Alterations in the Sensing and Transport of Phosphate and Calcium by Differentiating Chondrocytes*

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During endochondral bone formation and fracture healing, cells committed to chondrogenesis undergo a temporally restricted program of differentiation that is characterized by sequential changes in their phenotype and gene expression. This results in the manufacture, remodeling, and mineralization of a cartilage template on which bone is laid down. Articular chondrocytes undergo a similar but restricted differentiation program that does not proceed to mineralization, except in pathologic conditions such as osteoarthritis. The pathogenesis of disorders of cartilage development and metabolism, including osteochondrodysplasia, fracture non-union, and osteoarthritis remain poorly defined. We used the CFK2 model to examine the potential roles of phosphate and calcium ions in the regulatory pathways that mediate chondrogenesis and cartilage maturation. Differentiation was monitored over a 4-week period using a combination of morphological, biochemical, and molecular markers that have been characterized in vivo and in vitro. CFK2 cells expressed the type III sodium-dependent phosphate transporters Glvr-1 and Ram-1, as well as a calcium-sensing mechanism. Regulated expression and activity of Glvr-1 by extracellular phosphate and parathyroid hormone-related protein was restricted to an early stage of CFK2 differentiation, as evidenced by expression of type II collagen, proteoglycan, and Ihh. On the other hand, regulated expression and activity of a calcium-sensing receptor by extracellular calcium was most evident after 2 weeks of differentiation, concomitant with an increase in type X collagen expression, alkaline phosphatase activity and parathyroid hormone/parathyroid hormone-related protein receptor expression. On the basis of these temporally restricted changes in the sensing and transport of phosphate and calcium, we predict that extracellular phosphate plays a role in the commitment of chondrogenic cells to differentiation, whereas extracellular calcium plays a role at a later stage in their differentiation program.

Bones of the mammalian skeleton develop by two distinct mechanisms. Intramembranous bone, exemplified by the frontal and parietal bones of the cranial vault, is formed when mesenchymal stem cells differentiate directly into bone-forming osteoblasts. The bones at the base of the skull, including parts of the temporal and occipital bones, as well as the vertebral column and the appendicular skeleton, are formed by endochondral ossification. These “cartilage bones” are formed when mesenchymal precursors condense and differentiate into chondrogenic cells that deposit and mineralize a cartilage matrix (1), which is remodeled by catabolic cells (chondroclasts) brought in by the vasculature (2). The remodeled cartilage then forms the template on which bone is deposited by osteoblastic cells. The same process of endochondral ossification occurs in the adult during fracture healing. Although chondrocytes involved in endochondral ossification and those present in articular cartilage arise from the same lineage and are subject to similar regulatory mechanisms, those in articular cartilage do not proceed to mineralization competence under physiological conditions. The pathologic mineralization that occurs in disorders such as osteoarthritis has been attributed, in part, to dysregulation of pathways that maintain chondrogenic cells in a pre-mineralization state (3). The pathways that regulate chondrocyte maturation and matrix mineralization are linked to signaling by systemic hormones such as growth hormone, thyroid hormone, and insulin; matrix macromolecules such as collagen and proteoglycan; and locally derived factors such as insulin-like growth factor-1, transforming growth factor β, fibroblast growth factors (FGFs), and parathyroid hormone-related protein (PTHrP) (4). Defects in the genes encoding components of these signaling pathways, matrix molecules, or enzymes that remodel that matrix can lead to severe skeletal deformities that are collectively known as chondrodysplasia (5–8).

In cartilage destined to become bone, cells committed to chondrogenesis undergo a program of differentiation characterized by changing patterns of gene expression, matrix deposition and remodeling. For example, the major synthetic products of immature cells are type II collagen and sulfated proteoglycans, whereas differentiated cells express high levels of alkaline

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1 The abbreviations used are: FGF, fibroblast growth factor; CaSR, calcium-sensing receptor; CFK2, rat chondrogenic cell line; Glvr-1, gliob lastoma cell leukemia virus-1; Ihh, Indian hedgehog; Pit-1, Glvr-1, type III sodium-dependent phosphate transporter; Ram-1, type III sodium-dependent phosphate transporter; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; PTHrP, parathyroid hormone-related protein receptor; PBS, phosphate-buffered saline; FBS, fetal bovine serum; PCR, polymerase chain reaction; RT, reverse transcription; bp, base pair(s); Coll II, type II collagen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Phosphate and type X collagen prior to depositing mineral in the surrounding matrix. The changes in gene expression are accompanied by phenotypic alterations that include a progressive increase in cell size (hypertrophy) and the appearance of specialized outcroppings of the plasma membrane called matrix vesicles (9). Because of their central role in physiologic and pathologic mineralization, the biogenesis and release of matrix vesicles from chondrocytes has been a subject of intense investigation for more than 3 decades. Although the signals that initiate their budding and release from the cell remain undefined (10), they have been identified on proliferating and hypertrophic chondrocytes in vivo and shown to be released from both non-mineralizing and mineralizing chondrocytes in culture. Although many investigators believe that nucleation occurs exclusively in matrix vesicles (9), others have identified focal areas of mineralization in cartilage matrix in the absence of a surrounding vesicular membrane (11). Thus, the initial site at which mineral crystals are deposited in cartilage and the mechanisms that regulate their deposition remain a question of debate. Despite this uncertainty it is evident that, in vivo, matrix vesicles are concentrated in areas of cartilage that are destined to undergo mineralization and that they contain high levels of alkaline phosphatase, which is considered to be essential to generate inorganic phosphate from organic substrates (12). Additional studies suggest that proteoglycan can act as a focal nucleator for hydroxyapatite crystal formation, and that type II collagen cleavage by collagenase is a requisite step in the mineralization process, which allows for the release and accumulation of the C-propeptide (chondrocalcin), which binds avidly to hydroxyapatite (13).

Phosphate has long been recognized to play a central role in cartilage mineralization. In this context, its facilitated transport across the kidney tubule is necessary to maintain adequate circulating levels and its local concentration in cartilage is required for the initial phase of hydroxyapatite crystal formation. The homeostatic maintenance of extracellular phosphate is achieved primarily by re-absorption of phosphate in the proximal kidney tubule through the hormone- and phosphate-sensitive type II transporter (14). Inefficient re-absorption of phosphate by the kidney is associated with defective mineralization of the skeleton, which manifests as rickets in children and osteomalacia in adults. High doses of oral phosphate have been shown to be as effective as vitamin D in healing rachitic lesions in humans (15), as well as in rats (16) and mice (17). By adapting micropuncture techniques to sample cartilage lymph in the growth plates of rachitic chicks, it was shown that phosphate is preferentially sequestered relative to calcium in the zone of hypertrophic cells surrounded by unmineralized cartilage (18). These observations were supported by the work of Kakuta et al. (19), who suggested that regulation of the size of the inorganic phosphate pool may be the rate-limiting factor in cartilage mineralization. Furthermore, using a variety of different cell and organ culture systems, investigators have determined that the ideal medium for the culture of chondrogenic and osteogenic cells is high in phosphate and low in calcium, compared with conventional media, in which the opposite is true (20–23).

