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# Dietary zinc reduces osteoclast resorption activities and increases markers of osteoblast differentiation, matrix maturation, and mineralization in the long bones of growing rats☆

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**Abstract**

The nutritional influence of zinc on markers of bone extracellular matrix resorption and mineralization was investigated in growing rats. Thirty male weanling rats were randomly assigned to consume AIN-93G based diets containing 2.5, 5, 7.5, 15 or 30 μg Zn/g diet for 24 days. Femur zinc increased substantially as zinc increased from 5 to 15 μg/g diet and modestly between 15 and 30 μg/g ($P<.05$). By morphological assessment, trabecular bone increased steadily as dietary zinc increased to 30 μg/g. Increasing dietary zinc tended to decrease Zip2 expression nonsignificantly and elevated the relative expression of metallothionen-I at 15 but not 30 μg Zn/g diet. Femur osteoclastic resorption potential, indicated by matrix metalloproteinases (MMP-2 and MMP-9) and carbonic anhydrase-2 activities decreased with increasing dietary zinc. In contrast to indicators of extracellular matrix resorption, femur tartrate-resistant acid and alkaline phosphatase activities increased fourfold as dietary zinc increased from 2.5 to 30 μg Zn/g. Likewise, 15 or 30 μg Zn/g diet resulted in maximum relative expression of osteocalcin, without influencing expression of core-binding factor α-1, collagen Type 1 alpha-1, or nuclear factor of activated T cells c1. In conclusion, increased trabecular bone with additional zinc suggests that previous requirement estimates of 15 μg Zn/g diet may not meet nutritional needs for optimal bone development. Overall, the up-regulation of extracellular matrix modeling indexes and concomitant decrease in resorption activities as dietary zinc increased from 2.5 to 30 μg/g provide evidence of one or more physiological roles for zinc in modulating the balance between bone formation and resorption. Published by Elsevier Inc.

*Keywords:* Zinc; Bone; Extracellular matrix; Resorption; Rats

## 1. Introduction

A nutritional zinc (Zn) requirement for normal bone formation was first described in 1941 [1], from observations of reduced epiphyseal plate thickness related to impaired echondrogenesis and subnormal trabecular volume in Zn-deficient tibia from post-weanling rats. Later, O'Dell et al. [2] (1957) also reported defective hypertrophic chondrocyte proliferation related to low dietary Zn, providing additional evidence for an osteogenic Zn requirement. Bone formation in Zn-deficient animals is characterized by retarded growth plate activity [3–6] and is associated with reduced cancellous and trabecular volume [3,7,8]. The biophysical consequences of these events include decreased longitudinal and radial expansion of long bones, with increased susceptibility to breakage due to applied physical stress [3,9,10]. Positive effects of Zn on bone tissue growth and mineralization in rats [11,12], along with the Zn-specific inhibition of osteoclast-mediated bone resorption in vitro [13] provide evidence of an important role for nutritional Zn in bone tissue accumulation and retention.

Although Zn is the 23rd most abundant element in the earth's crust, it is the only trace mineral with a critical structural or enzymatic function in at least one enzyme in each of the six enzyme classes [14]. The integral role of Zn in numerous osteogenic enzymes, including alkaline phosphatase (ALP) [8], Type IV gelatinases [15,16], carbonic anhydrase II (CAII) [17] and tartrate-resistant acid phosphatase (TRAP) [18–20], is therefore not surprising. Nonetheless, the present understanding of skeletal Zn requirements for the synthesis, mineralization and remodeling of the extracellular matrix (ECM) during echondrogenesis remains unclear. Therefore, the effects of nutritional Zn on factors involved in endochondral bone formation were characterized by measuring various Zn-dependent biochemical activities from the metaphyseal–epiphyseal region of femurs of growing rats. Molecular indicators of osteoblast and osteoclast ECM synthesis and resorption potential related to bone mass accumulation

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were also evaluated to provide insight regarding the osteogenic and antiresorptive properties of Zn in bone.

## 2. Methods and materials

### 2.1. Animals and diet

Thirty 21-day-old male Sprague–Dawley rats (Charles River/Sasco) were randomly assigned to five dietary treatment groups ($n$=6). The diets were based on AIN-93G, modified as described by Reeves [21], and contained 2.5, 5, 7.5, 15 or 30 mg Zn/kg diet as $ZnCO_3$ with egg albumin as the protein source. Rats were housed individually in stainless steel, wire-bottom cages in a climate controlled room, with temperature and humidity settings at 72°C and 50%, respectively, with a 12-h light/dark cycle. Food and deionized water were provided ad libitum in glass containers and bottles fitted with stainless steel sippers fitted with Zn-free stoppers. Rats were anesthetized after 21 days using a 1.37:1 mixture of ketamine:xylazine (1.0 ml/kg body weight, intraperitoneal) and killed by exsanguination. Tibias and femurs were collected for analysis. The study was approved by the Animal Care Committee of the Grand Forks Human Nutrition Research Center, in accordance with the guidelines set by the National Research Council, for the care and use of laboratory rats.

