Published in final edited form as:

Bone. 2008 June ; 42(6): 1061–1071.

Modulation of Extracellular Matrix Protein Phosphorylation Alters Mineralization in Differentiating Chick Limb-bud Mesenchymal Cell Micromass Cultures

Adele L. Boskey, Stephen B. Doty, Valery Kudryashov, Philipp Mayer-Kuckuk, Rani Roy, and Itzhak Binderman

Hospital for Special Surgery, New York, NY 10021

Abstract

Protein phosphorylation and dephosphorylation are important regulators of cellular and extracellular events. The purpose of this study was to define how these events regulate cartilage matrix calcification in a cell culture system that mimics endochondral ossification. The presence of casein kinase II (CK2), an enzyme known to phosphorylate matrix proteins, was confirmed by immunohistochemistry. The importance of phosphoprotein phosphorylation and dephosphorylation was examined by comparing effects of inhibiting CK2 or phosphoprotein phosphatases on mineral accretion relative to untreated mineralizing controls. Specific inhibitors were added to differentiating chick limb bud mesenchymal cell micromass cultures during the development of a mineralized matrix at the times of cell differentiation, proliferation, formation of the mineralized matrix, or proliferation of the mineral crystals. The mineralizing media for these cultures contained 4mM inorganic phosphate and no organic-phosphate esters; control cultures had 1mM inorganic phosphate. Mineralization was monitored based on ⁴⁵Ca uptake and infrared characterization of the mineral; cell viability was assessed by three independent methods. Treatments that caused cell toxicity were excluded from the analysis.

Inhibition of CK2 activity with apigenin or CK2 inhibitor II reduced the rate of mineral deposition, but did not block mineral accretion. Effects were greatest during the time of mineralized matrix formation. Inhibition of phosphoprotein phosphatase activities with okadaic acid, calyculin A, and microcystin LR, at early time points also markedly inhibited mineral accretion. Inhibition after mineralization had commenced increased the mineral yield. Levamisole, an alkaline phosphatase inhibitor, had no effect on mineral accretion in this system, suggesting the involvement of other phosphatases. Adding additional inorganic phosphate to the inhibited cultures after mineralization had started, but not earlier, reversed the inhibition indicating the phosphatases were, in part, providing a source of inorganic phosphate.

To characterize the roles of specific phosphoproteins blocking studies were performed. Blocking with anti-osteopontin antibody confirmed osteopontin's previously reported role as a mineralization inhibitor. Blocking antibodies to bone sialoprotein added from day 9 or on days 9 and 11 retarded mineralization, supporting its role as a mineralization nucleator. Antibodies to osteonectin slightly stimulated early mineralization, but had no effect after the time that initial mineral deposition occurs. Taken together, the results of this study demonstrate the importance of the phosphorylation state of extracellular matrix proteins in regulating mineralization in this culture system.

Correspondence to: Adele L Boskey, Hospital for Special Surgery, 535 E 70th Street, New York, NY 10021, Phone 212 606 1453, Fax 212 472 5331, Email: boskeya@hss.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

micromass culture; avian; mineralization; casein kinase II; phosphoprotein phosphatase

Introduction

In cell-free mineralization studies the ability of extracellular matrix phosphoproteins such as osteopontin (OPN) [1,2], bone sialoprotein (BSP) [3,4], dentin matrix protein-1 (DMP1) [5] and phosphophoryn [6] to initiate hydroxyapatite (HA) mineral deposition and regulate the rate and extent of growth of the HA crystals is dependent on their state of phosphorylation. While the importance of the phosphate ion for appropriate mineral deposition is well recognized [7], it is only in recent years that attention has been paid to whether or not the extracellular matrix proteins were phosphorylated, as well as to their extent of phosphorylation and the enzymes involved in these posttranslational modifications [1–9]. The importance of phosphorylation and dephosphorylation has been suggested by the abnormal patterns of mineralization in mutant animals with defective phosphoprotein degradative enzymes [10–12], defective phosphate transport [13,14], or defective phosphorylation [15]. In cell culture, phosphorylation of osteopontin has been shown to be necessary for inhibition of vascular smooth muscle-mediated calcification [1], while inhibition of the enzyme responsible for extracellular matrix phosphoprotein phosphorylation (casein kinase II, CK2 [16]) by specific [17] and non-specific [18] inhibitors retards *in vitro* dentin and cartilage calcification, respectively. It is not clear, however, whether the presence of additional phosphate in the microenvironment, or the specific phosphorylation of extracellular matrix proteins is the controlling factor. It is our hypothesis that it is the phosphorylation of the anionic matrix proteins that is important, rather than the ability of enzymes such as alkaline phosphatase to release inorganic phosphate from these proteins. To test this hypothesis mineralization was monitored in a culture system that mimics endochondral ossification [19–26]. The cultures were challenged with inhibitors of CK2 and of phosphoprotein phosphatases, and with blocking antibodies to selected phosphorylated proteins.

Chick limb-bud mesenchymal cells plated in high-density micromass cultures differentiate to form a mineralizable matrix that resembles the growth plate in the chick [18–26]. Our earlier studies using 2,3-diphosphoglycerate and heparin, both non-specific inhibitors of CK2, showed inhibition of mineralization when protein phosphorylation was blocked [18]. However both these inhibitors have effects on other cell functions. How calcification is impacted by specific inhibition of phosphorylation of the matrix phosphoproteins has not yet been reported, nor has the effect of preventing phosphatase-mediated protein dephosphorylation at different stages of differentiation. Thus the purpose of the present study was to investigate the importance of extracellular matrix protein phosphorylation and dephosphorylation in a time dependent manner using the chick limb-bud mesenchymal cell micromass culture system.

Materials and Methods

Materials

All chemicals were reagent grade from Sigma Chemical (Saint Louis, MO) unless otherwise stated. Cell culture dishes (Falcon) were from Becton Dickinson (Franklin Lakes, NJ). ⁴⁵Calcium was from Perkin/Elmer (Boston, MA). Trypsin-EDTA was from CELLGRO (Herndon, VA) and Nitex filters from Tetko Inc (Ardsley, NY). Dulbecco's modified essential medium (DMEM) was acquired both from GIBCO (Grand Island, NY) and from the Media Laboratory (Memorial Sloan-Kettering, NY). Phosphate buffered saline (PBS) was from Abbott Laboratories (N Chicago, IL) or GIBCO (Grand Island, NY). Fetal bovine serum (FBS) was from GIBCO and GEMINI (Woodland, CA), with the GIBCO used for the first 7 days of

culture, and the GEMINI used after that. The CK2 antibody was from Santa Cruz Biotechnologies Inc (Santa Cruz, CA). The phosphatase inhibitors [27] that were used were okadaic acid (OA) and its inert form (okadaic acid tetraacetate), calyculin A, microcystin-LR, a bacteria derived phosphatase inhibitor that does not penetrate cell membranes [28], sodium vanadate, and levamisole or its inert form tetramisole. The two casein kinase II inhibitors used were apigenin and casein kinase II-inhibitor II (Calbiochem, La Jolla, CA). Polyclonal antibodies to chick osteopontin, osteonectin, and bone sialoprotein were generously provided by Dr Louis Gerstenfeld (Boston University). The total protein assay kit and pre-cast 10% Trisglycine gels were from BioRad (Hercules, CA); the protease inhibitor cocktail from Roche Applied Sciences (Indianapolis, IN), and the Diamond Q Phosphoprotein and Sypro Ruby gel staining kits were from Invitrogen (Carlsbad, CA). The Live-Cell assay kit was from (Molecular Probes, Invitrogen) and the mitochondrial tetronium salt (MTT) assay kit from ATCC (Manassas, VA). Phosphoprotein molecular size markers (peppermint stick) were from Invitrogen (Carlsbad, CA). Spectral grade Potassium Bromide (KBr) was from Fisher Chemical (Springfield, NJ) while barium fluoride windows were from Spectral Systems (Hopewell Junction, NY).