A type III sodium-dependent transport mechanism has been identified in chondrocytes and osteoblasts, as well as in other cells including those of the kidney epithelium. In 1991, the group of Caverzasio and Bonjour (24) identified and characterized a phosphate transport mechanism in matrix vesicles isolated from the growth plates of normal and rachitic chicks. The system was sodium-dependent, sensitive to hormone stimulation and phosphate deprivation, and up-regulated in the rachitic animals. The same group subsequently cloned the murine gene for gibbon ape leukemia virus (Glvr-1, now known as Pit-1) and demonstrated high levels of expression in the resting and proliferative zones of murine growth plates (25, 26). The amphotropic murine retrovirus receptor falls into the same family of type III phosphate transporters, although its regulation in skeletal tissues is less well defined (27). Taken together with evidence documenting the central role played by ambient phosphate in cartilage mineralization, these observations suggested that its focal accumulation inside the cell might also be necessary for chondrocyte differentiation and/or matrix mineralization.

Phosphate and calcium are the major constituents of the mineral phase of cartilage, bone, and dentin, which predicts that mechanisms for the regulated sensing and uptake of both ions might be present in the cells that are responsible for mineralizing these tissues. A cell surface calcium-sensing receptor (CaSR) was isolated and cloned from bovine parathyroid cells by Brown and colleagues in 1993 (28). The principal role of this polyvalent cation-sensing receptor lies in calcium homoeostasis, to transduce signals elicited by small changes in extracellular calcium into altered secretion of parathyroid hormone (PTH) by parathyroid cells. A CaSR is also expressed in cells lining the kidney tubule, where it can modulate renal cation handling (29). Naturally occurring inactivating and activating mutations in the CaSR gene cause familial hypercalciemic and hypocalcemic disorders, respectively (30). Calcium-sensing mechanisms have also been detected in cells other than parathyroid and kidney including neurons, pancreatic cells, keratinocytes, osteoblasts, and chondrocytes (31). The physiological roles that have been proposed for these sensors include promotion of mitogenic activity in osteoblasts (32) and stimulation of differentiation in keratinocytes (33). Recent evidence suggests that a calcium-sensing mechanism is also expressed in differentiated chondrocytes in rodent growth plates, where it may be involved in matrix mineralization (34).

The mechanisms that regulate chondrocyte function have been examined in primary cultures of growth plate cells by several different investigators (35–37). In the present work we have used a well characterized chondrogenic cell line, CFK2, to explore the potential roles of phosphate and calcium ions in regulating chondrocyte differentiation and cartilage maturation. The CFK2 and CFK1 cell lines were developed by limiting dilution and spontaneous transformation of cells released by collagenase digestion of fetal rat cranial bones. On the basis of morphological, histological and biochemical studies, the CFK2 cell line was designated chondrogenic and the CFK1 line osteogenetic (38). In view of their capacity to differentiate into hypertrophic chondrocytes under the appropriate culture conditions, the CFK2 cells have subsequently been used extensively as an in vitro model to investigate various aspects of chondrocyte biology at the cellular and molecular biochemical level (39–47).

In the present work, we demonstrate regulated expression and activity of the type III Glvr-1 phosphate transporter during early CFK2 differentiation in vitro, whereas expression and activity of a CaSR, which is responsive to changes in extracellular calcium, is up-regulated in differentiated CFK2 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**

All cell culture reagents were purchased from Life Technologies, Inc., and plasticware from Becton Dickinson Labware (Lincoln Park, NJ). Actinomycin D, cycloheximide, and colchicine, obtained from Sigma, were dissolved in 100% ethanol and diluted in culture medium to concentrations of 2 μg/ml actinomycin D, 50 μM cycloheximide, 0.3 and 0.5 μM colchicine. [32P]Orthophosphoric acid was obtained from PerkinElmer Life Sciences and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide from Sigma. All other reagents were of high chemical purity and purchased from standard laboratory suppliers.

**REFERENCES**

**EXPERIMENT**

**MATERIAL**

**METHOD**

**RESULT**

**CONCLUSION**

**IMPLICATIONS**

**ACKNOWLEDGEMENTS**

**REFERENCES**
**Methods**

**Cell Culture and Differentiation—**Cells were maintained in RPMI 1640, which contains 0.4 mM Ca\(^{2+}\) and 5 mM P\(_{\text{v}}\), supplemented with 10% FBS and antibiotic/antimycotic. Calcium was increased to the appropriate molar concentrations by addition of Ca\(^{2+}\), and the P\(_{\text{v}}\) decreased by diluting with phosphate-free RPMI 1640 at the indicated concentrations. Where indicated, CFFK2 cultures were treated with PTHrP-

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**Primer sequences and reference information**

| Name                        | S   | AS  | Sequence                                                                 | Reference   |
|-----------------------------|-----|-----|--------------------------------------------------------------------------|-------------|
| Type II collagen            | S   | AS  | 705CGACACTGCTCCTACCCGAGACAGT7050                                         | 74          |
| Type X collagen             | S   | AS  | 1103AGAGACACACGAGCTGCCAAAG7104                                           | Unpublished |
| Osteopontin                 | S   | AS  | 512TGGTGAAGCTTGTTAACAGGCT7120                                           | 75          |
| ALP                         | S   | AS  | 1183AGGCCAGGCTGACGAG7169                                                 | 66          |
| PTHIR                       | S   | AS  | 1603GCTGTTTCTGTCATGGA1620                                                 | 77          |
| Indian hedgehog             | S   | AS  | 1519GCGTCAGTCAGCCGCAA1529                                                 | 78          |
|                            |     |     | 1457GCTCTTCTGCTGAGAGA1492                                                |             |
|                            |     |     | 2021GCTGACAGGGAAGGTCTAT2038                                              |             |

a S, sense; AS, antisense.

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**Sodium-dependent Phosphate Transport—**Phosphate transport studies were performed as described previously (41) on confluent layers of CFFK2 cells subjected to the indicated culture conditions. Briefly, CFFK2 cells were seeded at a density of 10\(^4\) cells/well in 24-multwell plates and brought to confluence in RPMI with 10% FBS. Cultures were serum-starved overnight before subjecting them to phosphate deprivation and/or treatment with 2 \(\mu\)g/ml actinomycin D, 50 \(\mu\)g/ml cycloheximide, or 1 \(\mu\)g/ml colchicine for 2 h at 37 \(^o\)C. After rinsing three times with sodium-free buffer, P\(_{\text{v}}\) transport was initiated by the addition of 0.5 \(\mu\)M/well carrier-free \(^{32}\)P (orthophosphate (ICN Biomedicals, Costa Mesa, CA) in uptake solution at pH 6.4, 7.4, or 8.4 containing 150 mM NaCl, 1.0 mM CaCl\(_2\), 1.8 mM MgCl\(_2\), and 10 mM HEPES. There was no significant uptake of radiolabeled P, when the assay was performed in sodium-free buffer. P\(_{\text{v}}\) uptake was terminated after 5 min by the addition of 1 ml of ice-cold sodium-free uptake buffer. After extensive washing with the same buffer, the cell layers were solubilized with 0.5 ml of 2x NaOH and aliquoted for the determination of \(^{32}\)P. Counts were normalized to protein content using the Bio-Rad DC protein assay kit.

