is close to the ratio of 1. 55 which was calculated for calcified tibia bone under identical experimental conditions (Tab. 1).

For further characterisation of the mineralisation deposits electron diffraction pattern were generated from BMP-2 treated cultures after 10, 15 and 30 days. The diffraction patterns increased in intensity and complexity with culturing. At day 30 the patterns were similar to patterns observed with tibia and enamel, indicating their overall structure as crystalline apatite. The patterns observed from the control culture after 30 days revealed no significant amount of crystalline apatite.

Quantitative IR spectral analyses revealed a peak between 550 and 600 $cm^{-1}$ in samples of BMP-2 treated cultures at day 15 and day 30, that were similar to those found in samples of dentin, which is characteristic for crystalline CaP. Amorphous CaP showed a broad absorption at 575 $cm^{-1}$. The control culture without BMP-2 showed no signal. The percentage of crystallinity as calculated from the ratio of the integrated intensities to dentine with 100 % was found to be 93 % at day 15 and 97 % at day 30 of the total CaP in BMP-2 treated material. In conclusion, the results demonstrate an appearance of amorphous CaP intracellularly and exracellularly in early cultures and an increase of crystallinity along the mineralisation process.

## Discussion

The purpose of this study was to clarify the mineralisation process on osteoblasts *in vitro* and *in vivo* with respect to morphology, chemistry and maturation of crystal structure of the mineral. Bone marrow stroma cells have long been considered to be the source of osteoblast progenitors with an osteogenic potential similar as osteoprogenitors from periosteum and calvaria [16]. The time frame of ALP expression and mineral formation in our primary system in the presence of ascorbate and ß-glycerophosphate is similar as described by others on primary osteoprogenitor cells of different sources from different species [17] including humans [18]. The presence of different levels of ALP in cells and spots of mineral support the view that our BMSCs system recapitulates the successive stages of the osteoblast differentiation pathway comparable to the fetal rat calvaria system described by Malaval et al. [19].

For improving the cell culture system growth factors have been used. Apart from BMP-2 no other growth factor had any significant effect on ALP expression confirming other reports[20]. The expression of ALP, first seen after 4 days in culture, suggests that BMP-2 does not directly stimulate ALP activation or synthesis but rather induces the differentiation of progenitor cells. We further found that the mineralisation process requires ascorbate and ß-GP as supplements similar to described *in vitro* protocols by others. However, dexamethasone has no effect in our BMSCs system which differes from mammalian cell systems. Our gene expression data confirmed an uniform expression of a mature osteoblast like phenotype. Furthermore, the cell culture system improved in terms of chemically defined conditions thus limiting undesirable effects of the medium including unspecific mineralisation.

The biological relevance of the calcification process in BMSCs is supported by several critical observations. Mineralisation appears in a time dependent manner only in the culture expressing the osteogenic phenotype, including ALP. The absence of mineralisation in the matrix rich control culture without expressing ALP and also in the medium suggest that the mineral formed is dependent on the expression of an osteoblastic phenotype and not a result of a spontaneous calcium ion precipitation. The distribution of mineral deposits in each layer of the collagen matrix only in BMP-2 treated cultures and not in the layers of the control culture supports again an extensive role of the matured matrix on mineralisation. These data are consistent with the concept that collagenous matrix must undergo a maturation process

before it can support mineral induction and collagen I alone is not sufficient for ECM mineralisation even at high ionic concentrations as shown for mouse MC3T3-E1 cells [21, 22] Our additional finding of non-collagen associated mineral deposits increasing in size only in the BMP-2 induced cultures suggests that this mineral formation may also be associated with the presence of an osteoblastic phenotype. A similar result has been described in a study of a UMR10601 BSP cell-line. Extracellular mineral deposits of varying sizes have been found beneath and between cells and some located near, but rarely on, collagen fibrils upon addition of 5 m M ß-GP for the terminal 24 h of a 72 h culture period. Non-collagenous proteins are suggested to influence mineral formation and one of these glycoproteins, BSP, has been localized at the ultrastructural level within the early mineral accretions by UMR10601 BSP [23]. Furthermore, in the absence of collagen I mineral particles has been described in a subtype of ROS cells, suggesting that additional non-collagen products are involved in initiation and growth of mineral [24, 25].

In addition to these particles on the plastic surface we observed small mineral particles below the surface of the osteoblast *in vitro* in early culture phase and *in vivo* ,which makes an unspecific effect highly unlikely. We favour therefore a model of a collagen independent mineralisation within the early mineralisation accretions. In summary, the similarities between the mineralization process in short term cultures of cell-lines and in primary osteoblastic cultures may reflect a common phenomenon reflecting very early stages of apatite nucleation, which have not progressed sufficiently to transfer or seed mineral crystals onto collagen fibrils.