Methods

Cell Culture—Chick limb-bud mesenchymal cells were isolated from stage 21–24 [29] fertilized White Leghorn eggs (Truslow Farms, Chestertown, MD) as described in detail elsewhere [20]. The eggs were maintained in a humidified incubator at 37° C for 4.5 days. The embryos were then sterily withdrawn from the eggs and their limb-buds removed into cold 0.9% USP grade saline. Cells were released from the limb-buds by digestion with 5 ml 0.25 wt. % trypsin - 0.53 mM EDTA, and then were separated from debris by passage through two layers of 20 µm Nitex membrane. Cells were counted with a hemocytometer and checked for viability by trypan blue dye exclusion, and then pelleted at 4°C for 5 minutes at 2,300 rpm. Viability of the cells before plating in all cases exceeded 97%. Cells were resuspended in DMEM containing 0.3 mM Ca⁺² and plated using the micromass technique [30] at a density of 0.75 million cells per 20 µl drop in 35 mm Falcon dishes. Cells were allowed to attach for 2 hours in a humidified atmosphere of 5% CO₂ at 37° C. After 2 hours, 2 ml “complete media” made from DMEM supplemented with 1mM calcium chloride (final Ca concentration 1.3mM), 50-units/ml penicillin and 25 ug/ml streptomycin, 10% FBS, and 0.3 mg/ml L-glutamine, equilibrated to 37°C, was added and changed three times per week. The first day of culture was designated day 0. From day 2 onward, and with every change of medium, 25µg/ml sodium ascorbate was added to the cultures [20]. Mineralizing cultures were supplemented with 3 mM ammonium acid phosphate from day 2 onward, making the total inorganic phosphate (Pi) concentration 4mM. Non-mineralizing cultures were maintained with 1mM Pi. The concentrations of Ca and Pi in the media were verified by atomic absorption [31] and colorimetry [32], respectively, prior to each experiment. All experimental points (time and concentration) were repeated in triplicate for each individual study, and these individual studies were repeated three to five times. Data is expressed as means of the n individual studies (where the number of independent experiments, n, was between three and five), and multiple points within each independent experiment were averaged.

Studies of Effects of Inhibiting Kinase and Phosphatase Activities—To define concentrations of inhibitors for analysis, a series of pilot experiments were performed in which a wide range of concentrations for each inhibitor starting with those reported in the literature [18,21,33–37] were added either continuously from day 5, 7, 9 or 11, or once (acutely) at day 5,7,9,11, or 14. Where treatments caused visible cell death and cultures lifted off the culture dish no further analysis was done. Detailed assessment of less obvious toxicity of treatments was performed based on the Live-Cell assay (in which viable cells stain green and necrotic cells are stained red), the mitochondrial tetronium salt (MTT) assay, and/or measurement of

temporal changes in total DNA [38] in the presence and absence of the additive. Because phosphatase inhibition has been associated with apoptosis, chondrocyte apoptosis was checked in representative cultures using Flow cytometry on a FACS Calibur with CellQuest™ software (BD Bioscience, Inc.). The numbers of apoptotic cells were calculated by counting the number of events showing high Annexin V fluorescence [26]. Any concentration or addition time that caused necrosis or apoptosis greater than that seen in mineralizing controls was not included in the detailed studies. Concentrations that caused maximal changes in ⁴⁵Ca uptake (see below) were selected for evaluation. After concentrations were selected, the inhibitors or the carriers they were dissolved in were added either once at day 7, 9, 11, 14, or 16 (for 2 hrs or 2 days), or continuously (with each change of media) from these time points until day 21–22. In some experiments, to insure that serum was not affecting the action of the inhibitors, the media was replaced with serum free medium for 2 hours before the addition of the inhibitor, the inhibitor added for 2 hours, and then the media removed, the culture washed with fresh complete media, and complete media added until the end of the experiment.

To discriminate the effects of the phosphoproteins in the matrix and inorganic phosphate in the medium two different types of experiments were performed. In the first, based on earlier experiments, phosphatase activity was inhibited with okadaic acid from day 7–12, and then on day 13, after removal of the inhibitor, an extra 2 mM Pi (making the total 6mM) was added to half the untreated controls and half the phosphatase inhibited cultures and mineral accumulation was monitored in all cultures at days 16, 19, and 21. In the second experiments, the cells in “mineralizing” and control cultures were lysed by freeze-thawing at either day 7 (before mineralization had commenced) or at day 16 when abundant mineral was present. Mineralization parameters and protein profiles were assessed as detailed below.

Immunoblocking Studies—Chick specific antibodies directed against BSP, OPN, or ON were individually added to the cultures with 1:3000 or 1:1000 dilution as previously described [22]. These concentrations were selected based on pilot experiments that determined saturated binding. A previous study had shown that addition of nonspecific IgG at even higher titers did not affect ⁴⁵Ca accumulation. The antibody was either added acutely on day 9, 11, or 14, or continuously with each media change starting at each of these days. Mineralization was monitored as described below at days 16–22.

Mineralization Assays—To monitor mineral accretion, ⁴⁵Ca (0.5μCi/ml) was added to the culture dishes on day 7 and with every media change thereafter. At the indicated time points, cultures were briefly washed in PBS, and transferred to scintillation vials. The culture dish was rinsed with 100μL 4N hydrochloric acid, the wash added to the scintillation vials, and the contents of the vial hydrolyzed in a final volume of 200μl 4N HCl in a 60°C oven for 1 hr. Aquasol® solution (5mL) was then added to each cooled vial and vortexed until the liquid was free of any cloudiness. Counting was performed on a Beckman® scintillation counter. Accumulation of ⁴⁵Ca per micromass spot was calculated and data normalized to the accumulation in the mineralizing control culture at day 21 or 23. While historically we had corrected for ⁴⁵Ca uptake by the non-mineralizing controls [18–26], in initial experiments it was noted that this correction caused a difference of less than 1%; hence data are presented without this correction. However it should be noted that a single set of experiments for each inhibitor or blocking agent was performed to validate the fact that the controls treated with the agent in question were not binding more calcium than the untreated controls. We have previously shown that ⁴⁵Ca uptake is highly correlated with total calcium content as determined by atomic absorption spectroscopy [20]. Rates of mineralization were calculated from the initial slopes of ⁴⁵Ca uptake as a function of time from day 9–16. The time of initiation of mineral deposition (*t*₀) was calculated by extrapolating that line to zero.

At selected time points, cultures were sampled for histochemical visualization of calcium phosphate mineral using the phosphate-specific von Kossa technique. Briefly, each culture was split in half so that cross sections as well as sections parallel to the plate could be obtained from each culture. The cultures were washed with pH 8.0 water to remove excess phosphate, and then stained with 5% silver nitrate, with 2.5% acid fuchsin as the counter-stain. Additional cultures were fixed in glycerol and stained with Alizarin Red to identify the presence of calcium. To facilitate visualization, representative von Kossa-stained cultures not counter-stained, and instead were examined by back-scatter electron imaging using a FEI (Hillsboro, OR) Quanta 600, Environmental Scanning Electron Microscope. Because these and other staining techniques can neither distinguish calcium or phosphate not associated with mineral from mineral, nor identify the nature of the material reacting with the stain [39], X-ray diffraction and FTIR analyses were used to identify the presence of HA, the relative amount of HA present, and the average crystallinity of the HA in the culture [25].