**In Situ Staining for Proteoglycan and Alkaline Phosphatase—**Replicate cultures of CFFK2 cells were plated at a density of 5 \(\times\) 10\(^4\) in 35-mm dishes and removed at confluence (time 0) and at weekly intervals up to 4 weeks. Cells were rinsed with PBS, fixed with fresh 4% paraformaldehyde in PBS for 30 min, and stained with Alcian Blue, to identify proteoglycan deposition, or with naphthol AS-MX phosphate and Fast Red Violet LB (Sigma) to measure alkaline phosphatase activity as described previously (47). Stained dishes were overlaid with a 5% glycerol in PBS and photographed on a Nikon Labophot-2 microscope with a UFX-DX photomicrographic attachment.

**RNA Extraction, RT-PCR, and RNase Protection Analysis—**Total RNA was harvested from CFFK2 cells at timed intervals and under the indicated culture conditions, and from the rat PT cell line (48) using a standard guanidine isothiocyanate/phenol chloroform protocol (49). Neonatal mouse kidney and growth plate cartilage were homogenized with a Polytron homogenizer (Brinkmann) and RNA isolated from the homogenates using the same protocol. All RNA samples were stored in distilled water at -80 \(^o\)C until the time of assay. 5–10 \(\mu\)g of total RNA was treated with 1–2 units of DNase (Ambion) and reverse transcribed for first strand cDNA synthesis, which was subsequently used to generate PCR products as described previously (50). Primer sequences used to identify chondrocyte differentiation-associated gene expression are listed in Table I, and those for phosphate transporters and the CaSR in Table II. PCR products were fractionated on 1.2% agarose gels, visualized with ethidium bromide, and the relative band intensities assessed by scanning densitometry using the ChemiImager 4000 (Alpha Innotech Corp., San Leandro, CA).

The PCR products, amplified using primers derived from Glvr-1, type II collagen (Col II), and GAPDH, were cloned into pCR2.1 plasmid by using the TA cloning kit from Invitrogen (Carlsbad, CA) and sequenced (Glvr-1) at the Sheldon Biotechnology Institute (McGill University, Montreal, Quebec, Canada) or subcloned into pBluescript KS (Stratagene) for use as probes in the RNase protection assays. After linearization of the vector, the antisense probes were in vitro transcribed with T7 polymerase in the presence of [\(^{32}\)P]UTP (ICN) using the MAXIscript kit (Ambion, Inc.) and purified using gel electrophoresis. Ribonuclease protection assays were performed using the RPA III kit (Ambion, Inc.). The antisense probes (80,000 cpm) were hybridized overnight at 42 \(^o\)C with 5 \(\mu\)g of total RNA, digested with the ribonuclease A/T1 mix for 30 min at 37 \(^o\)C, and then protected fragment resolved on a 6% polyacrylamide urea denaturing gel. The dried gels were exposed to x-ray film overnight at 80 \(^o\)C.

**Southern Blot Analysis of PCR Products—**The primers derived from mouse CaSR sequences were used to amplify products from mouse kidney tissue, from rat CFFK2 chondrocytes, and from the rat PT cell line. The products were resolved on 1.2% agarose gels and transferred onto Zeta-PROBE membrane (Bio-Rad), which was probed with an internal oligonucleotide labeled with \(^{32}\)P using the Roche Molecular Biochemicals 5 End-labeling kit. Southern hybridization was performed according to standard protocols with the probe corresponding to bases 1457CGATATCTCATACGTTACCTGCTCT1494 of the murine CaSR gene. After hybridizing at 42 \(^o\)C overnight, membranes were rinsed thoroughly in 2X SSC, washed in two changes of 2X SSC for 30 min at 42 \(^o\)C and at 56 \(^o\)C for 15 min before exposing to x-ray film at -80 \(^o\)C for 2 h.

**Phosphate and Calcium Handling in Chondrocytes**

| Name                      | S/AS | Reference |
|---------------------------|------|-----------|
| Osteopontin               | S/AS | 512TGGTGAAGCTTGTTAACAGGCT | 75         |
| ALP                       | S/AS | 1183AGGCCAGGCTGACGAG   | 66         |
| PTHIR                     | S/AS | 1603GCTGTTTCTGTCATGGA | 77         |
| Indian hedgehog           | S/AS | 1519GCGTCAGTCAGCCGCAA | 78         |

**Data are expressed as the mean ± S.E. of triplicate determinations and are representative of at least three independent experiments. Statistical significance was determined by Student’s t test.***
RESULTS

The pathways that regulate progressive changes in phenotype and gene expression in chondrogenic cells during differentiation remain poorly defined, although they are known to include systemic hormones, locally derived growth factors, and components of the cartilage matrix. Production of a mature mineralized cartilage matrix also requires the presence of adequate levels of phosphate and calcium for hydroxyapatite crystal formation in the cartilage microenvironment. Increasing evidence suggests that these ions may also play a direct regulatory role during chondrocyte differentiation. To test this hypothesis, we examined the regulated expression and activity of type III phosphate transporters and a CaSR in differentiating CFK2 chondrogenic cells.

Changes in Phenotype and Gene Expression in CFK2 Cells Undergoing Differentiation in Culture—Fig. 1A shows regions of the rodent skull and hindlimb undergoing endochondral ossification at 18.5 days post coitum. Sequential staining with alcian blue and alizarin red differentiates cartilaginous elements (blue) of the chondrocranium and the growth plates from mineralized bone (red), seen in the cranial vault and in the shafts of long bones. During endochondral ossification, cells of the chondrogenic lineage undergo a program of differentiation that is characterized by sequential changes in phenotype and in gene expression. As shown in Fig. 1B, this orderly sequence of events is recapitulated by CFK2 chondrogenic cells when they are maintained for several weeks in a postconfluent state. Fig. 2 shows intense, widespread staining for proteoglycan at 2 weeks of differentiation (upper panels), whereas alkaline phosphatase activity is restricted to isolated clusters of cells undergoing differentiation (lower panels). At 4 weeks of differentiation, alcian blue staining is restricted to the periphery of differentiated nodules of cells, whereas alkaline phosphatase activity is more widespread and greatly increased in intensity in the focal areas of differentiated chondrocytes.

