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

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1 **Abstract**

2 The process of biomineralisation has been examined during osteoblastic differentiation of
3 bone marrow stroma cells (BMSC) from embryonic chick in culture and in periosteum itself
4 by a number of different techniques including transmission (TEM) and scanning (SEM)
5 electron microscopy. In cell culture of BMSCs at days 20-25 crystals were accumulated
6 extracellularly in the collagen matrix resulting in large plate-like crystallites and non-collagen
7 associated on the culture disk surface. In contrast up to days 10-18 mainly intracellular
8 mineralisation was visible by numerous needle-like crystal structures in the cell cytoplasm
9 and in vacuoles. After 20-30 days the crystal-content of these vacuoles is released -most
10 probably by membrane fusion to the outside of the cells. Energy Dispersive X-ray analysis
11 (EDX), Electron Spectroscopic Imaging (ESI) and Electron Energy Loss Spectroscopy
12 (EELS) demonstrated that Ca, O and P are located in the intra- and extracellular needle-like
13 crystals. From EDX-spectra a Ca/P ratio of 1.3 was estimated for the intracellular structures
14 and a Ca/P ratio of 1.5 for the extracellular material (for comparison the Ca/P ratio in tibiae is
15 1.6). X-ray diffraction and quantitative IR spectral (IRS) analysis also demonstrated an
16 increase of crystalline bone apatite along the mineralisation process. In addition to the finding
17 *in vitro*, the presence of intracellular needle-like crystals in vacuoles could also be
18 demonstrated *in vivo* in osteoblastic cells of the periosteum in tibia of day 11. The results
19 demonstrated are in favour of a novel model for mineralisation by osteoblasts, in which
20 amorphous Ca-P material is directly secreted via an exocytotic process from vacuoles of the
21 osteoblast and deposited extracellularly and propagated into the collagen fibril matrix and
22 matured to hydroxyapatite.

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1 **Introduction**

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

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

1 physiological regulatory factors such as BMP-2 and Vitamin D₃ under serum reduced
2 conditions. Our research focuses on two main goals 1) to investigate at different time points
3 during the calcification process the morphology, chemistry and structure of the CaP mineral
4 phase formed inside the cell and extracellularly in cell culture of BMSCs and 2) to compare
5 the *in vitro* data with the *in vivo* process in the osteoblast of the periosteum in tibia of chick.

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

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

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1 **Materials and Methods**

2 *Chemicals*

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

7 *Cell culture*

8 Fertilized eggs from chickens were obtained from a commercial supplier (Charles River,
9 Sulzfeld, Germany) and incubated in an egg incubator (Ehret, Emmendingen, Germany) with
10 a rotating time of 8 times/ day, at 37°C and 60% humidity. Tibiae and femora were dissected
11 from 17 day old embryonic chicks under sterile conditions and all adherent material removed.
12 The epiphyses were removed and the BMSCs flushed out of the marrow cavity with Dulbecco's
13 Modified Eagle's Medium (DMEM) supplemented with penicillin and streptomycin (100 µg/ml)
14 and glutamine (2mM) using a hypodermic syringe. The cells were then transferred to tissue
15 culture bottles and grown in DMEM containing the above supplements plus heat inactivated (56
16 °C for 30 min) 10 % FCS, the medium was changed every 2 days and the cells were allowed to
17 reach confluency. The cells were sub cultivated into 96 well, 12 well or 6 well plates at a cell
18 density of 10.000 cells/cm² in DMEM plus 10 % FCS and supplements. After 24 h, the medium
19 was replaced with DMEM containing supplements, 2 % FCS, ascorbic acid (50 µg/ml), β-
20 glycerophosphate (10 mM) and vitamin D₃ (10⁻⁹M). The cells were then left for another 48 h and
21 challenged with 20 ng/ml BMP-2. The culture without BMP-2 was used as negative control.
22 Culture with 10 % FCS in OI medium was used as positive control. During the course of
23 experiments medium was replaced every two days with fresh medium containing the above
24 supplements together with the inducing agent.