### 2.2. Femur tissue extraction of Zn-dependent enzymes

Freshly collected femurs were rinsed with ice-cold phosphate buffered saline and then stored on ice. Samples were sliced medially at the distal joint-to-femur neck, and then sections from the epiphysis to the limit of the diaphysis, including the epiphyseal growth plate and trabecular bone, were extracted three times in CNTN buffer (10 mM cacodylate/HCl, pH 6.0, 1M NaCl, 0.1% Triton-X100) at 4°C overnight [22]. Insoluble material was precipitated from each animal's pooled samples by centrifugation at 12,000×$g$ for 10 min, 4°C, and the remaining extracts were assayed for enzyme activity.

### 2.3. Gelatin zymography

Femur extract protein concentrations were determined using Bradford protein reagent [23]. Total protein from extracts was loaded onto a 10% gelatin, non-denaturing polyacrylamide gel (Invitrogen) and electrophoresed at 125 V for 1.5 h. After two 20 minute washes in gel renaturation buffer and 30 min equilibration in developing buffer, gels were incubated at 37°C for 21 h with gentle agitation in fresh developing buffer. Developed gels were stained with coomassie blue, and active protease bands were identified by negative staining after destaining in 10% methanol, 10% glacial acetic acid.

### 2.4. Carbonic anhydrase II activity

Femur extracts were diluted 1:1 with 40 mM bromopyruvic acid and 47 mM $NaHPO_4$ and incubated on ice for 1 h. Diluted extracts were incubated in 5 ml of ice-cold $H_2O$ and then saturated with $CO_2$ at a rate of 400 cc/min. The assay reaction was initiated by the addition of 2 ml of ice-cold, 50 mM barbital, pH 7.9, and CAII activity was determined by the electrometric change in pH to 6.7. One unit of CAII enzyme activity was determined by the difference in the pH end-point times between sample and blank reactions per mg protein.

### 2.5. Tartrate resistant acid phosphatase activity

TRAP activity was determined by the method of Lau et al. [24]. Extracts were incubated in 10 mM sodium citrate, pH 5.5 at 37°C for 1 h. TRAP activity assays were initiated by the addition of 100 mM $p$-nitrophenyl phosphate (PNPP), 200 mM NaCl, 80 mM sodium L-(+)-tatrate and 200 mM sodium citrate, pH 5.5 and then incubated at 37°C for 15 min before being terminated by adding an excess of 1 N NaOH. The amount of $p$-nitrophenol (pNP) produced was measured at 405 nm, and then TRAP activity was determined by a pNP (Sigma-Aldrich) standard curve. The results were reported as micromoles of PNPP hydrolyzed per milligram of protein.

### 2.6. Alkaline phosphatase activity

The ALP assay of Bessey et al. [25], was adapted for colorometric microtiter plate assay of femur ALP activity. Samples were diluted in 0.9% NaCl and incubated in the presence of 0.7 mM 2-Amino-2methyl-1-propanol, pH 10.5, at 37°C for 30 min in a humidified incubator. Enzymatic reactions were initiated by the addition of PNPP substrate to a final concentration of 5 mM at 37°C for 10 min then terminated by using an excess of 1 N NaOH, and the absorption was measured at 405 nm. Activity was determined based on a pNP standard curve and reported as micromoles of PNPP hydrolyzed per milligram of protein.

### 2.7. Real-time polymerase chain reaction analysis of gene expression

Rat femurs were cleaned and rinsed with phosphate-buffered saline, frozen in liquid nitrogen, and then stored at −80°C. Total RNA was extracted from sliced sections of disarticulated femur heads by using TRIzol Reagent (Invitrogen Life Technologies) according to the manufacturer recommendations. RNA concentration and purity were determined spectrophotometrically. For cDNA synthesis, 2.0 μg of total RNA was hybridized to 100 pmol of oligo $dT_{18}$ and then reverse-transcribed at 42°C for 60 min before terminating the reaction at 94°C for 5 min, according to the method of Ausubel et al. [26].

Real-time polymerase chain reaction (RT-PCR) was used to quantify gene expression. Briefly, 10–40 ng of reverse-transcribed RNA were used as template in 25-μl polymerase chain reaction (PCR) reactions that contained 5 mM Tris–HCl (pH 8.0), 10 mM NaCl, 0.01 mM EDTA, 0.1 mM DTT, 5% glycerol, 0.05% Nonidet-P40, 0.05% Tween-20, 3.0 mM $MgCl_2$, 200 μM of each dNTP, 1.25 U Taq polymerase (Promega), 0.4–2 μM of forward and reverse oligonucleotide primers and Sybr Green 1 dye (Molecular probes) from a master mix. PCR amplicon formation was quantified on a SmartCycler (Cepheid) instrument by using standard PCR cycle conditions. The second derivative maximum was used for the comparative cycle threshold (Ct) method [27]. Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) gene expression was used as an endogenous control and was not affected by any of the dietary treatments. The ratio of target gene expression among dietary treatment groups to gene expression levels of the 30 μg Zn/g diet treatment group are reported here as relative expression. Positive reaction results were based on the presence of a single peak on a melting point profile in addition to the visualization of a single ethidium bromide stained PCR product corresponding to the expected molecular weight. For a negative control, a blank PCR reaction without cDNA template was used to verify amplification specificity. