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Changes in Matrix Protein Gene Expression Associated With Mineralization in the Differentiating Chick Limb-bud Micromass Culture System

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ABSTRACT

Chick limb-bud mesenchymal stem cells plated in high density culture in the presence of 4 mM inorganic phosphate and vitamin C differentiate and form a mineralizable matrix, resembling that of the chick growth plate. To further elucidate the mechanism that allows these cultures to form physiologic hydroxyapatite deposits, and how the process can be manipulated to gain insight into mineralization mechanisms, we compared gene expression in mineralizing (with 4 mM inorganic phosphate) and non-mineralizing cultures (containing only 1 mM inorganic phosphate) at the start of mineralization (day 11) and after mineralization reached a plateau (day 17) using a chick specific microarray. Based on replicate microarray experiments and K-cluster analysis, several genes associated with the mineralization process were identified, and their expression patterns confirmed throughout the culture period by quantitative RT-PCR. The functions of bone morphogenetic protein 1, BMP1, dentin matrix protein 1, DMP1, the sodium phosphate co-transporter, NaPi IIb, matrix metalloprotease 13. MMP-13, and alkaline phosphatase, along with matrix protein genes (type X collagen, bone sialoprotein, and osteopontin) usually associated with initiation of mineralization are discussed. J. Cell. Biochem. 112: 607–613, 2011. © 2010 Wiley-Liss, Inc.

KEY WORDS: CHICK LIMB-BUD; MICROMASS CULTURE; MINERALIZATION; MICROARRAY; GENE EXPRESSION

Chick limb-bud mesenchymal stem cells plated in high density (micromass) culture under previously defined conditions [Binderman et al., 1979; Boskey et al., 1991; Boskey et al., 1992b; Boskey et al., 1996], differentiate and form a mineralizable matrix, that resembles that of the chick epiphysial growth plate [Boskey et al., 1992a]. This system has been used to study the effects of collagen [Boskey et al., 2000], proteoglycans [Boskey et al., 1997], and phosphoproteins [Boskey et al., 2008] on the mineralization process. To further elucidate the mechanism that allows these cultures to form physiologic hydroxyapatite deposits, and how the process can be manipulated to gain insight into mineralization mechanisms, we compared gene expression in mineralizing and non-mineralizing cultures at the start of mineralization (day 11) and after mineralization reached a plateau (day 17) [Boskey et al., 2008] using a chick specific microarray.

While gene expression profiling is not generally reported for the epiphyseal growth plate mediated calcification, there are reports for gene expression in the early differentiation of mesenchymal cells into chondrocytes [Cameron et al., 2009], in the reserve [Zhang et al., 2008], proliferating and hypertrophic zones of the growth plate [Wang et al., 2004b], and in healing fracture callus development [Rundle et al., 2006; Wang et al., 2006; Khan et al., 2008] a process that mimics endochondral ossification. Similarly there are reports of gene expression during intramembranous bone formation [Kuroda et al., 2005], and for the effects of different factors on growth plate chondrocytes [Ulici et al., 2010]. There is also a proteomics study of the mouse growth plate [Belluoccio et al., 2006] and of mineralizing extracellular matrix vesicles [Xiao et al., 2007]. Recently, James et al. [James et al., 2010] used microdissection of murine bones to characterize gene expression in a combination of proliferating and resting zone cells, prehypertrophic and hypertrophic chondrocytes,

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and calcified cartilage and bone and compared this data to micromass cultures of the murine cells. The emphasis in many of those studies was on signaling molecules and growth factors. The purpose of the present study was to identify genes associated with the mineralization process, and to select those extracellular matrix proteins and enzymes that modify them for further analysis.