25

26 *Alkaline phosphatase assay*

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

1 nitrophenol determined spectrophotometrically at 405 nm. Alkaline phosphatase was also stained
2 histochemically. In brief, a stock solution of 25 mg naphthyl phosphate in 10 ml formamide and
3 10 ml water was adjusted to pH 8 with 1 M sodium carbonate. The working solution was
4 prepared by adding 1 ml stock solution to 24 ml AP buffer, adding Fast Red TR-salt to a final
5 concentration of 1 mg/ml. The solution was then filtered. The cells were stained for 1 h at 20° C.

6

7 *Calcium determination*

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

13

14 *Protein determination*

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

18

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

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

1 recorded on a Perkin Elmer Fourier transform spectrometer model 1700 and Analect FX
2 6002, after embedding the dried sample in KBr pellets.

3

4 *Transmission electron microscopy and EDX analysis*

5 Samples were fixed with a fixation solution containing 2% glutaraldehyde and 5%
6 formaldehyde in cacodylate buffer (0.1 M cacodylate, 0.09 M sucrose, 0.01 M MgCl₂, 0.01 M
7 CaCl₂, pH 6.9) for 1 h on ice. Samples for morphological studies were further fixed and
8 contrasted with 1% aqueous osmiumtetroxide for 1 h at room temperature, washed and
9 dehydrated with a graded series of acetone (10, 30, 50%) for 30 min each step on ice. Another
10 contrasting step was introduced by dehydrating the samples further in 70% acetone containing
11 0.5% uranyl acetate at 4° C for overnight. Samples were then dehydrated with 90% and 100%
12 acetone on ice. The next 100% acetone step was performed at room temperature. Samples for
13 EDX analysis were not treated with osmiumtetroxide and uranyl acetate to minimize possible
14 line overlapping during EDX analysis. All samples were then embedded in the low viscosity
15 epoxy resin according to Spurr [15]. After heat polymerisation (8 h at 70°C) samples were cut
16 with glass knives (Reichert Ultratome S, Wien, Austria) and collected onto formvar covered
17 300 mesh copper grids. Samples for morphological studies were counterstained with 4%
18 aqueous uranyl acetate for 2 min and lead citrate for 1 min. Ultrathin sections (40 nm in
19 thickness) for EELS/ESI analysis were cut with a diamond knife and not counterstained as
20 well as sections for the EDX analysis. Samples were observed in a Zeiss transmission electron
21 microscope EM910 at an acceleration voltage of 80 kV. The EM910 was attached with an
22 Oxford ISIS 300 EDX-analysis system (Oxford, Wiesbaden, Germany) and a digital scanning
23 unit for elemental mapping. Collecting of spectra and processing of data was done according
24 to the manufacturers guidelines. ESI and EELS analysis were performed with an Zeiss CEM902
25 transmission electron microscope with the SIS EFTEM software package (Muenster,
26 Germany) at an accelerating voltage of 80 kV.

27

28 *Field emission scanning electron microscopy (FESEM)*

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

1 during the last 100% acetone step and then critical-point dried with liquid CO₂. For
2 morphological studies samples were sputter coated with an appr. 10 nm thin gold film.
3 Samples for EDX analysis were carbon coated. To gain access to the underlying collagen
4 matrix with mineralisation deposits half of the cellular material after critical-point drying was
5 blown away with pressured air and then carbon coated. Samples were examined in a Zeiss
6 field emission scanning electron microscope DSM982 Gemini at an acceleration voltage of 5
7 kV for morphological studies using the Everhart-Thornley detector and the in-lens detector in
8 a 50:50 ratio. EDX analysis was performed with an acceleration voltage of 15 kV. The
9 microscope was equipped with an Oxford ISIS300 EDX-analysis system (Oxford, Wiesbaden,
10 Germany).

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12 *Statistical analysis*

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

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1 **Results**

2 *Synchronized cellular osteogenic differentiation by osteogenic compounds using bone* 3 *marrow derived mesenchymal stem cells from embryonic chick*

4 In order to clarify the calcification process by osteoblasts *in vitro* BMSCs from embryonic
5 chick were used. The choice of culture conditions is obviously of critical importance for the
6 kinetics of the osteogenic process. Therefore, we reinvestigated the culture conditions to
7 determine optimal conditions for induction the mineralisation process. To achieve this, firstly
8 the induction of ALP activity was used to measure the inductive role of compounds in
9 osteogenesis. In a basal medium (DMEM in the presence of β -GP and ascorbic acid in 2%
10 FCS) no ALP activity was detectable. Vitamin D₃ at a concentration of 10^{-7} M, however,
11 slightly increased ALP activity, whereas dexamethason had no effect. Of the growth factors
12 only BMP-2 was found to be positive with maximum effect at 20 ng/ml, whereas TGF- β 1,
13 IGF-II, bFGF, EGF had no significant effect on ALP induction. Furthermore, the induction of
14 osteocalcin as a late marker of osteogenesis was found to be induced by BMP-2 was higher
15 compared to VitD₃ at day 10. Finally, in regards to their effect on cell proliferation VitD₃ was
16 found to cause an inhibition, whereas BMP-2, slightly induced cell proliferation. We found
17 that both ascorbate (50 μ g/ml) and β -GP (10 mM) are required for mineralisation, however
18 dexamethasone showed no effect. From this data combinations of 1,25-(OH)₂ D₃ (10^{-9} M) and
19 FCS (2%) in the presence of β -GP and ascorbic acid with 20 ng/ml BMP-2 were used. As
20 seen in Fig. 1 over the culture period of 30 days the ALP expression was found to be transient
21 and mineralisation increased from day 15 in the BMP-2 treated culture continuously. In situ
22 staining for ALP revealed nearly homogenous ALP positive cells in the BMP-2 treated
23 culture at a confluent state. ALP activity and mineralisation was not seen in the control
24 culture without BMP-2. Furthermore, no mineral particles has been seen in the absence of
25 cells even at incubation for 30 days of the medium. These data suggest that the expression of
26 mineralisation is strictly dependent on the presence of an osteoblastic phenotype.

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

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

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

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

29 *Formation of non collagen and collagen associated mineralisation as seen by high resolution* 30 *FESEM*

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

1 methods like high resolution FESEM, Electron Spectroscopic Imaging (ESI) and Electron
2 Energy Loss Spectroscopy (EELS) as well as energy-dispersive x-ray analysis (EDX). When
3 applying high resolution FESEM to our in vitro mineralisation system we observed at higher
4 magnifications small island-like deposits on the culture disk surface after 18 days which were
5 formed out of a multitude of smaller mineralisation deposits (Fig. 3, a-c). At day 18-20
6 sphere-like material was deposited on the top of these mineralisation islands (Fig. 3, d and e)
7 and with time larger aggregates have been formed (Fig. 3, f-h). These aggregates on the
8 culture disc surface were not embedded in collagen fibrils. The collagen fibrils were first
9 observed in the following layer of mineralisation aggregates (see also Fig. 5). EDX- analysis
10 revealed that even in the small island-like deposits as well as in the larger aggregates Ca, P
11 and O could be detected (Fig. 4, a). The signals for all three elements were more pronounced
12 in the large aggregates suggesting more deposition of these elements in the large
13 mineralisation areas. Analysis of the plain culture disk surface gave no signal for Ca and P
14 (data not shown). We then applied EDX elemental mapping analysis to detect colocalisation
15 of Ca, P and O. Results of the mapping analysis revealed that all three elements are
16 colocalised in the mineralisation deposits (Fig. 4, b-e). After 25 days of BMP-2 induction a
17 very pronounced formation of an extracellular matrix comprised of collagen fibres was
18 detectable. Within this matrix we could detect different sized mineralisation aggregates (Fig.
19 5, a). Even on the collagen fibres we were able to detect some small mineralisation deposits
20 (Fig. 5, b-d).

21 *Visualisation of intracellular mineralisation needles by ultrathin section analysis*

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

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

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

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

21 *Electron Spectroscopic Imaging (ESI) and Electron Energy Loss Spectroscopy (EELS) of* 22 *intracellular mineralisation needle aggregates and extracellular mineralisation deposits*

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

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

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

12 *Analysis of intracellular and extracellular mineralisation in vivo in osteoblasts of the* 13 *periosteal regions in tibia*

14 The osteoblast of the periosteal region of chick tibia was used for comparing our *in vitro*
15 findings with the *in vivo* situation in the chick bone. Morphological analysis of ultrathin
16 sections revealed that under *in vivo* conditions mineralisation aggregates which are located in
17 membrane-surrounded vacuoles are also formed (Fig. 10 a-c). Furthermore, mineralisation
18 deposits are also located in the extracellular space in near vicinity to the calcified bone (Fig.
19 10, a and b, arrow heads). One striking feature observed was the identical morphological
20 appearance of the early mineralisation needles as seen in cell culture (compare Fig. 6, d with
21 Fig. 10, d). When EDX analysis of the mineralisation aggregates was performed, again
22 colocalisation of Ca, P and O was obvious (Fig. 10, e). Therefore, our results give strong
23 evidence that mineralisation *in vitro* using BMSCs exactly mimics the *in vivo* situation of
24 bone mineralisation in the periosteum.