The pathway of maturational changes of the mineralisation with an incomplete mineralisation of ECM after 30 days, as we documented here, has been reported by others [17]. This may reflect the low kinetik of the osteogenic progression in comparison to the fast process in osteoblast cell-lines as ROS 17/2-8, UMR and the *in vivo* process in embryonic bone.

For further evaluation of the mineral transport we analysed the culture for the presence of matrix vesicles. We were not able to isolate matrix vesicles with mineral particles from our BMSCs as has been reported for newly synthesized bone [26]. However, by applying the identical isolation protocol we could detect matrix vesicles from the epiphysis. Thus the mineralisation particles we detect in our cell culture system and *in vivo* in the osteoblasts of the periostal regions in tibia are not derived from matrix vesicles which are characteristic for endochondral mineralisation suggests that mineral particles are most probably extruded from

the osteoblast. Our report therefore favours a novel matrix independent process of mineralisation and we demonstrate for the first time a direct visualisation of intracellular residing mineralisation particles.

The process by which intracellular mineral particles are formed and transported outside is completely unknown. For balancing bone mineralization it is a widely accepted view that osteoblast specific gene products can control the formation of mineral crystals by two different mechanisms either to initiate or inhibit the ECM mineralisation. There are evidences that the intracellular calcium pool of the osteoblast is involved in mineralisation, since calcium and phosphor deposition depends on the activity of a specific ATPase and mitochondrial oxidative phophorylation. Furthermore, the mineralisation response of the osteoblast was found to be dependent on calcium and phosphate supplementation. [27] However, there is no mechanism known, how mineral particles are formed and released from the osteoblast.

We present here evidence for an accumulation of Ca/P particles in the cytoplasm of the osteoblast. We found that mineral particles are present in the cytoplasm and were surrounded by a membrane *in vitro* and *in vivo*. In both situations we see underneath the Ca/P needles an luminescent part representing a proteinaceous structure. From EDX, X-ray analysis and IR spectroscopical analysis this mineral seems to be amorphous Ca/P. Amorphous precursor phases are currently widely discussed as preceding the formation of actual mineral crystals. Very young crystals in BMSCs from chick have been reported as a poorly crystalline carbonated apatite [14].From our finding of an association of mineral crystals raises the possibility of different functions as neutralisation of the ions or vectors for accumulation, or inhibitor for mineral maturation. After fusion these large membrane-bound compartments move to the cell membrane and by fusion with the cell membrane the mineral particles were extruded. For procollagen, and lipoprotein droplets, and algal scales it has been suggested that transport occurs through progressive maturation of the Golgi cisternae [28, 29]. There is some evidence that the transport of mineral particles may follow this model. As we have not seen any collagen fibers in our vacuoles, we suggest that the mineral transport may be an additional, independent osteoblastic activity. It has already been suggested that cisternal maturation may coexist with other intro-Golgi traffic mechanisms [30]. From this it can be assumed that the osteoblast has different transport mechanisms in addition to that of known function for small proteins like phosphoproteins, alkaline posphatase, matrix gla proteins, osteopontin, osteonectin, and osteocalcin which are carried across the Golgi stacks and in

general along the secretory pathway by transport vesicles. Our view of a secretory process involved in mineral transport is supported by a recent report demonstrating that the assembly of nucleators takes place within intracellular locations and these structures appear to nucleate apatite crystals soon after they emerge from the cells [23].

The mineral structure revealed a change in morphology and composition along the culture period . Small internal and extruded mineral particles contain both calcium and phosphate, as analysed by EDX- analysis and the Ca/P ratio suggests poorly crystalline particles. On the mineral phase of the plastic surface a crystal growth as revealed by SEM is paralleled by increase of Ca/P ratio quantified by EDX analysis. The highest crystalline material was observed in late cultures as determined by EDX, X-ray analysis and IR spectroscopical analysis, suggesting maturational changes within the crystal growth *in vitro*. These results are consistent with reports on the maturation of newly deposited calcium crystals in chicken osteoblast cultures [14].

Based on our results, we propose the following model for osteoblastic mineralisation (Fig.11): The intracellular calcium concentration increase provided by the internal calcium pool of endoplasmatic reticulum and mitochondria precipitated out in the presence of phosphate resulting in visible intracellular mineralisation needle structures. Single needles form small aggregates (Fig. 11, a 1). These aggregates are then surrounded by a membrane (Fig. 11, a 2). The membrane-bound vacuoles can fuse which each other and move to the cell membrane were by another fusion event the mineralisation aggregates are secreted to the outside (Fig. 11, a 3-5). At early culture time points small mineralization aggregates are deposited onto the culture dish surface and with time more mineralisation aggregates are deposited onto the first non collagen associated mineralisation islands. With time the first collagen fibres are detectable in these mineralisation deposits (fig. 11, b 1-2). After longer induction time more and more mineralisation deposits are placed into the growing collagen matrix (Fig.11, b 3-5) This process is accompanied by a maturation of the crystallinity of the deposits.