Total RNA was harvested for RT-PCR analysis from cells cultured in parallel with those used for in situ staining, shown in Fig. 2. Fig. 3A shows the PCR products resolved on agarose gels that were amplified with primers derived from genes encoding collagenous and non-collagenous proteins associated with chondrocyte differentiation. Fig. 3B shows the results of densitometric analysis of the band intensities relative to that for GAPDH. Coll II is the major collagenous protein expressed by prehypertrophic chondrocytes in vivo. Its expression in CFK2 cells peaked at 2 weeks of differentiation, concomitant with peak staining for proteoglycan (Fig. 2), and declined thereafter. Collagen X (type X collagen) is a recognized marker for hypertrophic chondrocytes in vivo. Its expression showed a dramatic increase in 3- and 4-week-old cultures, coincident with the appearance of circumscribed nodules of cells that stained intensely for alkaline phosphatase (Fig. 2). Osteopontin reached its peak level of expression at 2 weeks, whereas the progressive increase in alkaline phosphatase was reflected in the increase in its activity shown in Fig. 2. These expression profiles are consistent with the predicted in vivo roles of osteopontin in the earliest stages of matrix mineralization and of alkaline phosphatase in later stages of mineral deposition.

Papers:

- Table II: Primer sequences for phosphate transporters and calcium-sensing receptor

| Name   | S/AS | Sequence | Product size | Reference |
|--------|------|----------|--------------|-----------|
| Glvr-1 | S    | 1463CCTGAAGGAGGAGACCAGCA | 539 | Unpublished data |
| Ram-1  | S    | 4015TGAACCGCATGATTCTCTG | 619 | 79 |
| NaPi-1 | S    | 224GACAGTTCTTCTACCCACAA | 619 | 62 |
| NaPi-2 | S    | 1440CTGGTGGTCTTAAACCTGCA | 228 | 62 |
| CaSR   | S    | 1553CTATGTGTTATGCTTCCAG | 257 | Unpublished data |
| GAPDH  | S    | 1651CTTGAGTGTCAGTGCCCCTA | 496 | 80 |

a S, sense; AS, antisense.
b NaPi-1 and NaPi-2, sodium P, transporters type 1 and 2, respectively.

dent's t test, or analysis of variance, with a probability of p < 0.05 taken to be statistically significant.

| In Vivo Bone Growth | Time in Culture | Differentiation Markers |
|---------------------|-----------------|-------------------------|
| Committed Progenitor | 0 Time          | Collagen II              |
| Proliferation        | 0-2             | Protoproglycan           |
| Transition           | 2-3             | Alk Phos                 |
| Differentiated nodules | 3-4           | Collagen X               |

Fig. 1. Endochondral ossification and the CFK2 chondrogenic model. A, the skeleton of a wild type fetal rat, removed by caesarian section at 18.5 days post coitum (dpc), was stained with alizarin red and alcian blue to differentiate cartilage (blue, *) from bone (red). The cartilaginous template on which endochondral bone will be formed can be seen at the base of the skull and in the growing plates of the femur (f) and tibia (t). B, the sequence of changes in phenotype and gene expression observed in CFK2 cells maintained in postconfluent culture for 4 weeks.
proliferation, rather than in differentiation. This series of experiments established the time frame for differentiation-associated alterations in phenotype and gene expression in our CFK2 model and provided the context in which to look for regulated expression of genes encoding phosphate and calcium sensors/transporters.

**Expression of Sodium-dependent Phosphate Transporters in CFK2 Cells**—Many investigators believe that initiation of cartilage mineralization is regulated by prehypertrophic chondrocytes and that the process may be driven by phosphate accumulation within the cell. We therefore examined expression levels for phosphate transporters in CFK2 cells over the 4-week period of differentiation. Using primers derived from the rodent genes for type I and type II sodium-dependent phosphate transporters, the expected 619- and 228-bp products were amplified from mouse kidney but not from total RNA harvested from undifferentiated or differentiated CFK2 cells (data not shown). In contrast, Fig. 4A demonstrates that products of 539 and 619 bp were amplified from CFK2 cells throughout differentiation with primers derived from the genes encoding the type III transporters, Glvr-1 and Ram-1, respectively. A transient increase in the expression of both genes was observed at 1 week of differentiation, which preceded maximum expression of any of the differentiation markers shown in Fig. 3. The RT-PCR signals for Glvr-1 and Ram-1 were normalized to those for GAPDH and expressed as a ratio. Phosphate transport activity was also assessed at 1 week of differentiation by incubating the cells for 5 min in transport medium containing 150 mM NaCl and 2 μCi/ml [32P]orthophosphate at varying pH, or at pH 7.4 in the presence or absence of additional extracellular phosphate (P).

In C, it can be seen that sodium-dependent phosphate uptake by CFK2 cells was decreased at alkaline pH and increased at acidic pH. D shows a significant increase in phosphate transport activity within 5 min of phosphate deprivation. Results are expressed as percentage of change from control (pH 7.4 in B and time 0 in C) and represent the mean ± S.E. of measurements from triplicate wells. The data are representative of four different assays. *, *p < 0.05; **, **p < 0.01 compared with control.

**CFK2 Cells**—Many investigators believe that initiation of cartilage mineralization is regulated by prehypertrophic chondrocytes and that the process may be driven by phosphate accumulation within the cell. We therefore examined expression levels for phosphate transporters in CFK2 cells over the 4-week period of differentiation. Using primers derived from the rodent genes for type I and type II sodium-dependent phosphate transporters, the expected 619- and 228-bp products were amplified from mouse kidney but not from total RNA harvested from undifferentiated or differentiated CFK2 cells (data not shown). In contrast, Fig. 4A demonstrates that products of 539 and 619 bp were amplified from CFK2 cells throughout differentiation with primers derived from the genes encoding the rodent type III transporters, Glvr-1 and Ram-1, respectively. A transient increase in the expression of both genes was observed at 1 week of differentiation, which preceded maximum expression of all markers of differentiation, including type II collagen (see Fig. 3). The RT-PCR results were confirmed by performing RNase protection analyses, shown in Fig. 4B. Fig. 4 (C and D) depicts sodium-dependent phosphate transport activity in CFK2 cells at 1 week of differentiation, measured as uptake of [32P]orthophosphate into the cell layer. Transport activity,
which was corrected for protein content of the cells, was increased in an acidic environment (Fig. 4C) and also by an acute decrease in the concentration of extracellular phosphate (Fig. 4D).

Regulation of Type III Phosphate Transporter Expression and Activity in CFK2 Cells—Type III transporters are expressed in a wide variety of tissues, where their physiological role in sodium-dependent phosphate transport remains poorly defined, although recent reports suggest that Glvr-1 may be involved in osteoblast and chondrocyte function. Taken together with our data indicating that their expression is upregulated during early differentiation in CFK2 cells, we proceeded with an in depth evaluation of Glvr-1 expression and activity in response to changes in extracellular phosphate. The PCR product was first confirmed to be identical to a segment of the mouse Glvr-1 gene.