MATERIALS AND METHODS

THE CULTURE SYSTEM

Chick limb-buds were obtained at stage 21–24 [Hamburger and Hamilton, 1951] from White Leg Horn chick embryos. Mesenchymal cells released by digestion in 0.05% trypsin-EDTA (Invitrogen) were separated from debris by passage through a 20 μm Nitex membrane, counted with a hemocytometer, checked for viability by trypan blue dye exclusion, and pelleted at 1100 rpm. Cells, resuspended in DME (Sloan Kettering Media Lab) containing 1 g/l glucose supplemented with 10% FBS (Invitrogen) and antibiotic/antimycotic (Invitrogen; 100 units penicillin, 100 μg streptomycin, 0.25 μg amphotericin B/ml), were plated using the micromass technique [Ahrens et al., 1977] at a density of 0.75×10^6 cells per 20 μl drop in 35mm Falcon tissue culture dishes. The micromass cultures were allowed to attach for 1.5 h in a humidified atmosphere of 5% CO_2 at 37°C, and then flooded with the same DME media. From day 2 onward, 25 μg/ml Vitamin C (Sigma) was added to the media along with 0.3 mg/ml L-glutamine (Invitrogen). At day 2, the calcium concentration of the media was adjusted to 1.3 mM using calcium chloride and the phosphate concentration was adjusted to 1.0 mM using monobasic sodium phosphate. For all mineralizing cultures, the total phosphate concentration (Pi) was adjusted to 4.0 mM with potassium acid phosphate (Fisher Chemicals, New Jersey). Control non-mineralizing cultures received no acid phosphate addition and were in 1.0 mM Pi. Cultures were incubated at 37° with 95% air, 5% CO_2 with media changed every 48 h. The overview of the experiment is presented schematically in Figure 1.

RNA ISOLATION

Following washing with phosphate buffered saline, total cellular RNA was isolated from mineralizing and non-mineralizing cultures on days 11 and 17 using the single step Qiagen RNeasy kit (Chatworth, CA), and used for gene expression profiling using the Affymetrix GeneChip chicken genome array containing 32,773 transcripts. Six cultures were used per time point and each culture system was repeated three times, providing triplicate RNA samples for analysis. The MessageAmp Premier RNA Amplification Kit (Ambion/Applied Biosystems, Austin, TX), a one-round in vitro transcription (IVT) system was used to amplify nanogram amounts of total RNA from 6 samples and labeled cRNA for microarray hybridization. The quantity and quality of the amplified cRNA were assessed using NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and Agilent Bioanalyzer (Santa Clara, CA). The biotin labeled cRNA was fragmented and hybridized to the Chicken Genome arrays (Santa Clara, CA). After Hybridization, GeneChip arrays were washed, stained, and scanned by GeneChip Scanner 3000 7G according to the Affymetrix Expression Analysis technical manual.

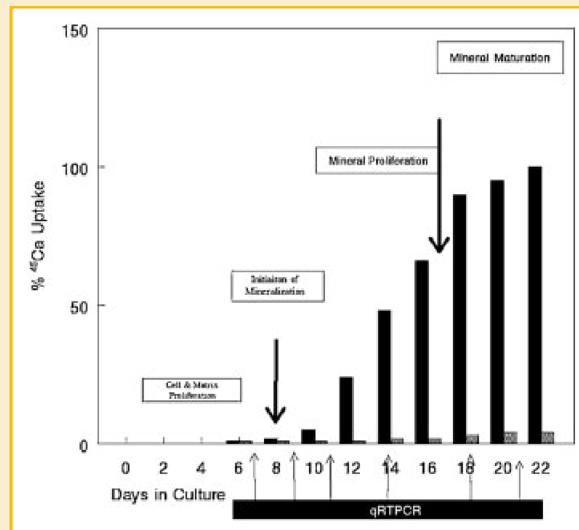

Fig. 1. Schematic showing stages of matrix and mineral development in the chick limb-bud micromass culture system with typical ^{45}Ca uptake data for mineralizing (dark bars) and non-mineralizing (light bars) as % of day 21 values in mineralizing culures. The titles in boxes define the stages; the dark arrows indicate the times sampled for micro-array analysis, and the small arrows indicate the times mineralized and non-mineralized cultures were analyzed by qRT-PCR.

Affymetrix GeneChip Operating Software was used for image acquisition. The target signal intensity from each chip was scaled to 500. Analyses were done in the CUMC Genomics Resources Core Facility.

MICROARRAY ANALYSES

GeneChip chicken genome arrays (#900590) were acquired from Affymetrix (Affymetrix Inc., Santa Clara, CA), and were used according to the manufacturer's instructions. Three replicates of each experiment were analyzed for the data normalization, statistical analysis, and pattern study using GeneSpring software (Agilent, Redwood City, CA). Data was sorted for expression level based on p value ($P > 0.05$ was excluded) and fold increase/decrease. Data was plotted as K-clusters indicating genes that increased and or decreased in expression with time or with mineralization.