Two distinct methodologies were used for FTIR analyses of mineral content and composition. For bulk analyses, unstained mineralizing cultures were recovered from the dish, washed with ammoniated water, air dried, and homogenized in a mortar and pestle by mixing with spectral grade KBr, and made into pellets. The KBr pellets were examined using a Thermo-Nicolet Spectrometer 4700 under nitrogen purge. The background-corrected spectra were base-lined (BioRad's Win-IR Pro 3.1), and areas under the phosphate ν_1, ν_3 peak ($\sim 900\text{--}1200\text{ cm}^{-1}$) and amide I peak ($\sim 1585\text{--}1720\text{ cm}^{-1}$) calculated using Win-IR Pro. Mineral/matrix ratio was calculated as the ratio of the above two integrated areas. For cultures containing mineral, crystallinity was estimated from the peak height intensity of the 1030 and 1020 cm^{-1} subbands. For representative studies mineral accumulation was documented by FTIR imaging spectroscopy (FTIRI) as described previously for this culture system [25] was used to display the distribution of the mineral to matrix ratio and the distribution of crystallinity within the culture. For FTIRI, the washed cultures were lifted from the dish with a cell scraper and transferred to a barium fluoride window. The windows were desiccated over night, and then the cultures were flattened by sandwiching them between a second window. Because the micromass cultures were thicker in the center, and the absorbance in the culture centers were extremely high, only areas along the edges, and adjacent to the edges of the culture were examined using a Perkin Elmer Spotlight Imaging system (Perkin Elmer Instruments, Shelton, CT) at 4 cm^{-1} spectral resolution. The spatial resolution was $\sim 7\mu\text{m}$. Parameters as described above were calculated for the images using ISYS software (Spectral Dimensions, Olney, MD).

Protein Identification—To identify proteins made in the culture system, cultures at different time points were washed three times with ice cold PBS and cell pellets from ~ 20 dishes for each condition were combined and extracted in (4M or 2M) guanidinium hydrochloride with protease inhibitors as described elsewhere [40]. In brief, guanidine HCL was made in 10mM Tris buffer containing 5mM EDTA, a protease inhibitor cocktail, phenyl methylsulfonyl fluoride, and dithiol threitol; cultures were extracted three times at 4°C with stirring (~ 24 hrs/ extraction). Extracts were recovered by centrifugation and dialyzed against PBS for 48 hrs. In the first set of experiments, the dialyzed 4M extracts were immunoprecipitated with chick specific antibodies to osteopontin, osteonectin, or bone sialoprotein. Precipitates were resuspended in 0.05M Tris buffer containing 2% SDS and subjected to Western blot analyses [40]. To identify variations in protein phosphorylation, while minimizing cellular protein constituents, 2M extracts were used as those extracts are enriched in extracellular matrix proteins (AM Malfait, personal communication). For these studies, only the control, apigenenin, and Microcystin-LR studies were tested in non-mineralizing conditions at day 15. The protein concentrations of the dialysates were determined using the Bio-Rad Total Protein Assay kit. The samples were then cleaned using chloroform precipitation as described in the Diamond Q Phosphoprotein staining instructions. SDS-PAGE was then performed using precast 10% Tris-glycine gels with $15\mu\text{g}$ of protein per lane from each sample as determined

from the total protein assay. The gels were then fixed and stained according to Diamond Q protocol and imaged on a BioRad Molecular Imager FX at excitation and emission of 532nm and 555nm respectively. The gels were restained with Sypro Ruby protein gel stain and imaged on a standard gel box to detect total proteins.

Results

Characterization of Cultures

As previously reported [18–26], the mesenchymal cells differentiated in culture and formed alcian blue positive chondrocyte nodules. At day 14 (figure 1a), the cultures began to show trace amounts of von Kossa positive staining. By day 16 (figure 1b) and day 21 (figure 1c, d) the nodules were surrounded by increasing amounts of von Kossa staining indicative of the presence of phosphate, and Alizarin red staining (figure 1e) indicative of the presence of calcium, showing the progressive mineralization of the cultures. Casein kinase II was detected by immunohistochemistry as early as day 9 in the periphery of the cultures (not shown). This staining decreased as the mineral accumulated, but persisted in the center of the cultures around mature chondrocytes even at day 21 (figure 1f).

Casein Kinase II (CK2) Inhibition

We first asked what would happen to mineralization if phosphorylation of matrix proteins was inhibited. Pilot studies were used to determine the concentration and times of addition at which apigenin (4',5,7-trihydroxyflavone), a selective CK2inhibitor, blocked ⁴⁵Ca uptake without causing cell necrosis or apoptosis. Based on these pilot studies, apigenin was then added to cultures at a final concentration of 40 μM. At this concentration there was no detectable effect on cell viability; in fact the treated cultures showed less cell death than the untreated cultures (figure 2a, b). Inhibition of CK2 from day 7 or 9 significantly delayed the start of mineralization, the rate of ⁴⁵Ca uptake and the yield of mineral at 21 days (figure 2c, Table 1). The extent of mineralization was stimulated when continuous addition began at day 11, 14, or 16 (figure 2d). Lesser effects were noted with single additions, but the trends were similar (data not shown).

The mineral formed in all the cultures was a poorly crystalline hydroxyapatite, as demonstrated by FTIR spectroscopy and FTIR imaging spectroscopy (figure 3). The presence of mineral and the relative yield of mineral (based on mineral/matrix ratio) of the entire culture was confirmed by the FTIR analyses on day 21. The distribution of mineral (mineral/matrix ratio) and crystallinity in cultures in which CK2 was inhibited from day 9 shows reduced mineral formation and more uniform crystallinity as compared to the cultures without apigenin (figure 3, Table 1).

The specific CK2 inhibitor, casein kinase II-inhibitor II, evaluated in pilot studies at concentrations from 0.16–64 μM, was used at 16 μM where it also blocked initial mineralization without effects on cell viability, and stimulated it when added at later time points (figure 4, Table 1). The yield of mineral at day 21, and the mineral crystallinity was decreased relative to control for continuous addition from day 7 or day 9 (Table 1). The observed effect was not due to a reaction between the inhibitor and an agent in the serum, as the culture's matrices all looked similar and had similar ⁴⁵Ca uptakes when the inhibitor was added to serum free media for brief time periods (data not shown).

Phosphatase Inhibitor Studies

We next examined how mineralization would be affected by preventing breakdown of phosphorylated proteins by phosphatases. Only one of the agents (sodium vanadate) caused cell toxicity at all concentrations tested, independent of the time of addition (Table 2). The data

in table 2, which shows % cell death relative to control cultures at day 16, includes toxicity studies with the casein kinase inhibitors as well as the phosphatase inhibitors.

Okadaic acid, which blocks serine phosphatases [34,35] was toxic when added at day 5. When added from day 7, mineral accretion was significantly blocked in a dose dependent manner (figure 5a). The inert tetra-acetate form of okadaic acid had similar ^{45}Ca uptake as the untreated cultures. The okadaic acid inhibition was less pronounced when the inhibitor was added from day 9 and non-detectable when it was added from day 11. In fact, addition from day 11 stimulated mineral accretion. The initial rates of mineral accretion in the presence of okadaic acid increased in these cultures once mineralization commenced; however the time for mineralization to start was markedly delayed with additions of okadaic acid (Table 1). Calyculin A which also blocks serine or threonine phosphatases [34] when added at day 7 was inhibitory, but when added after day 11 or day 14 it stimulated mineral accretion (figure 5b and Table 1). Where mineral accretion was retarded, crystals were smaller (Table 1) than those observed in control mineralizing cultures.