The rapidity with which phosphate transport was modulated (within minutes) following a reduction in extracellular phosphate suggested that the effect was not mediated through transcriptional or translational mechanisms, but rather by recruitment of functional transporters from an intracellular pool. This hypothesis was tested by the experiment presented in Fig. 5A, which shows that pretreatment of the cells with an inhibitor of transcription (actinomycin D) or of translation (cycloheximide) failed to alter the early increase in transport activity, whereas pretreatment with colchicine, shown in Fig. 5B, completely abrogated the response. This colchicine-sensitive increase in phosphate transport activity was not accompanied by any change in Glvr-1 mRNA expression and appeared to be maintained throughout the 24-h time frame of the experiment (data not shown). Fig. 5C shows the result of a tetracium-based metabolic assay, which was used to ensure that the decrease was not an artifact due to altered metabolic activity induced by shape change in the colchicine (microtubule inhibitor) treated cells. No difference was observed in the metabolic activity between untreated and colchicine-treated cultures.

Sodium-dependent phosphate transport in CFK2 chondrogenic cells, therefore, appeared to be regulated by inorganic phosphate in a maturation stage-dependent manner at a post-transcriptional level. It has been established that relatively high concentrations of extracellular phosphate are necessary for initiation of chondrification mineralization in vivo and in vitro. Fig. 6 provides confirmation of the physiological relevance of extracellular phosphate to CFK2 differentiation. Chronic (24-h) exposure of cells at time 0 and at 1 week of differentiation to graded reductions in extracellular phosphate resulted in concomitant, dose-dependent decreases in Glvr-1 and type II collagen expression during early differentiation but not in undifferentiated cells, as shown in Fig. 6 (A–C). This stage-specific response to phosphate depletion was reflected in transport activity, shown in Fig. 6D, which was increased following chronic phosphate deprivation at 1 week but not at time 0. Further support for the physiological relevance of adaptive phosphate transport during chondrocyte differentiation is the data presented in Fig. 7. PTHrP has been shown to stimulate the proliferation of chondrogenic cells and to inhibit their passage from a proliferative to hypertrophic phenotype in vivo (52, 53) and in vitro (40, 41). We now show that treatment of CFK2 cells at 1 week of differentiation with increasing concentrations of amino-terminal PTHrP resulted in a dose-dependent decrease in the expression (Fig. 7A) and activity (Fig. 7B) of Glvr-1, and in the expression of type II collagen, in phosphate-deprived cultures. These results suggest that facilitated transport of phosphate into chondrocytes is not only stage-specific but is also subject to regulation by a factor that plays a pivotal role in chondrocyte maturation and endochondral bone formation.

Expression of CaSR in Differentiating CFK2 Cells—Whereas phosphate ions may be instrumental in driving chondrocytes through the early stages of differentiation, it is self-evident that calcium ions must also play a major role in hydroxyapatite crystal formation in more mature cells. Recent reports have suggested that mature chondrocytes have a calcium-sensing mechanism that may play a role in matrix mineralization. Fig. 8A shows a 257-bp PCR product amplified from CFK2 cells using intron-spanning primers derived from the mouse CaSR gene sequence. The band reached a maximum intensity at 2 weeks of differentiation and was maintained at a high level thereafter. Fig. 8B shows the results of Southern blot analysis of PCR products amplified from mouse kidney tissue and rat
CFK2 and PT cells with the same primer pair. An internal oligonucleotide probe annealed under stringent hybridization conditions to the 257-bp products, which were expressed in kidney and to a much lesser extent in CFK2 and PT cells. The result of immunoblot analysis of total lysate prepared from rat tissues and cells is shown in Fig. 8C. The affinity-purified antiserum, raised against a sequence in the extracellular domain of the CaSR, recognized proteins between 120 and 160 kDa, which represent nonglycosylated and glycosylated monomeric forms (54), as well as multimeric forms greater than 200 kDa in rat parathyroid and kidney. A predominant species of 120 kDa was detected in CFK2 cell lysates. To determine at what stage of differentiation the CFK2 calcium-sensing mechanism was responsive to changes in extracellular calcium, the cells were exposed to graded increments in calcium for 24 h at time 0 and at 2 weeks of differentiation, when expression of the CaSR transcript reached a maximum. The data in Fig. 9A show that maximum sensitivity to extracellular calcium occurs at 2 weeks of differentiation and peaks at a concentration of 1.4 mM calcium. Fig. 9B shows that calcium influx into the cell layer, measured by the uptake of $^{45}$Ca, was also regulated by the concentration of extracellular calcium at 2 weeks of differentiation, which reinforces the hypothesis that calcium regulates its own import into differentiating chondrocytes. Type X collagen expression was also up-regulated by extracellular calcium at 2 weeks of differentiation in a dose-dependent manner.

**DISCUSSION**

During endochondral bone formation, cells committed to the chondrogenic lineage undergo a program of differentiation during which they manufacture, remodel and mineralize an extensive extracellular matrix. Articular chondrocytes undergo a similar, but restricted, program of differentiation that does not proceed to the mineralization stage, except in pathologic states such as osteoarthritis. Many years of intensive research have shown that cartilage mineralization is mediated by chondrocytes through complex pathways linked to signaling by growth factors, cytokines, and extracellular matrix proteins, as well as by the availability of phosphate and calcium for hydroxyapatite crystal formation. An extensive literature documents the potential mechanisms that regulate the later stages of chondrocyte differentiation and matrix mineralization. However, the regulatory pathways that mediate the commitment of these cells to a hypertrophic, differentiated phenotype remain poorly defined. To better understand the roles played by the regulated transport of phosphate and calcium into chondrogenic cells during cartilage maturation, we used the CFK2 cell line to...
sequences encoding CaSR. RT-PCR analysis using intron-spanning primers derived from mouse same RNA samples that were used for Figs. 2 and 3 were subjected to pendent experiments. was raised (data not shown). Results are representative of three inde-

The bands were demonstrated to be specific by staining a replicate blot with the same antiserum preadsorbed with the peptide against which it was raised (data not shown). Results are representative of three independent experiments.

explore changes in expression and activity of a type III phosphate transporter and a calcium-sensing mechanism during chondrocyte differentiation. Although the regulated transport of inorganic phosphate into CFK2 cells was transiently up-regulated at the time of commitment to terminal differentiation, facilitated calcium transport was increased at a later time point. In addition to predicting a regulatory, as well as a permissive, role for phosphate and calcium in chondrocyte maturation and matrix mineralization, these studies suggest that dysregulation of phosphate transport into chondrogenic cells could contribute to the pathogenesis of defective cartilage mineralization.

Clonal Cell Lines as Models for Chondrogenesis and Matrix Mineralization—The spontaneous maturation of CFK2 cells from a chondroprogenitor to a differentiated chondrocyte phenotype was first demonstrated in 1993 using molecular markers of differentiation that included type I and type II collagen, link protein, and aggrecan (39). After 2 weeks of postconfluent culture, the cells formed focal nodules of polygonal cells that stained heavily for collagen II and proteoglycan and exhibited a brisk cAMP response to PTH-(1–34). Furthermore, six consecutive passages of the differentiated cells were sufficient to return them to the chondroprogenitor phenotype. The dramatic increase in PTH-sensitive adenylate cyclase activity was later shown to be a function of up-regulation of the PTH1R at 2 weeks of differentiation (41). These cells have subsequently been used by a number of different investigators under a vari-

eity of culture conditions, primarily to investigate stage-specific alterations in signaling by growth factors and cytokines. Other useful cell lines that have been developed recently for the investigation of chondrogenesis and matrix mineralization include ATDC5, which was derived from a mouse embryonal carcinoma (55, 56) and the conditionally immortalized cell lines derived from the growth plate of fetal PTH1R-null mice (57, 58). To date these cells have been used primarily to examine the later stages of chondrocyte differentiation and matrix mineralization, rather than the transition to hypertrophy and terminal differentiation.