SELECTION OF GENES FOR qRTPCR

Genes were sorted into categories: signaling molecules and growth factors, genes associated with metabolism, inflammation, translation and transduction, matrix proteins and enzymes that modulate matrix proteins. Several of the latter were selected based on their postulated roles in the mineralization process, discussed later, for quantification by qRTPCR. Additionally we did qRTPCR on genes known to be involved in growth plate mineralization from earlier studies [Boskey et al., 1992b].

qRTPCR

RNA was isolated from the chick system at days 4, 7, 9, 11 or 12, 14, 16 and 21. Mineralizing (4P) and non-mineralizing cultures (1P)

TABLE I. Primers Used in this Study

Gene	Forward primer	Reverse primer
BMP I	QIAGEN CAT#: QT01491812	
NaPi IIb	CTGGATGCACTCCCTAGAGC	TTATCTTTGGCACCCTCCTG
MMP13	CAACCCAAAACATCCCAAAAC	CCATTCATAGCCCAAACCTTC
DMP1	QIAGEN CAT#: QT01138354	
Col X	AGTGCTGTCATTGATCTCATGGA	TCAGAGGAATAGAGACCATTGGATT
Col II	GACCTCGTGGTGACAAAGGT	CATGCCGTTAGAGCCATCTT
Col I	GCCGTCACCTCAGACTTAGC	TTTTGTCCTTGGGGTTCTTG
OPN	CCAGCTCTGAAGAAAAATAC	CTAGGAATGTCAGGAAAGTC
OCN	TCGCGGCGCTGCTCACATTCA	TGGCGGTGGGAGATGAAGGCTTTA

were included for each time point. In the first two experiments, collection was based on multiple batches of eggs. In the third and fourth experiment, the same batch of eggs and embryos provided all the cultures for all analysis. Expression of selected genes was analyzed by quantitative real-time qRTPCR using 5ng cDNA with the QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA) on a DNA Engine Optican 2 System (MJ Research, Waltham, MA). Specific primers for these genes were obtained from Qiagen (Valencia, CA) using the chicken sequences with β-actin or GAPDH mRNA as a reference for quantification. The primers and their sources are summarized in Table I. Relative transcript levels are reported as "fold change" (x in the following formula) in gene expression and calculated using the threshold cycle (Ct) and the following formula, in which ctl = control, exp = experimental, and GAPDH or β-actin = the housekeeping gene: $x = 2^{\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta E - \Delta C$, $\Delta E = Ct(exp) - Ct(GAPDH)$, and $\Delta C = Ct(ctl) - Ct(GAPDH)$. The change for ΔΔCt was used in the calculation. Fold change was calculated relative to non-mineralizing controls.

STATISTICS

Mean and standard deviations for each group of genes analyzed in triplicate or quadriplucate were calculated (GraphPad, Instat) and compared to the 1.0 value indicating equivalence in the culture. Prior to these comparisons, single outliers for fold change were excluded using the Q-test [Dean and Dixon, 1951]. Significance was assumed with $P < 0.05$.

RESULTS

Microarray analysis uncovered 1814 genes that were differentially expressed in mineralizing and control cultures at day 11 and 17. Only those genes that were elevated/suppressed more than two fold and had significant ($P < 0.05$) alterations in expression based on replicate microarrays from separate culture experiments were considered.

K-cluster analyses indicated genes that were significantly up or down regulated as mineralization progressed (Fig. 2) as opposed to those that were increased initially and then decreased or those that increased or decreased temporally rather than with mineralization. These genes had distinct functions, summarized in the pie chart in