Microcystin-LR, which is reported to not cross the cell membrane [28], did not cause any cell toxicity if left in the cultures for one day or less. Cultures treated with 1 or 10 nM at days 7, 9, or 11 did not show a dose dependent difference, thus the results in figure 5c are shown only for 10 nM. When Microcystin-LR, which is reported to act like okadaic acid [41,42], was added on day 7, although there was a slight decrease in the rate of mineral accretion, the yield at day 21 was not altered. When added on day 9 the yield was decreased, while the rate of mineral accretion was comparable to that in the controls. Similarly, when added on day 11, the yield was also decreased, but the rate of mineral accretion was not affected (Table 1).

Levamisole, a specific inhibitor of alkaline phosphatase, had no effect on mineral accretion in this study in which inorganic phosphate was provided directly, and yields were equivalent to those in cultures with tetramisole (Figure 5d, Table 1). The ^{45}Ca uptake in the tetramisole treated cultures and the levamisole treated cultures differed from the control mineralizing cultures by less than 1% (not shown).

To determine the relative importance of the phosphorylated matrix and the inorganic phosphate released from the matrix, the effects of cell lysis before and after matrix protein proliferation and initial mineralization were compared. When cells were lysed by freeze thawing before the mineralization had commenced (on day 7) there was a significant delay in the initiation of mineral accretion and the yield of mineral was markedly reduced. In contrast, when cells were disrupted at day 16 there was very little change in the extent of ^{45}Ca uptake, although the rate of mineral accretion was increased (figure 6a).

To test the hypothesis that the observed effects of phosphatase inhibition were due to a decrease of phosphate release from phosphoproteins, an additional 2 mM inorganic phosphate was added to control and inhibited cultures on day 12. Data in figure 6b shows the effects on cultures with 1.6 nM okadaic acid added from day 9–11, with the additional phosphate added to the mineralizing control and mineralizing okadaic acid treated cultures on day 11. Increasing the local phosphate concentration increased the mineral yield in the cultures without okadaic acid. The inhibited cultures had a decreased mineral accumulation at day 14, but the yield was equivalent to controls by day 16. With addition of an extra 2 mM Pi , the okadaic acid inhibited cultures showed a significantly greater yield of mineral by day 14, but the yield did not differ significantly from the okadaic acid -free cultures with the same 6 mM inorganic phosphate.

Protein Identification

Western analyses showed that osteopontin was present in the cultures from day 11 on (figure 7a). Because the antibody could not distinguish phosphorylated and dephosphorylated proteins

one cannot state that this was the phosphorylated protein being affected by the antibody, although the blot was positive to a P-tyrosine antibody (figure 7a). This was not the only protein that could have been affected by the inhibitors as both Osteonectin and BSP were detected in the immunoprecipitation studies although their staining with the P-tyrosine antibody was lower (data not shown). Phosphorylated protein distribution was reduced in cultures treated with apigenin and enhanced or equal to control in cultures in which phosphatases were inhibited with microcystin while total protein distributions were comparable (figure 7b). Data is shown for the first of three 2M guanidinium-HCl extracts from day 14 cultures, as the majority of the protein was present in the first extracts. Similar findings (majority of phosphorylated bands in control and microcystin treated cultures, and non detectable bands for apigenin treated cultures) were seen in the second and third extractions (data not shown). The osteopontin band (~61 kD) showed the decreased phosphorylation; other bands showing similar variation were not identified due to a lack of availability of anti-chick matrix protein antibodies.

Antibody Blocking

Blocking phosphoprotein epitopes with polyclonal antibodies had distinctive effects on mineral accretion (figure 8). Results at 1:1000 and 1:3000 dilutions were comparable thus data is shown in figure 8 for the 1:3000 dilutions. Anti-osteopontin resulted in increased mineral accumulation when added on days 9, 11, and 14 (figure 8a), anti bone sialoprotein resulted in decreased ⁴⁵Ca uptake (figure 8b), and anti-osteonectin had no significant effect (figure 8c) at these time points.

Discussion

This study has demonstrated that both extracellular matrix phosphorylation and dephosphorylation can influence the rate and extent of mineralization in chondrocyte cultures derived from differentiating chick limb-bud mesenchymal cells. We hypothesized that specific proteins were phosphorylated and dephosphorylated during the stages of initiation and proliferation of mineral crystals based on solution free studies of the effects of these proteins [2–6] and on our unpublished micro-array results showing upregulation of kinases during early stages of mineralization and down-regulation of phosphatases at the same time. We also hypothesized that the functions of the phosphatases was not simply to elevate the local inorganic phosphate concentration.

The phosphorylated proteins synthesized by mature chick chondrocytes and present in the mineralizing chick cartilage matrix include osteopontin, bone sialoprotein, and osteonectin [43,44]. It is likely that other matrix proteins (e.g., DMP1, DSP, etc) present in the murine growth plate [45] are also present in the chick, as suggested in our unpublished microarray data. In general, these proteins are phosphorylated by specific kinases [16] prior to the initiation of mineralization and CK2 has been identified as the enzyme responsible for phosphorylation of mineralized tissue proteins in other systems [16,46,47]; we verified the presence of this enzyme in the chondrocytes nodules formed in the micromass cell cultures used in this study.

The analysis of the changes in phosphorylation of the proteins, a majority of which should have been extracellular based on the use of 2M guanidine hydrochloride, demonstrated that the inhibitors tested altered the phosphorylation profile. Especially cultures treated with apigenin showed a reduced phosphorylation of several distinct protein bands. Because of the objective of this study we have not identified the proteins or phosphorylation sites affected by a specific inhibitor. However, a comparison between the sizes of chick OPN (~60 kD), BSP (~70kD), and ON (~30kD) and the reported murine dentin matrix protein 1 (85–90 kD [48]) with the protein bands affected by phosphorylation inhibitors prompts us to speculate that phosphorylation of these proteins may have been reduced. This suggests that the inhibitors were functioning as predicted, and also that extracting with 2M guanidine hydrochloride

enriched the extracts with these phosphorylated proteins. Each of these proteins can be nucleators of and/or inhibitors of hydroxyapatite formation and crystal growth, depending on their concentrations, posttranslational modifications, and interactions with other molecules [9].

In the differentiating chick limb-bud mesenchymal cell system, inhibition of casein kinase II activity prior to the start of mineralization inhibited the initiation of mineralization, the rate of mineral accretion, and the yield of mineral at 21 days. The mineral crystals were smaller, on average, than those formed in cultures without kinase inhibitors. Together these data suggest that phosphorylation of one or more proteins in the extracellular matrix is important for induction of mineralization. Candidate proteins are BSP (confirmed in this system as a nucleator by anti-body blocking) and DMP1 for which chick cross-reactive antibodies are not yet available. While highly phosphorylated OPN can act as a nucleator [5], the antibody blocking studies confirmed it was acting as an inhibitor in the mineralizing chick micromass system, in agreement with previous reports [4,49,50], as when OPN was blocked the extent of mineralization increased. It is interesting to note that in the same differentiating chick limb-bud mesenchymal cell micromass culture system, Barak-Shalom et al [43] reported that phosphorylated OPN is released into the media when micromass cultures are treated with ascorbic acid, and that OPN gene expression and OPN phosphorylation are regulated by different mechanisms. They did not investigate mineralization in their studies.