In vitro, chondrocyte differentiation is characterized phenotypically by the adoption of a non-proliferative, hypertrophic phenotype and by a switch from primarily type II collagen and sulfated proteoglycan synthesis to expression of high levels of alkaline phosphatase, type X collagen, and calcium-binding proteins such as osteocalcin and collagen II C-propeptide (2). Sequential changes in phenotype and gene expression have been successfully monitored in several in vitro systems using primary chondrocytes harvested from rabbit (37), chick (35), and bovine tibia (36). Changing patterns of gene expression and matrix composition were also recapitulated in our CFK2...
chondrogenic model, where maximum expression of type II collagen and staining for proteoglycan occurred at 2 weeks. This was in contrast to markers of the fully differentiated phenotype such as type X collagen, alkaline phosphatase, and osteopontin, which were poorly expressed until after 2 weeks of culture. Ihh has been proposed to act as a negative regulator of chondrocyte differentiation in the growth plate through a long distance feedback loop that involves PTHrP (59, 60). In the current work, Ihh expression was significantly down-regulated by 2 weeks of differentiation, whereas expression of PTH1R was abruptly up-regulated at this time. These observed reciprocal changes in Ihh and PTH1R expression raise the possibility that PTH1R, as well as PTHrP per se, could be a direct molecular target for Ihh signaling and that repression of PTH1R by Ihh could represent an alternative mechanism whereby Ihh inhibits chondrocyte differentiation. In a recent report by Guo et al. (58), a similar panel of molecular markers was used to distinguish between the morphologically distinct hC1–5 and nhC2–27 cell lines that had been established from the growth plates of PTH1R mutant mice. Type II collagen and Ihh were well expressed in the nonhypertrophic nhC2–27 cells, whereas type X collagen, alkaline phosphatase, and osteopontin were absent from nhC2–27 but strongly expressed in the hypertrophic hC1–5 cells. The alterations in gene expression over the time course of CFK2 maturation were also accompanied by a progressive change in phenotype from undifferentiated chondroprogenitor cells to focal condensations of rounded chondrocytes that stained heavily for proteoglycan and alkaline phosphatase. Taken together, these studies exemplify the usefulness of clonal cell lines and in vitro markers of chondrocyte maturation to study the regulation of chondrogenesis and matrix mineralization in vitro.

Stage-specific Changes in Type III Phosphate Transport in Chondrogenic Cells—The type III sodium-dependent phosphate transporters are expressed in numerous tissues, including kidney, bone, and cartilage, where they appear to be differentially regulated. They have a higher affinity for phosphate than the renal type II transporter, which may account for their preference for an acidic environment (61). In MDCT mouse kidney distal tubular cells, the activity of type III transporters was enhanced in an acidic environment and their expression level was up-regulated by a chronic reduction in extracellular phosphate (62). Similar adaptive responses to pH and phosphate deprivation were seen in CFK2 cells at the time of commitment to terminal differentiation, as evidenced by the expression of the recognized molecular markers described above. Guicheux et al. examined Glvr-1 expression and activity in the previously characterized ATDC5 chondrogenic cell line derived from a mouse teratocarcinoma (63). Similar to our observations in CFK2 cells, Glvr-1 expression in intact ATDC5 cells was maximal prior to their transition to a fully differentiated phenotype and its activity was inhibited in an alkaline environment. In contrast to CFK2 cells, in which Glvr-1 expression and activity were transiently elevated at 1 week of differentiation, maximum sodium-dependent phosphate transport matrix vesicles isolated from ATDC5 peaked after 3 weeks of differentiation, concomitant with the onset of type X collagen expression. This difference may reflect an intrinsic difference in sodium-dependent transport in cells derived from the rat chondrocranium and from a mouse teratocarcinoma or a difference in the phosphate transporters expressed in the cell membrane and in the matrix vesicle membrane. Alternatively, mature chondrocytes may express yet another phosphate transporter, as was suggested by Mansfield et al. (64), who linked an increase in intracellular phosphate to apoptosis of terminally differentiated primary chick chondrocytes during matrix mineralization. Despite demonstrating the presence of both type III (Glvr-1) and type II transporters in these cells, it was concluded that they most probably did not mediate the rise in intracellular phosphate in cells cultured in high phosphate medium. An apparent discrepancy between maximal Glvr-1 expression and activity has also been noted in MC3T3 osteoblastic cells. Similar to our own observations, sodium-dependent transport was transiently elevated in MC3T3 cells prior to the onset of mineralization (65), whereas Glvr-1 mRNA expression increased concomitant with osteocalcin expression and matrix mineralization (66). Of additional interest to the current discussion is the fact that a transient increase in Glvr-1 expression was noted in MC3T3 cells 3 days after seeding at high density, which corresponds to an early time point in the differentiation of these cells. Taken together, this work documenting time-dependent alterations in the expression and activity of sodium-dependent phosphate transporters in cells destined for matrix mineralization support a biphasic role for Glvr-1, first during the commitment to terminal differentiation and second during matrix mineralization.

In addition to the adaptive response to extended phosphate deprivation, an acute increase in sodium-dependent phosphate transport was noted in CFK2 cells within minutes of the withdrawal of phosphate from the culture medium. The rapidity of the response and its sensitivity to colchicine, which binds to tubulin, led to the conclusion that the activity was a function of the retrieval of functional transporters from an intracellular pool. This acute response to phosphate deprivation is characteristic of the type II transporters that are expressed primarily in the kidney proximal tubule, where they act as the major regulators of systemic phosphate homeostasis (14). Its presence in CFK2 chondrocytes at a restricted point in their differentiation program implies a functional necessity to maintain the level of intracellular phosphate in the face of fluctuating extracellular levels at this time. Given the temporal relationship between the increase in Glvr-1 activity and alterations in markers of chondrocyte maturation, the observations support the hypothesis that intracellular accumulation of inorganic phosphate is related to the onset of terminal differentiation in chondrogenic cells.

Potential Role of Facilitated Phosphate Transport in Chondrogenic Cells—The mechanisms that have been proposed for initiation of cartilage mineralization include the biosynthesis of matrix vesicles as nucleation sites, the removal of inhibitory compounds such as pyrophosphate, and the accumulation of calcium and phosphate ions. The transient increase in Glvr-1 expression and activity in maturing CFK2 chondrocytes could ultimately impact on each of these mechanisms, as well as meeting the additional metabolic needs of a rapidly growing cell. In this context, the type III sodium-dependent phosphate transporters were originally thought to be constitutively active, to accommodate the continuous requirements for inorganic phosphate in metabolically active cells (67). Thus, it is reasonable to expect that chondrocytes undergoing hypertrophy, which are situated in a relatively avascular and anoxic environment, might respond to small changes in the ambient phosphate with an increase in transport activity, to ensure a steady supply during this time of increased need. However, this does not explain the restricted time during which adaptive phosphate transport occurs, or its regulation by factors like PTHrP that are known to influence chondrocyte maturation.

In addition to the general increase in biosynthesis and metabolism that occurs during the transition to hypertrophy, this stage marks the onset of hydroxypatite crystal deposition in matrix vesicles adjacent to the longitudinal cartilage septa (9). It has been demonstrated both in vivo and in vitro that matrix
vesicles arise from the membranes of late proliferative and early hypertrophic chondrocytes, which do not mineralize their surrounding matrix (10, 68). Although the phospholipid membrane of these non-mineralizing vesicles appears similar to that of vesicles derived from terminally differentiated, mineralizing cells, they appear qualitatively different in their content of alkaline phosphatase and annexin V which mediates the influx of calcium prior to hydroxyapatite precipitation (35). It is interesting to note that, in previous work, annexin V mRNA was shown to be transiently up-regulated in CFK2 cells during their commitment to differentiation (43). The observations lend support to the previous suggestion regarding qualitative differences in sodium-dependent phosphate transport in matrix vesicles derived from immature versus mature chondrocytes. Thus, the transient increase in intracellular phosphate in transitional chondrocytes could be to meet the initial requirements for matrix vesicle biosynthesis. It is, however, surprising that the increased level of transport is not maintained to accommodate the continuous increase in matrix vesicles as the cells continue to differentiate. An alternative hypothesis worth considering is that the newly synthesized vesicles must be pre-loaded with phosphate before calcium loading and release from the parent cell. This conjecture is supported by the current work, as well as that of Montessuit et al. (24), who examined the regulated transport of radiolabeled phosphate and calcium into vesicles isolated from the growth plates of normal and rachitic chickens. The first significant observation in their studies was that sodium-dependent phosphate transport was up-regulated in vesicles from the rachitic birds, which was attributed to the fact that they represented a less mature species of matrix vesicle. The second major finding was that a relatively slow, steady increase in phosphate transport preceded a sharp incline in phosphate and calcium uptake. Taken together with our own observations that accumulation of intracellular phosphate precedes that of calcium, these data suggest a critical role for the accumulation of intracellular phosphate during matrix maturation.

In addition to producing mineralization competent vesicles, mature chondrocytes must generate a matrix that is conducive to mineralization. Conversely, premature precipitation of hydroxyapatite crystals must be prevented in the chondrocytes and matrix of immature cartilage. This is accomplished in part by the intracellular and extracellular enzymatic conversion of ATP to inorganic pyrophosphate (69). In chondrogenic cells, intracellular pyrophosphate is released along with type II collagen, or is generated by cell surface enzymes from ATP released from the cell (70). Thus, the regulated production and release of inorganic pyrophosphate prevents the premature precipitation of hydroxyapatite in matrix vesicles and inhibits the outgrowth of preformed crystals in the matrix. It is possible that the transient increase in intracellular phosphate in CFK2 cells at 1 week of differentiation is required for oxidative phosphorylation and ATP production to meet this demand for pyrophosphate to inhibit hydroxyapatite precipitation.

Regulation of Type III Phosphate Transport by PTHrP in CFK2 Cells—In vivo, Ihh has been identified as a negative regulator of chondrocyte differentiation through a mechanism that involves PTHrP (59, 60). Amino-terminal fragments of PTH and PTHrP, via their action on PTH1R, have been shown to stimulate the proliferation of chondrogenic cells and to either inhibit or stimulate their differentiation in a stage-specific manner (71). In the current work we showed that Glvr-1 was transiently up-regulated during early differentiation in CFK2 cells, when PTH1R expression was low and Ihh expression high. A reciprocal decrease in Ihh and an increase in PTH1R expression occurred between 1 and 2 weeks. PTH1R is also up-regulated in the transition zone of chondrocytes in fetal mouse growth plates in vivo (72). The temporal relationship between up-regulation of Glvr-1 activity and subsequent changes in gene expression of differentiation markers led us to examine the regulation of this transporter by amino-terminal PTHrP. In view of the literature that documents a stimulatory role for PTH in sodium-dependent phosphate transport in skeletal cells (61), our observation that physiologic concentrations of PTHrP-1 (34) dose-dependently inhibited the adaptive response of CFK2 cells to phosphate deprivation was somewhat surprising. However, if the maintenance of an intracellular phosphate pool is necessary for the onset of terminal differentiation as discussed above, and PTHrP inhibits terminal differentiation, then our observations that PTHrP inhibits adaptive phosphate transport by Glvr-1 as well as expression of type II collagen seem less surprising. They also support the observations of other investigators (55), who noted a marked decrease in differentiation of ATDC5 chondrogenic cells cultured in the presence of amino-terminal PTHrP. Of particular interest in the later studies was the observation that PTHrP production by these cells apparently preceded expression of PTH1R and decreased throughout the differentiation period, whereas PTH1R expression increased over the same time frame. Taken together, these observations suggest a biphasic mechanism of PTHrP action in chondrogenic cells, with a relative abundance of ligand over receptor during the commitment to differentiation and the converse being true at a later stage of development. In fact, these findings could account for some of the previously reported discrepancies in the literature regarding the effect of PTHrP on chondrocyte differentiation. Whether or not the inhibitory influence of PTHrP on Glvr-1 is direct, or mediated indirectly by inhibition of FGF signaling remains to be defined, although preliminary work supports an indirect effect (46, 73).

Potential Role of a Calcium-sensing Mechanism in Chondrogenic Cells—Polyvalent cation-sensing mechanisms have been identified in diverse tissues, including skin, where extracellular calcium has been shown to regulate biological function in a tissue-specific manner. Keratinocytes in culture express a receptor homologous to CaSR, which is up-regulated in a differentiation-dependent manner. The observation that these cells respond to an increase in extracellular calcium by stimulating calcium influx through the cell membrane (33) is consistent with the predicted role of increased calcium in keratinocyte differentiation. A CaSR was also identified in hypertrophic growth plate chondrocytes and in C5.18 chondrogenic cells (34). Chronic exposure of the C5.18 cells to high extracellular calcium also results in increased intracellular calcium, which is accompanied by an apparent decrease in differentiation. In the present studies a CaSR transcript was identified by RT-PCR, and the receptor protein by immunoblot analysis, in the well characterized CFK2 chondrogenic cells. The predominant CFK2 CaSR species was a monomeric, non-glycosylated form, which is in contrast to the abundant glycosylated species in kidney and parathyroid. Expression and activity of the calcium-sensing mechanism appeared to be optimal in 2-week differentiated CFK2 cell cultures in the presence of 1.4 mM extracellular calcium, which is the value reported for cartilage lymph extracted from the hypertrophic zone of mineralizing chick growth plates (18). In contrast to C5.18 cells, where type X collagen was decreased by chronic exposure to high levels of extracellular calcium (34), type X collagen expression was up-regulated by calcium in a dose-dependent manner in differentiated CFK2 cells. This discrepancy most probably arises from the difference in the cell culture models, the stage in their differentiation program at which they were treated, and also
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The length of time to which they were exposed to high calcium. In summary, we have demonstrated regulated expression of a type III phosphate transporter and a CaSR in differentiating CFK2 chondrogenic cells. The regulated influx of phosphate into CFK2 cells was coincident with changes in phenotype and gene expression that are associated with the commitment of chondrocytes to terminal differentiation in vivo. Regulated expression and activity of a CaSR was also evident in CFK2 cells at a later stage of differentiation. Taken together, our observations predict a role for phosphate in the commitment of chondrocytes to mineralization competence, whereas regulated calcium sensing may be required at a later stage for the precipitation of calcium phosphate as hydroxyapatite crystals in matrix vesicles.