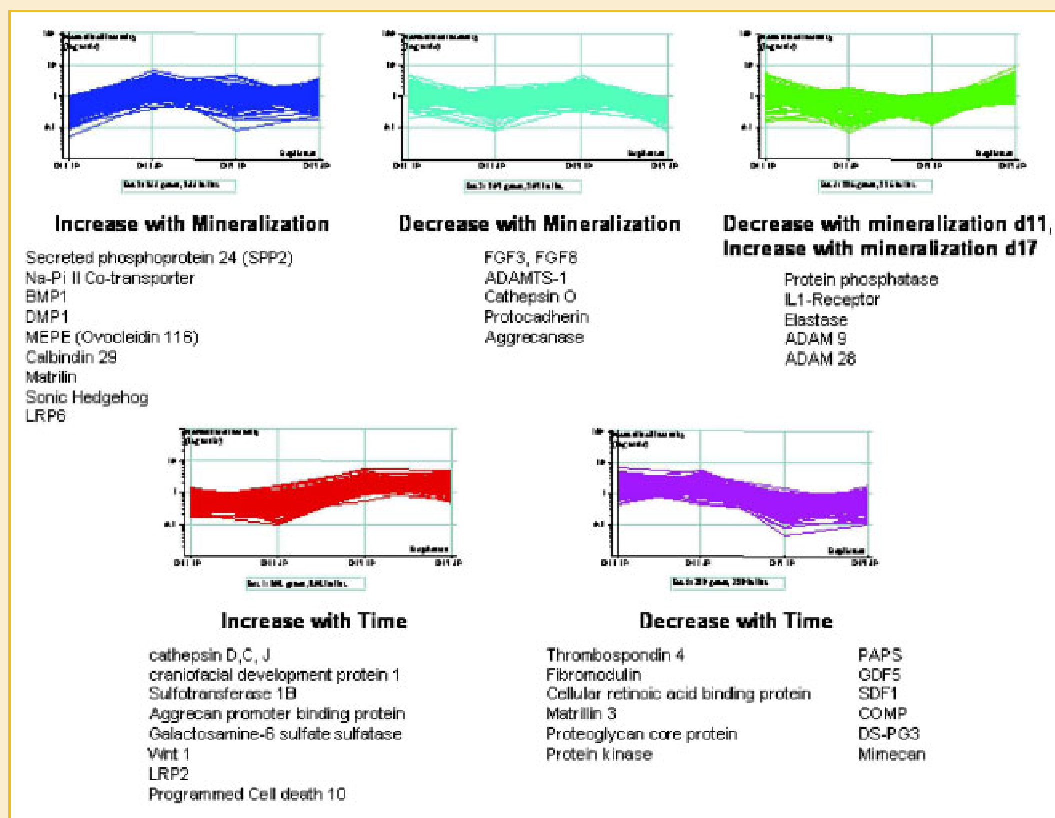

Fig. 2. K cluster analyses indicating variation in gene expression with mineralization and time. The genes listed under each figure are those which have been implicated in other studies to the progression of endochondral ossification and/or growth plate mineralization.

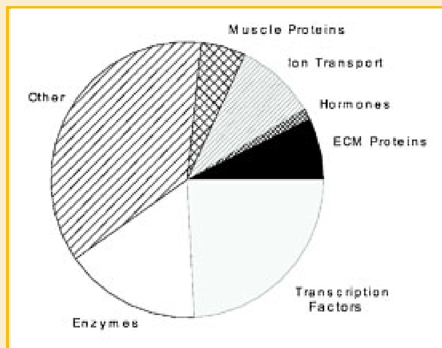

Fig. 3. Distribution of up- and down- regulated genes at day 11 by category.

Figure 3. The fold change in selected genes based on microarray that we hypothesized were important for mineralization are shown in Table II.

Several of these genes hypothesized to be important for the mineralization process based on level of expression were selected for confirmation by qRTPCR. The genes that were selected as of interest for the mineralization process were extracellular matrix proteins, the enzymes that modify them, and a sodium phosphate co-transporter.

Bone morphogenic protein 1 (BMP1), a metalloproteinase that activates collagen, several growth factors, and the extracellular matrix protein dentin matrix protein 1 (DMP1) [Reynolds et al., 2000; Ge and Greenspan, 2006] showed increasing gene expression (Fig. 4A) relative to non-mineralizing cultures throughout the culture period, although the increase only reached statistical significance at day 21. DMP-1, a Small Integrin-Binding Ligand N-Glycosylated (SIBLING) protein associated with initiation of mineralization, showed significantly increased expression on days 9–12, just as mineralization commenced (Fig. 4B). The metalloproteinase MMP-13, important for cartilage modification, and believed to be important for preparing the growth plate for vascular invasion [Malemud, 2006] was highest in expression prior to the commencement of mineralization, and then gradually approached equivalence with values in the non-mineralizing cultures (Fig. 4C). Alkaline phosphatase gene expression was significantly elevated relative to the non-mineralizing controls at days 7 and 9, and then gradually decreased so that by day 21 expression was higher in the non-mineralizing controls (Fig. 4D from 3D). The sodium-phosphate co-transporter, NaPi IIb (Fig. 4E) was elevated before mineralization commenced, remained elevated during initial mineralization and was equivalent to the non-mineralizing controls at later time points (days 16 and 21).