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**Table 1**

| time of culture | EDX analysis<br>Ca/P ratio | X-ray analysis<br>average intensities | IR-spectroscopy<br>% crystallinity |
|---|---|---|---|
| **10 days + BMP-2** | **NT** | **- - -** | **-** |
| **15 days + BMP-2** | **NT** | **52 ss - 64 ss** | **93** |
| **25 days + BMP2** | **intra. extra.**<br>**1,21±0,09 1,38±0,11** | | |
| **30 days + BMP-2** | **intra. extra.**<br>**1,28±0,12 1.45 ±0.07** | **52 ss 57 ss 64 ss** | **97** |
| **tibia** | **1.55 ± 0.03** | **52 m 57.5s 63.5 i** | **NT** |
| **enamel** | **1.65 ± 0.12** | **52 s 57.5 s 63.5 m** | **100** |
| **30 days control** | **- -** | **- - -** | **-** |

EDX- analysis, X-ray analysis and IR- spectroscopy were performed on samples from BMSC +BMP-2 and BMSC as control (C) from embryonic chicks at the indicated time points in culture. EDX analysis were performed with samples from 18 well plates, X-ray analysis and IR spectroscopical analysis were performed with samples from six well plates. Note - = no intensity, ss = very weak, m = middle, i = intensive intensities of reflections, intra = intracellular, extra = extracellular. NT not determined.

## Figure legends

**Fig. 1:**

Time dependent expression of ALP and mineralisation on mesenchymal stem cells (BMSCs) from embryonic chick. Secondary passage of BMSCs isolated from embryonic chicks at day 18 were plated in DMEM 10 % FCS at a density of 10. 000cells / $cm^2$. After 24 h, the medium was replaced with DMEM containing supplements, 2 % FCS, ascorbic acid (50 µl/ml), ß-glycerophosphate (10 mM) and vitamin $D_3$ ($10^{-9}$M). The cells were then left for another 48 h and then challenged with 20 ng/ml BMP-2. ALP was measured with PNPP and mineralisation by measuring calcium after HCl treatment at the indicated time points. The results are expressed as the percentage to the pre-treated control. ◆ BMP-2 ALP; ▲ control ALP; △ medium ALP, **X** BMP-2 mineralisation, ■ control mineralisation. □ medium mineralisation. Bars represent means ± SEM, n = 4 wells of 24 well. The experiment was repeated 3 times. The *P* values for mineralisation versus control are indicated ***$P < 0.001$, *$P < 0.05$. Inlet left represents PCR-products of transcripts for BMP-2, Alk-3, BMP-R II, Alk-6, OPN and OCN during the cultivation period. Inlet right represents in situ staining for ALP (dark areas) at day 15.

**Fig. 2:**

Morphological analysis of the mineralisation process; extracellular mineralisation deposits in the extracellular matrix as seen by TEM after 30 days of BMP2 induction. a, mineralisation deposits can be found outside the BMSCs after 21 days. b, after longer induction times these deposits are found embedded in a collagen fibre matrix (marked with c). c and d, additionally, we could repeatedly detect a typical organization pattern in the collagen matrix; specificaly that collagen fibres run perpendicular to each other; 1 in c represents a cross section of the collagen fibres whereas 2 shows a longitudinal section and 3 depicts again a cross section. d, after 30 days control BMSCs (no BMP-2 induction) also exhibit a collagen fibre matrix in the extracellular space, but no mineralisation deposits are detectable. Bars represent 5 µm in a, 0.5 µm in b and c, 1 µm in d.

**Fig. 3:**

FESEM analysis of growing crystalline deposits after BMP-2 induction. a-c, small island-like mineral deposits can be found underneath the BMSCs after 18 days. d-e, these mineralisation islands function as a basis for additional deposits by the BMSCs at longer induction times (21 days), note that no further deposit of crystalline structures can be detected around the islands on the culture disk, g and h, after 30 day of induction the complete area underneath the BMSCs is covered with mineralisation deposits. Bars represent 1 µm in a-f, 2 µm in g and 0.2 µm in h.