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REFERENCES

1. Jee, W. S. S. (1983) in Histology Cell and Tissue Biology (Weiss, L., ed.) Vol. 5, pp. 251–276, Alan R. Liss, New York.
2. Poole, A. R., Laveret, S., and Mwale, F. (2000) in The Osteoporosis Primer (Henderson, J. E., and Goltzman, D., eds) pp. 3–17, Cambridge University Press, Cambridge, United Kingdom.
3. Terkelson, R., M., Johnson, K., Deng, D., Hashimoto, S., Goldenberg, M. B., Burton, D., and Defos, L. J. (1998) Arthritis Rheum. 41, 2152–2164
4. Henderson, J. E., and Goltzman, D. (2000) in The Osteoporosis Primer (Henderson, J. E., and Goltzman, D., eds) pp. 113–129, Cambridge University Press, Cambridge, United Kingdom.
5. Warman, M. L., Abbott, M., Apte, S. S., Hefferdon, T., McIntosh, I., Cohn, D. H., Hecht, J. T., Olson, B. R., and Francomano, C. A. (1993) J. Cell. Biol. 120, 79–82.
6. Tavormina, P. L., Shiang, R., Thompson, L. M., Zhu, Y. Z., Wilkinson, D. J., Lachman, R. S., Wilcox, W. K., Rimon, D. L., Cohn, D. H., and Wasmuth, J. J. (1995) Nat. Genet. 10, 321–328.
7. Schipani, E., Kruse, K., and Jumper, H. (1995) Science 268, 98–101.
8. Yao, Y., Shippley, J., M., Bergers, G., Berger, J. E., Helms, J. A., Brown, H., Bonjour, J., and Caverzasio, J. (1998) Cell 95, 411–422.
9. Henderson, J. E. (1999) Clin. Orthop. Rel. Res. 314, 266–280.
10. Glaser, J. H., and Conrad, H. E. (1981) J. Biol. Chem. 256, 12607–12611.
11. Gerstenfeld, L., and Landis, W. J. (1991) J. Cell. Biol. 112, 501–513.
12. Poole, A. R. (1991) in Cartilage: Molecular Aspects (Hall, B., and Newman, S., eds) pp. 179–211, CRC Press, Boca Raton, FL.
13. Billinburth, R., Dahlberg, L., Innesco, M., Reiner, A., Bourke, A., and Horacek, M. (1983) J. Clin. Endocrinol. Metab. 57, 260–273.
14. Karp, S. J., Schipani, E., Reader, C. R., Reiner, A. M., Goldenberg, R. H., and Rosenbrough, A. (1972) N. Engl. J. Med. 287, 481–487.
15. Simmons, D. J., and Kunin, A. (1970) Clin. Orthop. Rel. Res. 85, 252–260.
16. Marie, P. J., and Glorieux, F. H. (1981) Clin. Endocrinol. 67, 911–914.
17. Mitchell, P., and Hambor, J., Diekmann, O., Tschesche, H., Chen, J., and Miller, A. D. (1994) J. Biol. Chem. 269, 2753–2763.
18. Howell, D., Pita, J., Marquez, J., and Madruga, J. (1968) J. Clin. Invest. 47, 142–145.
19. Johnson, K., Vaingankar, S., Chen, Y., Moffa, A., Goldring, M. B., Sano, K., and Friedman, P. A. (1994) J. Cell Biol. 124, 1827–1835.
20. Karp, S. J., Reiner, A. M., Goldenberg, R. H., and Rosenbrough, A. (1972) N. Engl. J. Med. 287, 481–487.
21. Howell, D., Pita, J., Marquez, J., and Madruga, J. (1968) J. Cell Biol. 47, 142–145.
22. Mitchell, P., Diekmann, O., Tschesche, H., Chen, J., and Miller, A. D. (1994) J. Biol. Chem. 269, 2753–2763.
23. Johnson, K., Vaingankar, S., Chen, Y., Moffa, A., Goldring, M. B., Sano, K., and Friedman, P. A. (1994) J. Cell Biol. 124, 1827–1835.
24. Krebsbach, P. H., Nakata, K., Bernier, S. M., Hatano, O., Rhodes, C. S., and Caverzasio, J. (1994) J. Biol. Chem. 269, 2721–2728.
25. Palmer, G., Manen, D., Bonjour, J., and Caverzasio, J. (2000) Bone 26, 430–433.
26. Glaser, J. H., and Conrad, H. E. (1981) J. Biol. Chem. 256, 12607–12611.
27. Gerstenfeld, L., and Landis, W. J. (1991) J. Cell. Biol. 112, 501–513.
28. Brown, E. M., Gamba, G., Lombardi, M., Rorabeck, C. H., and Bonjour, J. P. (1991) J. Biol. Chem. 266, 575–580.
29. Bapty, B. W., Dai, L.-J., Ritchie, G., Jurik, F., Canaff, L., Hendy, G. N., and Quamme, G. A. (1998) Kidney Int. 53, 583–592.
30. Hendy, G. N., D’Souza, L., Yang, B., Canaff, L., and Cole, D. E. C. (2000) Hum. Mutat. 16, 281–296.
31. Yamaguchi, T., Tsuchiyadaiy, N., and Brown, E. M. (2000) Advances in Pharmacology 47, 209–252.
32. Pin, M., Gamba, G., Puthney, R., and Quamme, G. A. (2000) J. Biol. Chem. 275, 3256–3263.
33. Bock, D., Ratnam, A., Mauro, T., Harris, J., and Piliari, S. (1999) J. Cell. Biol. 138, 1085–1093.
34. Chang, W., Tu, C., Bajra, R., Komuevs, L., Miller, S., Strewler, G., and Shoback, D. (1999) Endocrinology 140, 1911–1919.
35. Kirsch, T., Nah, H.-D., Shapiro, I., and Pacitti, M. (1997) J. Cell. Biol. 138, 1085–1093.