TABLE II. Microarray Results

Gene	Day 11	Day 17
Extracellular matrix molecules		
DMP1	6.3	2.5
Ovocleidin 116 (MEPE)	5.3	2.4
SPP24 (SPP2)	4.5	0.24
Type IIA collagen precursor		5.1
Type XIV collagen		2.5
Type XII collagen		0.48
Osteoglycin		24
Phosphate Regulation		
Na Pi II Co-transporter	3.1	2.6
Phosphatases (5 distinct genes)	4–12x	2–4
Spingosine phosphatase (also called Phospho1)		8.2
Enzymes (Proteinases & Aggrecanases)		
BMP1	2.6	3.1
MMP15	3	0.5
Stromelysin 3	2.1	
MT3-MMP	0.36	
ADAMTS-2 (procollagen III N-proteinase)		0.2

Type X collagen, a marker of chondrocyte hypertrophy [Nurminskaya and Linsenmayer, 1996] was elevated relative to non-mineralizing cultures at days 9,11, and 14 (Fig. 5A), returning to equivalent levels as in the controls by day 16 (Fig. 5A). Types II (Fig. 5B) and I (Fig. 5C) collagen gene showed few significant changes associated with mineralization although there were temporal changes, and type II collagen gene expression was significantly elevated in the mineralizing cultures at day 12. Similarly, bone sialoprotein (BSP) expression was also elevated at day 16 (Fig. 5D) as was osteocalcin (OCN) expression (Fig. 5E). In contrast, osteopontin (Fig. 5F) showed a marked elevation just prior to the time of detectable mineral deposition, and remained elevated through day 16.

DISCUSSION

New information on biomineralization of the avian growth plate was provided by these analyses, specifically the lack of major variation in collagen I and II gene expression when mineralizing and non-mineralizing cultures were compared at the same time points, the early upregulation of the osteopontin gene, and the upregulation of the sodium-phosphate co-transporter, the protease BMP1, and one of its substrates, DMP1. Confirmatory information relative to earlier results in the chick system was given for Type X collagen [Boskey et al., 1992b], and, in other growth plate systems by the up-regulation of the matrix metalloproteinases [Wang et al., 2004b]. Similar to other reports [James et al., 2010] we found correspondence between our microarray and qRTPCR data. In this light it is interesting to note that our microarray data, similar to other studies describing gene regulation in the hypertrophic zone of the growth plate (not mineralization), also reported up-regulation of type I collagen, lysyl oxidase, ADAMSTS1, pro col V, and I, BMP2 and 6, ADAM17, calmodulin I, FGFRI, and Annexin I compared to the proliferating zone [Wang et al., 2004b]. Novel among our findings was the upregulation of the Na Pi II b co-transporter during the mineralization process.

The Na Pi IIb cotransporter, upregulated during both the start of mineral deposition and during mineral proliferation, was first isolated from kidney membranes [Debiec et al., 1992], and is the most abundant transporter in murine kidneys [Miyamoto et al., 1997]. Type II co-transporters, in general, play a major role in the regulation of renal Pi reabsorption by dietary Pi and parathyroid hormone, which in turn regulate the endocytosis/exocytosis of the transporters [Takeda et al., 1999]. In chondrocytes, Mansfield et al. [2001] using terminally differentiated hypertrophic chick chondrocytes and a competitive inhibitor of the transporter, phosphonoformic acid, blocked anion-induced cell death and prevented an