**Fig. 4:**

Energy dispersive x-ray microanalysis (EDX) of mineralisation deposits 25 days after BMP-2 induction and EDX elemental mapping for Ca, P and O on 21 days BMP-2 induced BMSCs. a, EDX analysis clearly shows the presence of Ca, P and O in the large crystalline aggregates (spectrum 2) whereas lower signals of these three elements were detectable on the small island-like deposits (spectrum 1); FESEM image depicts the crystalline deposits; EDX analysis points are on a very fine deposit of mineral (1) and on the growing crystalline aggregates (2). The Ca/P ratio was calculated to be between 1.4 and 1.45. b, depicts the mineralisation area on which the mapping was performed; c-e, exhibits the signals of the elemental mapping analysis which clearly demonstrates the presence of Ca, P and O in the mineralisation deposits as evident from the signals of every element in the deposits. No signal was detectable from the surrounding plastic area of the culture disk.

**Fig. 5:**

Mineralisation deposits in the extracellular collagen matrix as seen by FESEM. a, after 25 days of BMP-2 induction the formation of a collagen fibre matrix was obvious in the samples. Within these matrix crystalline deposits could be identified. b-d, on single collagen fibres some mineralisation deposits were also detectable. Bars represent 2 µm in a, 0.2 µm in b and 0.1 µm in c and d.

**Fig. 6:**

Morphological analysis of the mineralisation process; early events as seen by TEM. a and b, analysis of ultrathin sections reveals areas of mineralisation needles in the cytoplasm of BMSCs at day 18 after BMP2 induction (arrow heads). The mineralisation needles aggregate to form larger complexes without being surrounded by a membrane (arrow). c and d, the mineralisation needles consist of two different phases (circles in c), one phase represents the mineralisation deposit (arrow heads in d) which is accompanied by a white hallow which most properly represents a proteinaceous character (arrows in d). Bars represent 0.5 µm in a, 0.25 µm in b and 50 nm in c. R, ribosomes

**Fig. 7:**

Morphological analysis of the mineralisation process; intracellular maturation as seen by TEM after 21-25 days after BMP2 induction. a, free in the cytoplasm aggregated mineralisation needle complexes are engulfed by a membrane. b and c, two membrane surrounded mineralisation complexes can fuse intracellularly with each other (arrow head) forming a larger membrane engulfed vesicle within the BMSCs cytoplasm. d and e, the content of the vesicles can then be released to the extracelluar space in an exocytotic process. Bars represent 0,25 µm in a-d and 0.5 µm in e.

**Fig. 8:**

Energy dispersive x-ray analysis (EDX) of ultrathin sections of BMP-2 induced BMSCs applying TEM. a, after 18 days of BMP-2 induction BMSCs start to form mineralisation needles and aggregates. EDX spectra demonstrate that Ca, P and O can be detected within these aggregated complexes (spectrum 2, spot 2), whereas analysis of the ribosome rich region in the cytoplasm of the BMSC reveals no significant signals for the three elements (spectrum 1). b, analysis of the intracellular and extracellular deposited mineralisation aggregates clearly shows that both spectra show the presence of Ca, P and O. The visible dominat Ni signal results from the Ni grid used as a carrier for the ultrathin sections and the Cl signal is due to the relative high content of Cl in the used Spurr embedding resin.

**Fig. 9:**

Detection of Ca, P and O in the intracellular mineralisation needle aggregates applying Electron Energy Loss Spectroscopy (EELS) and element distribution of Ca in extracellular mineralisation deposits applying Electron Spectroscopic imaging (ESI). a, depicted are the background corrected spectra for Ca, P and O demonstrating the colocalisation of these elements; FESEM image depicts the measured area. b, ESI analysis clearly demonstrated that Ca is localized in the extracellular mineralisation deposits. The image at the carbon edge (250 eV) shows the morphology of the analysed sample area, the Ca nett distribution was deduced by applying the 2-window method, i.e. taken two images, one image before the Ca-edge (341 eV) and the second image behind the Ca-edge (358 eV). From these images the Ca content (nett Ca) of the sample was calculated.

**Fig. 10:**

Comparison of the *in vitro* mineralisation process with the *in vivo* process in osteoblasts of the periostal regions in tibia. a-c, depict ultrathin sections of osteoblasts of the periostal regions in chick tibia; mineralisation needle aggregates can be found in membrane-bound vacuoles intracellularly (arrows) and mineralisation deposits are located in the extracellular matrix space (arrow heads in b); d, the mineralisation needles have identical morphology compared to needles in BMP-2 induced BMSCs (compare with Fig. 6, d). e, EDX analysis of the mineralisation aggregates show the colocalisation of Ca, P and O. Bars represent 0,5 µm in a and b, 0,25 µm in c.

**Fig.11:**

Model of the mineralization process in BMSCs after BMP2-induction. a, depicts the intracellular mineralisation process, b, presents the formation of the extracellular mineralisation deposits.