25 *Physical characterisation of mineralisation during osteogenesis*

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

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

7 Quantitative IR spectral analyses revealed a peak between 550 and 600 cm^{-1} in samples of BMP-
8 2 treated cultures at day 15 and day 30, that were similar to those found in samples of dentin,
9 which is characteristic for crystalline CaP. Amorphous CaP showed a broad absorption at 575
10 cm^{-1} . The control culture without BMP-2 showed no signal. The percentage of crystallinity as
11 calculated from the ratio of the integrated intensities to dentine with 100 % was found to be 93 %
12 at day 15 and 97 % at day 30 of the total CaP in BMP-2 treated material. In conclusion, the
13 results demonstrate an appearance of amorphous CaP intracellularly and extracellularly in early
14 cultures and an increase of crystallinity along the mineralisation process.

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1 **Discussion**

2 The purpose of this study was to clarify the mineralisation process on osteoblasts *in vitro* and
3 *in vivo* with respect to morphology, chemistry and maturation of crystal structure of the
4 mineral. Bone marrow stroma cells have long been considered to be the source of osteoblast
5 progenitors with an osteogenic potential similar as osteoprogenitors from periosteum and
6 calvaria [16]. The time frame of ALP expression and mineral formation in our primary system
7 in the presence of ascorbate and β -glycerophosphate is similar as described by others on
8 primary osteoprogenitor cells of different sources from different species [17] including
9 humans [18]. The presence of different levels of ALP in cells and spots of mineral support the
10 view that our BMSCs system recapitulates the successive stages of the osteoblast
11 differentiation pathway comparable to the fetal rat calvaria system described by Malaval et al.
12 [19].

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

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

1 before it can support mineral induction and collagen I alone is not sufficient for ECM
2 mineralisation even at high ionic concentrations as shown for mouse MC3T3-E1 cells [21,
3 22] Our additional finding of non-collagen associated mineral deposits increasing in size only
4 in the BMP-2 induced cultures suggests that this mineral formation may also be associated
5 with the presence of an osteoblastic phenotype. A similar result has been described in a study
6 of a UMR10601 BSP cell-line. Extracellular mineral deposits of varying sizes have been
7 found beneath and between cells and some located near, but rarely on, collagen fibrils upon
8 addition of 5 m M β -GP for the terminal 24 h of a 72 h culture period. Non-collagenous
9 proteins are suggested to influence mineral formation and one of these glycoproteins, BSP,
10 has been localized at the ultrastructural level within the early mineral accretions by
11 UMR10601 BSP [23]. Furthermore, in the absence of collagen I mineral particles has been
12 described in a subtype of ROS cells, suggesting that additional non-collagen products are
13 involved in initiation and growth of mineral [24, 25].

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

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

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

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

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

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

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

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

14 Based on our results, we propose the following model for osteoblastic mineralisation (Fig.11):
15 The intracellular calcium concentration increase provided by the internal calcium pool of
16 endoplasmatic reticulum and mitochondria precipitated out in the presence of phosphate
17 resulting in visible intracellular mineralisation needle structures. Single needles form small
18 aggregates (Fig. 11, a 1). These aggregates are then surrounded by a membrane (Fig. 11, a 2).
19 The membrane-bound vacuoles can fuse which each other and move to the cell membrane
20 were by another fusion event the mineralisation aggregates are secreted to the outside (Fig.
21 11, a 3-5). At early culture time points small mineralization aggregates are deposited onto the
22 culture dish surface and with time more mineralisation aggregates are deposited onto the first
23 non collagen associated mineralisation islands. With time the first collagen fibres are
24 detectable in these mineralisation deposits (fig. 11, b 1-2). After longer induction time more
25 and more mineralisation deposits are placed into the growing collagen matrix (Fig.11, b 3-5)
26 This process is accompanied by a maturation of the crystallinity of the deposits.