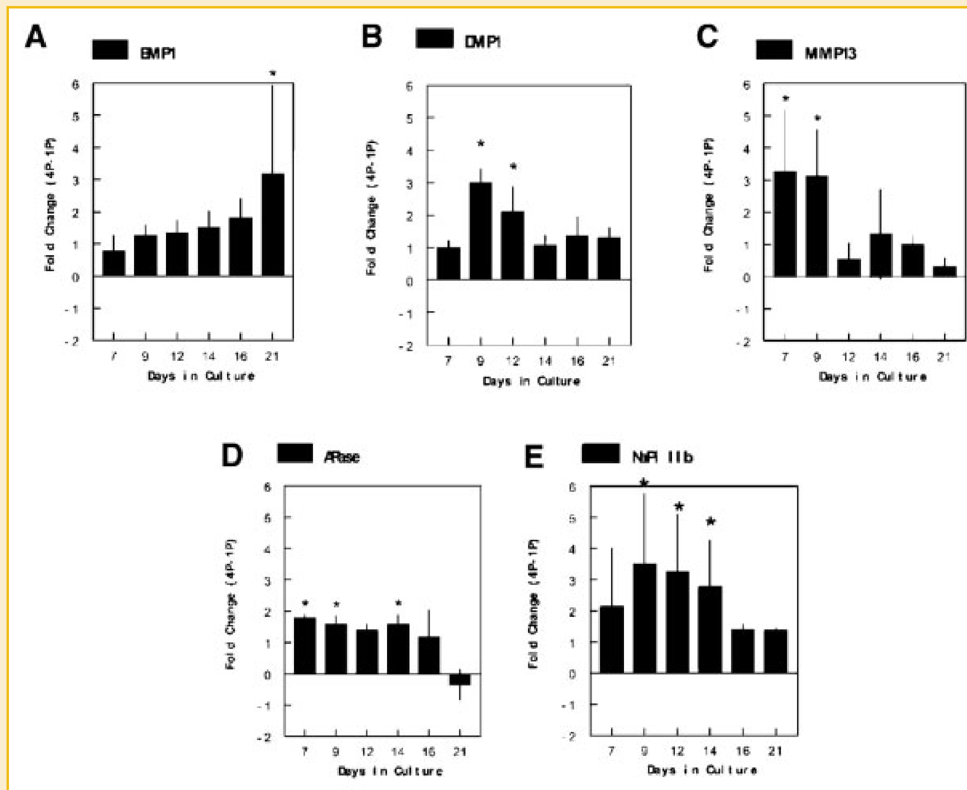

Fig. 4. Relative expression of A) BMP1, B) DMP-1, C) MMP-13, D) alkaline phosphatase, and E) NaPi IIb. Values are fold change relative to non-mineralizing controls, mean ± SD, n = 3 or 4. $^{*}P < 0.05$ relative to equivalence (constant = 1).

increase in the cell Pi content. They suggested that at the mineralization front, cell death was linked directly to the elevation in environmental anion concentration and the concomitant rise in intracellular Pi levels. In a murine mesenchymal cell line Denison et al. [2009] showed this NaPi co-transporter was involved in chondrocyte differentiation. The NaPi IIb co-transporter is also important in other mineralized tissue forming cells. Both rat odontoblasts [Lundquist et al., 2002] and osteoblast-like cell lines [Lundquist et al., 2007] were shown to be dependent for mineralization on the activity of a NaPi-II cotransporter. In the rat, NaPi II transporter expression is both regulated by Pi in a manner consistent with their playing a role in transcellular Pi flux during mineralization and appears to be involved in the mineralization process. The cellular Pi is presumably used for ATP synthesis, which in turn may promote apoptosis of growth plate chondrocytes through activation of the caspase signal pathways [Roy et al., 2010]. Extracellular matrix vesicles also use phosphate transport to accelerate mineralization, demonstrated by inhibiting phosphate transport in chick growth plate cultures [Wu et al., 2002].

It is also important to comment on the up-regulation of the BMP1 gene with increasing mineral proliferation. BMP1 (bone morphogenetic protein 1 or tolloid-like metalloproteinase) [Ge and Greenspan, 2006] is the enzyme responsible for the cleavage of dentin matrix protein-1, DMP-1, among other non-collagenous and collagenous matrix proteins into their component fragments [Steiglitz et al., 2004; Von Marschall and Fisher, 2008]. Although relative upregulation of DMP-1 expression preceded BMP1 elevation in the mineralizing vs. non-mineralizing cultures, BMP-1 is likely to be upregulated in both culture systems, as it is needed for processing collagen and lysyl-oxidase, as well as proteins more associated with mineralization. Animals with a mutated BMP1 cleavage site have decreased mineralization (Qin, personal communication), as do hypophosphatemic DMP-1 knockout mice [Liu et al., 2008]. This suggests that BMP1 is needed to prepare the newly formed matrix for mineralization.