After initiation of mineralization, inhibition of CK2 activity had little effect on the yield of mineral, although the rates of mineral proliferation tended to be higher. This could be explained if the proteins have a lesser effect once the initial mineral crystals are nucleated. The proteins may play additional roles binding to crystal surfaces and modulating the shapes of the crystals, as suggested by the different crystal sizes detected by FTIR analyses.

In the initial time periods before mineralization commenced, inhibiting phosphatases also retarded mineral accretion, either because of the early expression of OPN [43], an effective inhibitor of mineralization, or because there was an unidentified effect on the cells that did not result in cell death. It is also possible that inhibition of mineral accretion could be attributed to a lower inorganic phosphate concentration in the media as demonstrated when additional exogenous phosphate rescued the mineralization deficit in the cultures with phosphatase inhibitors. The smaller size of the mineral crystals formed in these cultures may also reflect the lower inorganic phosphate concentrations. After mineralization commenced, inhibiting phosphatases had the opposite effect, accelerating mineralization suggesting that it is the stabilization of phosphoproteins rather than the release of inorganic phosphate that is most important at this time. Inhibition of alkaline phosphatase activity had no effect on mineral accretion, suggesting that other phosphatases such as those discussed above may be of more relevance in this system.

It is useful to comment on why addition of agents that block CK2 or phosphatases had to commence on or after day 7. Phosphatases that affect chondrogenesis [51–53] and mineralization [54] in similar cultures also have been reported, and it is likely that at the early stages of differentiation the effects are on intracellular events as most of the phosphatases signal across the membrane. There may also be effects of blocking CK2 on such events. Before chondrocyte nodules are formed, inhibitors that block phosphorylation affect cell viability, mitosis, chondrocyte condensation, and the cytoskeleton [33–37]. Thus phosphorylation and dephosphorylation play major roles in regulating the differentiation and proliferation of mesenchymal cells in culture, hence the studies reported here focused on times after the cells had differentiated and the matrix was formed.

The studies reported here also verified the findings that BSP is a nucleator of hydroxyapatite formation, since blocking antibodies decreased initial mineral accretion while having lesser effects on mineral accumulation, similar to what is seen *in vitro* [55,56]. Perhaps BSP must be phosphorylated by CK2 during the early stages of mineralization, and OPN must be dephosphorylated to facilitate further mineral proliferation. Studies where either OPN or BSP is knocked down in this culture system will be required to confirm that hypothesis.

Osteonectin blocking had very little effect, and as previously suggested may be more important for regulating matrix formation than mineralization [57]. It is important to note that the decreases observed in the blocking experiments were less than that observed when inhibiting phosphorylation of these proteins with the CK2 inhibitors or dephosphorylation with phosphoprotein phosphatases. This implies that other phosphoproteins, e.g., matrix extracellular phosphoprotein (MEPE), DMP1, and bone acidic glycoprotein (BAG-75), all expressed by chondrocytes [58–61] may be involved in cartilage calcification. These will either have to be examined in future studies when antibodies that cross react with chick are available or in cultures from a different species for which such antibodies are available.

While these studies have not pin-pointed which specific phosphoproteins are regulating cartilage calcification they do indicate that modulating the extent of phosphorylation of the phosphoproteins present has significant effects on the nature and amount of the mineral that is formed in this system. There have been no other studies in which the effects of phosphorylation or dephosphorylation of matrix proteins in chondrocyte cultures have been examined by characterization of the rate of mineral formation or the nature of the mineral formed. In fact, the only studies in which phosphorylation was considered were those related to signaling mechanisms [51,62,63], although a recent report by Murshed et al. stresses the importance of regulating phosphate concentrations in mineralizing systems [64]. In the future we hope to use siRNA techniques (developed for long-term silencing) in chick systems to further evaluate which proteins and enzymes are crucial.

Acknowledgements

These studies were supported by NIH grant AR037661. The investigation was conducted in a facility constructed with support from Research Facilities Improvement Program Grant Number C06-RR12538-01 from the National Center for Research Resources, National Institutes of Health. FT-IR Imaging studies were performed in the NIH-sponsored Musculoskeletal Repair and Regeneration Core Center (AR046121). The authors are indebted to Linda Mobula and Dalina Stiner for their assistance in the early stages of this project, to Dr. Jeffrey Gorski for his advice on phosphoprotein identification, and to Dr. Lisa K. Denzin who allowed us to use her fluorescent plate reader.

References

1. Jono S, Peinado C, Giachelli CM. Phosphorylation of osteopontin is required for inhibition of vascular smooth muscle cell calcification. *J Biol Chem* 2000;275:20197–20203. [PubMed: 10766759]
2. Gericke A, Qin C, Spevak L, Fujimoto Y, Butler WT, Sorensen ES, Boskey AL. Importance of Phosphorylation for Osteopontin Regulation of Biomineralization. *Calcified Tissue Int* 2005;77:45–54.
3. Razzouk S, Brunn JC, Qin C, Tye CE, Goldberg HA, Butler W. Osteopontin posttranslational modifications, possibly phosphorylation, are required for *in vitro* bone resorption but not osteoclast adhesion. *Bone* 2002;30:40–47. [PubMed: 11792563]
4. Tye CE, Rattray KR, Warner KJ, Gordon JA, Sodek J, Hunter GK, Goldberg HA. Delineation of the hydroxyapatite-nucleating domains of bone sialoprotein. *J Biol Chem* 2003;278:7949–7955. [PubMed: 12493752]
5. Tartaix PH, Doulaverakis M, George A, Fisher LW, Butler WT, Qin C, Salih E, Tan M, Fujimoto Y, Spevak L, Boskey AL. *In vitro* effects of dentin matrix protein-1 on hydroxyapatite formation provide insights into *in vivo* functions. *J Biol Chem* 2004;279:18115–18120. [PubMed: 14769788]