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2 **References**

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2 **Table 1**

time of culture	EDX analysis Ca/P ratio	X-ray analysis average intensities	IR-spectroscopy % crystallinity
10 days + BMP-2	NT	- - -	-
15 days + BMP-2	NT	52 ss - 64 ss	93
25 days + BMP2	intra. extra. 1,21±0,09 1,38±0,11		
30 days + BMP-2	intra. extra. 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	- -	- - -	-

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

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2 **Figure legends**

3 **Fig. 1:**

4 Time dependent expression of ALP and mineralisation on mesenchymal stem cells (BMSCs)
5 from embryonic chick. Secondary passage of BMSCs isolated from embryonic chicks at day
6 18 were plated in DMEM 10 % FCS at a density of 10. 000cells / cm². After 24 h, the medium
7 was replaced with DMEM containing supplements, 2 % FCS, ascorbic acid (50 µl/ml), β-
8 glycerophosphate (10 mM) and vitamin D₃ (10⁻⁹M). The cells were then left for another 48 h and
9 then challenged with 20 ng/ml BMP-2. ALP was measured with PNPP and mineralisation by
10 measuring calcium after HCl treatment at the indicated time points. The results are expressed as
11 the percentage to the pre-treated control. ◆ BMP-2 ALP; ▲ control ALP; △ medium
12 ALP, X BMP-2 mineralisation, ■ control mineralisation. □ medium mineralisation. Bars
13 represent means ± SEM, n = 4 wells of 24 well. The experiment was repeated 3 times. The *P*
14 values for mineralisation versus control are indicated ****P* < 0.001, **P* < 0.05. Inlet left
15 represents PCR-products of transcripts for BMP-2, Alk-3, BMP-R II, Alk-6, OPN and OCN
16 during the cultivation period. Inlet right represents in situ staining for ALP (dark areas) at
17 day 15.

18 **Fig. 2:**

19 Morphological analysis of the mineralisation process; extracellular mineralisation deposits in
20 the extracellular matrix as seen by TEM after 30 days of BMP2 induction. a, mineralisation
21 deposits can be found outside the BMSCs after 21 days. b, after longer induction times these
22 deposits are found embedded in a collagen fibre matrix (marked with c). c and d, additionally,
23 we could repeatedly detect a typical organization pattern in the collagen matrix; specifically
24 that collagen fibres run perpendicular to each other; 1 in c represents a cross section of the
25 collagen fibres whereas 2 shows a longitudinal section and 3 depicts again a cross section. d,
26 after 30 days control BMSCs (no BMP-2 induction) also exhibit a collagen fibre matrix in the
27 extracellular space, but no mineralisation deposits are detectable. Bars represent 5 µm in a,
28 0.5 µm in b and c, 1 µm in d.

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2 **Fig. 3:**

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

10 **Fig. 4:**

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

22 **Fig. 5:**

23 Mineralisation deposits in the extracellular collagen matrix as seen by FESEM. a, after 25
24 days of BMP-2 induction the formation of a collagen fibre matrix was obvious in the samples.
25 Within these matrix crystalline deposits could be identified. b-d, on single collagen fibres
26 some mineralisation deposits were also detectable. Bars represent 2 μm in a, 0.2 μm in b and
27 0.1 μm in c and d.

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2 **Fig. 6:**

3 Morphological analysis of the mineralisation process; early events as seen by TEM. a and b,
4 analysis of ultrathin sections reveals areas of mineralisation needles in the cytoplasm of
5 BMSCs at day 18 after BMP2 induction (arrow heads). The mineralisation needles aggregate
6 to form larger complexes without being surrounded by a membrane (arrow). c and d, the
7 mineralisation needles consist of two different phases (circles in c), one phase represents the
8 mineralisation deposit (arrow heads in d) which is accompanied by a white hallow which
9 most properly represents a proteinaceous character (arrows in d). Bars represent 0.5 μm in a,
10 0.25 μm in b and 50 nm in c. R, ribosomes

11 **Fig. 7:**

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

19 **Fig. 8:**

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

29 **Fig. 9:**

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

11 **Fig. 10:**

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

20 **Fig.11:**

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

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