The expression of osteocalcin genes were also significantly enhanced after mineralization had commenced, during the stage when mineral crystals are proliferating most actively. Both of these genes have been associated with the presence of mineral in other systems [Owen et al., 1991]; their expressions are parallel in other systems [Wang et al., 2004a], however, their precise roles in cartilage calcification are not well defined.

In conclusion, these studied enhanced our understanding of the mechanism that allows the chick limb-bud mesenchymal cell cultures to form physiologic hydroxyapatite deposits, and suggest how the process can be further examined (by knockdown or overexpression experiments) to gain additional insights into mineralization mechanisms, pointing out the potential roles of specific enzymes and phosphate transport systems in the mineralization process. Limitations of the study include the fact that avian and mammalian growth plate mineralization may not be identical and the lack of information on many of the up- and down-regulated

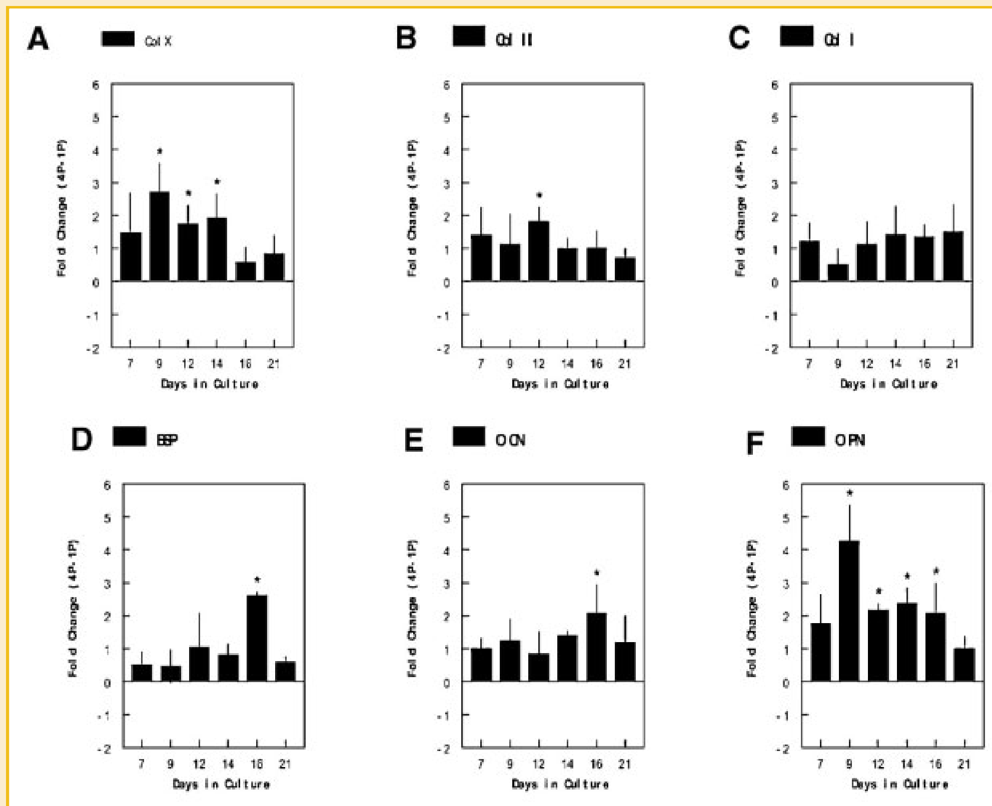

Fig. 5. Relative expression of collagens A) type X, b) type II, c) type I, and extracellular matrix proteins D) bone sialoprotein, E) osteocalcin and F) osteopontin. Values are fold change relative to non-mineralizing controls, mean ± SD, n = 3 or 4. $^{*}P < 0.05$ relative to equivalence (constant = 1).

genes on the chick microarray. None the less this study indicated a list of factors and extracellular matrix components that can be further studied to define their roles in the mineralization process.

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