6. He G, Ramachandran A, Dahl T, George S, Schultz D, Cookson D, Veis A, George A. Phosphorylation of phosphophoryn is crucial for its function as a mediator of biomineralization. *J Biol Chem* 2005;280:33109–33114. [PubMed: 16046405]
7. Laroche M. Phosphate, the renal tubule, and the musculoskeletal system. *Joint Bone Spine* 2001;68:211–215. [PubMed: 11394620]
8. Stewart AJ, Roberts SJ, Seawright E, Davey MG, Fleming RH, Farquharson C. The presence of PHOSPHO1 in matrix vesicles and its developmental expression prior to skeletal mineralization. *Bone* 2006;39:1000–1007. [PubMed: 16837257]
9. Qin C, Baba O, Butler WT. Post-translational modifications of sibling proteins and their roles in osteogenesis and dentinogenesis. *Crit Rev Oral Biol Med* 2004;15:126–136. [PubMed: 15187031]
10. Kay MA, Meyer MH, Delzer PR, Meyer RA Jr. Changing patterns of femoral and skeletal mineralization during growth in juvenile X-linked hypophosphatemic mice. *Miner Electrolyte Metab* 1985;11:374–380. [PubMed: 4069087]
11. Anderson HC, Harmey D, Camacho NP, Garimella R, Sipe JB, Tague S, Bi X, Johnson K, Terkeltaub R, Millan JL. Sustained osteomalacia of long bones despite major improvement in other hypophosphatasia-related mineral deficits in tissue nonspecific alkaline phosphatase/nucleotide pyrophosphatase phosphodiesterase 1 double-deficient mice. *Am J Pathol* 2005;166:1711–1720. [PubMed: 15920156]
12. Johnson K, Goding J, Van Etten D, Sali A, Hu SI, Farley D, Krug H, Hessle L, Millan JL, Terkeltaub R. Linked deficiencies in extracellular PP(i) and osteopontin mediate pathologic calcification associated with defective PC-1 and ANK expression. *J Bone Miner Res* 2003;18:994–1004. [PubMed: 12817751]
13. Cecil DL, Rose DM, Terkeltaub R, Liu-Bryan R. Role of interleukin-8 in PiT-1 expression and CXCR1-mediated inorganic phosphate uptake in chondrocytes. *Arthritis Rheum* 2005;52:144–154. [PubMed: 15641067]
14. Wang W, Xu J, Du B, Kirsch T. Role of the progressive ankylosis gene (ank) in cartilage mineralization. *Mol Cell Biol* 2005;25:312–323. [PubMed: 15601852]
15. Abe T, Nomura S, Nakagawa R, Fujimoto M, Kawase I, Naka T. Osteoblast differentiation is impaired in SOCS-1-deficient mice. *J Bone Miner Metab* 2006;24:283–290. [PubMed: 16816922]
16. Sfeir C, Veis A. The membrane associated kinases which phosphorylate bone and dentin extracellular matrix phosphoproteins are isoforms of cytosolic CKII. *Connect tissue Res* 1996;35:215–222. [PubMed: 9084660]
17. Torres-Quintana MA, Lecolle S, Goldberg M. Effects of inositol hexasulphate, a casein kinase inhibitor, on dentine phosphorylated proteins in organ culture of mouse tooth germs. *Arch Oral Biol* 1998;43:597–610. [PubMed: 9758042]
18. Boskey AL, Guidon P, Doty SB, Stiner D, Leboy P, Binderman I. The mechanism of beta-glycerophosphate action in mineralizing chick limb-bud mesenchymal cell cultures. *J Bone Miner Res* 1996;11:1694–1702. [PubMed: 8915777]
19. Boskey AL, Camacho NP, Mendelsohn R, Doty SB, Binderman I. FT-IR microscopic mappings of early mineralization in chick limb-bud mesenchymal cell cultures. *Calcif Tissue Int* 1992;51:443–448. [PubMed: 1451012]
20. Boskey AL, Stiner D, Doty SB, Binderman I, Leboy P. Studies of mineralization in tissue culture: optimal conditions for cartilage calcification. *Bone Miner* 1992;16:11–36. [PubMed: 1371424]
21. Boskey AL, Doty SB, Binderman I. Adenosine 5'-triphosphate promotes mineralization in differentiating chick limb-bud mesenchymal cell cultures. *Microsc Res Tech* 1994;28:492–504. [PubMed: 7949395]
22. Boskey AL, Stiner D, Binderman I, Doty SB. Type I collagen influences cartilage calcification: an immunoblocking study in differentiating chick limb-bud mesenchymal cell cultures. *J Cell Biochem* 2000;79:89–102. [PubMed: 10906758]
23. Boskey AL, Doty SB, Stiner D, Binderman I. Viable cells are a requirement for in vitro cartilage calcification. *Calcif Tissue Int* 1996;58:177–185. [PubMed: 8852573]
24. Boskey AL, Stiner D, Binderman I, Doty SB. Effects of proteoglycan modification on mineral formation in a differentiating chick limb-bud mesenchymal cell culture system. *J Cell Biochem* 1997;64:632–643. [PubMed: 9093912]

25. Boskey AL, Paschalis EP, Binderman I, Doty SB. BMP-6 accelerates both chondrogenesis and mineral maturation in differentiating chick limb-bud mesenchymal cell cultures. *J Cell Biochem* 2002;84:509–519. [PubMed: 11813256]
26. Pourmand EP, Binderman I, Doty SB, Kudryashov V, Boskey AL. Chondrocyte apoptosis is not essential for cartilage calcification: Evidence from an in vitro avian model. *J Cell Biochem* 2006;100:43–57. [PubMed: 16888817]
27. Cohen P. The structure and regulation of protein phosphatases. *Annu Rev Biochem* 1989;58:453–508. [PubMed: 2549856]
28. Gehringer MM. Microcystin-LR and okadaic acid-induced cellular effects: a dualistic response. *FEBS Lett* 2004;557:1–8. [PubMed: 14741332]
29. Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. *J Morphol* 1951;88:49–52.
30. Ahrens PB, Solursh M, Reiter RS. Stage-related capacity for limb chondrogenesis in cell culture. *Dev Biol* 1977;60(1):69–82. [PubMed: 198274]
31. Willis JB. Determination of metals in blood serum by atomic absorption spectroscopy. I Calcium. *Spectrochim Acta* 1980;16:259–272.
32. Heinonen JK, Lahti RJ. A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphates. *Anal Biochem* 1981;113:313–317. [PubMed: 6116463]
33. Zakany R, Bako E, Felszeghy S, Hollo K, Balazs M, Bardos H, Gergely P, Modis L. Okadaic acid-induced inhibition of protein phosphatase 2A enhances chondrogenesis in chicken limb bud micromass cell cultures. *Anat Embryol (Berl)* 2001;203:23–34. [PubMed: 11195086]
34. Morimoto Y, Ohba T, Kobayashi S, Haneji T. The protein phosphatase inhibitors okadaic acid and calyculin A induce apoptosis in human osteoblastic cells. *Exp Cell Res* 1997;230:181–186. [PubMed: 9024777]
35. Cruz TF, Mills G, Pritzker KP, Kandel RA. Inverse correlation between tyrosine phosphorylation and collagenase production in chondrocytes. *Biochem J* 1990;269:717–721. [PubMed: 1697163]
36. Sarno S, Reddy H, Meggio F, Ruzzene M, Davies SP, Donella-Deana A, Shugar D, Pinna LA. Selectivity of 4,5,6,7-tetrabromobenzotriazole, an ATP site-directed inhibitor of protein kinase CK2 ('casein kinase-2'). *FEBS Lett* 2001;496:44–48. [PubMed: 11343704]
37. Pagano MA, Andrzejewska M, Ruzzene M, Sarno S, Cesaro L, Bain J, Elliott M, Meggio F, Kazmierczuk Z, Pinna LA. Optimization of protein kinase CK2 inhibitors derived from 4,5,6,7-tetrabromobenzimidazole. *J Med Chem* 2004;47:6239–6247. [PubMed: 15566294]
38. Kim YJ, Sah RLY, Doong JYH, Grodzinsky AJ. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. *Anal Biochem* 1988;174:168–176. [PubMed: 2464289]
39. Bonewald LF, Harris SE, Rosser J, Dallas MR, Dallas SL, Camacho NP, Boyan B, Boskey A. von Kossa staining alone is not sufficient to confirm that mineralization in vitro represents bone formation. *Calcif Tissue Int* 2003;72:537–547. [PubMed: 12724828]
40. Butler WT. Dentin -specific proteins. *Methods Enzymol* 1987;145:290–301. [PubMed: 3600395]
41. Honkanen RE, Codispoti BA, Tse K, Boynton AL, Honkanen RE. Characterization of natural toxins with inhibitory activity against serine/threonine protein phosphatases. *Toxicon* 1994;32:339–350. [PubMed: 8016855]
42. Ishihara H, Martin BL, Brautigan DL, Karaki H, Ozaki H, Kato Y, Fusetani N, Watabe S, Hashimoto K, Uemura D, Hartshorne DJ. Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. *Biochem Biophys Res Commun* 1989;159:871–877. [PubMed: 2539153]
43. Barak-Shalom T, Schickler M, Knopov V, Shapira R, Hurwitz S, Pines M. Synthesis and phosphorylation of osteopontin by avian epiphyseal growth-plate chondrocytes as affected by differentiation. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 1995;111:49–59. [PubMed: 7656184]
44. Gerstenfeld LC, Shapiro FD. Expression of bone-specific genes by hypertrophic chondrocytes: implication of the complex functions of the hypertrophic chondrocyte during endochondral bone development. *J Cell Biochem* 1996;62:1–9. [PubMed: 8836870]
45. Ye L, Mishina Y, Chen D, Huang H, Dallas SL, Dallas MR, Sivakumar P, Kunieda T, Tsutsui TW, Boskey A, Bonewald LF, Feng JQ. Dmp1-deficient mice display severe defects in cartilage formation

- responsible for a chondrodysplasia-like phenotype. *J Biol Chem* 2005;280:6197–6203. [PubMed: 15590631]
46. Veis A, Wu CB. Phosphorylation of the proteins of the extracellular matrix of mineralized tissues by casein kinase-like activity. *Crit Rev Oral Biol* 1997;8:360–379.
 47. Salih E, Ashkar S, Gerstenfeld LC, Glimcher MJ. Protein kinases of cultured osteoblasts: selectivity for the extracellular matrix proteins of bone and their catalytic competence for osteopontin. *J Bone Miner Res* 1996;11:1461–1473. [PubMed: 8889846]
 48. Steiglitiz BM, Ayala M, Narayanan K, George A, Greenspan DS. Bone morphogenetic protein-1/Tolloid-like proteinases process dentin matrix protein-1. *J Biol Chem* 2004;279:980–986. [PubMed: 14578349]
 49. Hunter GK, Kyle CL, Goldberg HA. Modulation of crystal formation by bone phosphoproteins: structural specificity of the osteopontin-mediated inhibition of hydroxyapatite formation. *Biochem J* 1994;300:723–728. [PubMed: 8010953]
 50. Pampena DA, Robertson KA, Litvinova O, Lajoie G, Goldberg HA, Hunter GK. Inhibition of hydroxyapatite formation by osteopontin phosphopeptides. *Biochem J* 2004;378:1083–1087. [PubMed: 14678013]
 51. Zakany R, Bako E, Felszeghy S, Hollo K, Balazs M, Bardos H, Gergely P, Modis L. Okadaic acid-induced inhibition of protein phosphatase 2A enhances chondrogenesis in chicken limb bud micromass cell cultures. *Anat Embryol (Berl)* 2001;203:23–34. [PubMed: 11195086]
 52. Zakany R, Szucs K, Bako E, Felszeghy S, Czifra G, Biro T, Modis L, Gergely P. Protein phosphatase 2A is involved in the regulation of protein kinase A signaling pathway during in vitro chondrogenesis. *Exp Cell Res* 2002;275:1–8. [PubMed: 11925100]
 53. Bang OS, Kim EJ, Chung JG, Lee SR, Park TK, Kang SS. Association of focal adhesion kinase with fibronectin and paxillin is required for precartilaginous condensation of chick mesenchymal cells. *Biochem Biophys Res Commun* 2000;278:523–529.
 54. Houston B, Stewart AJ, Farquharson C. PHOSPHO1-A novel phosphatase specifically expressed at sites of mineralisation in bone and cartilage. *Bone* 2004;34:629–637. [PubMed: 15050893]
 55. Hunter GK, Goldberg HA. Nucleation of hydroxyapatite by bone sialoprotein. *Proc Natl Acad Sci U S A* 1993;90:8562–8565. [PubMed: 8397409]
 56. Boskey AL. Osteopontin and related phosphorylated sialoproteins: effects on mineralization. *Ann N Y Acad Sci* 1995;760:249–256. [PubMed: 7785899]
 57. Boskey AL, Moore DJ, Amling M, Canalis E, Delany AM. Infrared analysis of the mineral and matrix in bones of osteonectin-null mice and their wildtype controls. *J Bone Miner Res* 2003;18:1005–1011. [PubMed: 12817752]
 58. Rowe PS. The wickkened pathways of FGF23, MEPE and PHEX. *Crit Rev Oral Biol Med* 2004;15:264–281. [PubMed: 15470265]
 59. Ye L, Mishina Y, Chen D, Huang H, Dallas SL, Dallas MR, Sivakumar P, Kunieda T, Tsutsui TW, Boskey A, Bonewald LF, Feng JQ. Dmp1-deficient mice display severe defects in cartilage formation responsible for a chondrodysplasia-like phenotype. *J Biol Chem* 2005;280:6197–6203. [PubMed: 15590631]
 60. Midura RJ, Wang A, Lovitch D, Law D, Powell K, Gorski JP. Bone acidic glycoprotein-75 delineates the extracellular sites of future bone sialoprotein accumulation and apatite nucleation in osteoblastic cultures. *J Biol Chem* 2004;279:25464–25473. [PubMed: 15004030]
 61. Gorski JP, Kremer EA, Chen Y. Bone acidic glycoprotein-75 self-associates to form large macromolecular complexes. *Connect Tissue Res* 1996;35:137–143. [PubMed: 9084651]
 62. Johnson KA, Terkeltaub RA. External GTP-bound transglutaminase 2 is a molecular switch for chondrocytes hypertrophic differentiation and calcification. *J Biol Chem* 2005;280:15004–15012. [PubMed: 15691824]
 63. Ijiri K, Zerbini LF, Peng H, Correa RG, Lu B, Walsh N, Zhao Y, Taniguchi N, Huang XL, Otu H, Wang H, Wang JF, Komiya S, Ducy P, Rahman MU, Flavell RA, Gravalles EM, Oettgen P, Libermann TA, Goldring MB. A Novel Role for GADD45{beta} as a Mediator of MMP-13 Gene Expression during Chondrocyte Terminal Differentiation. *J Biol Chem* 2005;280:38544–38555. [PubMed: 16144844]

64. Murshed M, Harmey D, Millan JL, McKee MD, Karsenty G. Unique coexpression in osteoblasts of broadly expressed genes accounts for the spatial restriction of ECM mineralization to bone. *Genes Dev* 2005;19:1093–1104. [PubMed: 15833911]

1.
Histochemical Characterization of the Differentiating Chick Limb bud Mesenchymal Cells.
 All sections shown are parallel to the surface of the culture dish: (a–c) Back scatter imaging of von Kossa (silver-stained) cultures at days 14 (a), 16 (b), and 21 (c). Magnification bar is 1 mm. (d) von Kossa staining of d21 plastic-embedded mineralizing cultures showing the distribution of mineral (dark brown) around the edges of the nodules, original magnification 1.25X. (e) Alizarin red staining of d21 glycerol fixed cultures showing accumulation of Ca on the edges of the nodules and around the periphery of the culture, original magnification 1.25X. (f) Casein kinase immunolocalization in d21 cultures, original magnification 8X. Note the majority of the activity is in the mature chondrocytes in the center of the culture.

2.

Apigenin inhibits the rate of ^{45}Ca uptake without affecting cell viability. a) Live-dead cell assay shows no effect of apigenin on cell viability at day 21 with 40uM added continuously from d7 as contrasted with b) mineralizing cultures without apigenin at d21, magnification 15x. c) Kinetics of ^{45}Ca accretion: All data were normalized to the ^{45}Ca uptake of control mineralizing cultures at day 21. Data points shows mean $\pm$ SD (n=4) of non-mineralizing and mineralizing controls (not treated with apigenin), and mineralizing cultures treated from day 9 with 40 μM apigenin. The curve represents best-fits to the data ($r^2 > 0.97$). The dotted line shows the slope of the maximum rate of ^{45}Ca accretion used to calculate rates, with the intercept indicating the induction time. (d) The effect of apigenin depends on time and duration of addition. Apigenin added continuously from day 7 or day 9 to mineralizing cultures inhibits ^{45}Ca accretion, while continuous addition after day 11 stimulates ^{45}Ca uptake. Untreated mineralizing controls (4P) are contrasted with cultures given 40 μM apigenin continuously from day shown (e.g., d7c)

3.

Typical FTIR images of mineral-to-matrix ratio (Mineral/matrix) and crystallinity (1030/1020) at day 21 mineralizing cultures with and without 40 uM apigenin added from day 9. The spectrum at the top corresponds to the pixel indicated by the arrow. In the images, x and y axis scales are in pixels; each pixel is ~7um × 7um. Color scales for each parameter are presented adjacent to the figure, and mean ± SD for all pixels in the image are shown in bold below the images.

4. Effects of 16uM casein kinase II inhibitor II on ^{45}Ca accumulation with continuous addition from day indicated to day 23. Values are mean $\pm$ SD, n=3-5. All data was normalized to the ^{45}Ca uptake of untreated control mineralizing cultures (4P) at day 23.

5.

Effects of Inhibition of Phosphatase Activity on mineral accretion (^{45}Ca uptake) relative to untreated (0) mineralizing controls. a) Inhibition by okadaic acid (OK) or its inert tetra acetate form (OKTA) added at different concentrations from different days in culture. b) Effects of calyculin A added once on day 7 or 14 (a), or continuously (c) from day 7 or 14. c) Inhibition by 10 nM Microcystin-LR added from different time points results in different rates of mineral accrual and different initiation times d) Continuous addition of 100 uM Levamisole from day indicated has no effect on ^{45}Ca uptake. Values are mean $\pm$ SD, n=4. All data was normalized to the ^{45}Ca uptake of control mineralizing cultures at day 21.

6.

Importance of Viable Cells and Phosphate Concentration in Cell-mediated Mineralization: a) Mineral accretion is retarded to different extents by cell lysis before (day 7) and after (day 16) initiation of mineralization. b) Comparative ^{45}Ca uptake in mineralizing cultures with phosphatase activity inhibited by addition of 1.6 nM okadaic acid from day 9–11 in the presence and absence of additional 2 mM inorganic phosphate (6P) from day 12. All data was normalized to the ^{45}Ca uptake of control mineralizing cultures at day 21. Values are mean $\pm$ SD, $n=3$

7.

Protein analyses: a) Western blot showing the presence of osteopontin at multiple time points in the cultures. Three different sequential time points (day11, 16, 19) are shown, with 20 ug total protein loaded at each point. The band at 61.3 kD corresponds to OPN. b) Diamond Q staining for phosphoproteins showing ECM extracts from untreated (control), apigenin treated (Ap) and microcystin treated (MC) cultures at day 14; the same gel stained with Sipro Ruby for total protein.

8. Effects of blocking antibodies on ^{45}Ca accumulation. Antibodies (AB) used were directed against; a) osteopontin, b) bone sialoprotein, and c) osteonectin and added on the days shown. Values are mean $\pm$ SD, n=4. All data was normalized to the ^{45}Ca uptake of control mineralizing cultures at day 21.

Table 1
Effect of Casein Kinase and Phosphatase Inhibitors on Mineralization as a Function of Concentration and Time of Addition (Mean (SD) *)

Inhibitor **	% Yield	Rate	t ₀	Mineral/matrix	crystallinity
CK2 inhibitors					
D7 Apigenin	49(38)	2.2	8.0	0.86	0.9
D7 CK2-inh II	36(3)	2.5	10.0	0.94	0.9
D9 Apigenin	116(16)	14.5	13.2	1.12(0.05)	1.14(0.4)
D9 CK2-inh II	48(1)	3.8	10.2	1.55(0.08)	1.08(0.03)
D11 Apigenin	124(14)	22	12.8	1.172(0.08)	1.11(0.03)
D11 CK2-inh II	88(9)	14.5	13.0	1.70(0.67)	1.09(0.04)
D14 Apigenin	99(6)	20	14	1.7(0.1)	1.10(0.05)
D14 CK2-inh II	103(10)	19	14	1.71(0.15)	1.09(0.06)
None	100	16.1(1.8)	12.5(1.3)	1.43(0.03)	1.09(0.05)
(non-mineralizing control)	...			1.46±0.05	1.0±0.05
Phosphatase Inhibitors					
D7 OK 1.6nM c	5.4 (3)	30	18.6	0.38(0.06)	0.6±0.08
1.6nM a	5.2	30	19.2		
D7 OK 3.2nM c	8.9(3)	30	15.6		
3.2nM a	8.4	31	15.2		
D7Calv.1.0nM c	14	36	18.5		
1.0 nM a	88	38.5	19.8		
D7Calv.10nM c	86	30	18.4		
10nM a	88	25	18.1		
D9 OK 1.6 nM c	94	15	14	0.36(0.09)	0.58(0.03)
D14 OK 1.6 nM c	114	14	16.3	1.5	1.1
D14Calv.1.0nM c	132	36	16.3		
1.0 nM a	89	37	16.2	1.5	1.08
D14 OK 3.2nM c	103(13)	13	13		
3.2nM a	109	13	14		
D14 Calv.10nM a	170	13	13		
D7 Mic 10nM a	100	7.4	13.0		
D9 Mic 10nM a	87.3	12.8	11.0		
D11 Mic 10nM a	78	10.3	13.1		
D7,9,11,14 Lev c	103±6	7.6±3	10	1.46(0.05)	1.0(0.05)

* Means for 3–5 independent experiments. If no SD is shown for continuous addition of CK2 inhibitors, but acute (a) and continuous addition (c) of phosphatase inhibitors is included. Since kinetic data (rate of initial calcium uptake (rate) and induction time (t₀)) were calculated from plots including data from all experiments mean and SD cannot be shown. Infrared parameters (mineral/matrix and crystallinity) were determined by FTIR of selected cultures and represent mean (SD) for 3 experiments. Where no SD is shown n=2.

** Apigenin concentration for all studies was 40 uM; CK2 inhibitor II (CK2-Inh II) was 16 uM. Ok=okadaic acid; Calv= calyculin A, Mic= Microcystin LR; Lev= levamisole (100uM); nd=not measurable.

Table 2

Cell Viability in the Presence of Phosphatase Inhibitors and Other Agents

Agent and concentration	% Viability (Experimental/Control) mean	SD
Apigenin 40 μ M	100.9	10.5
CKII inh 16 μ M	98.9	13.2
NaVO ₄ 1.5 nM	5.91	1.75
Okadaic acid 1.6 nM	95.8	11.6
Microcystin 10 μ M	108.8	12.1
anti Osteopontin 1:1000	107.6	16.0
anti Bone Sialoprotein 1:1000	98.8	14.0
anti Osteonectin 1:1000	100.4	8.4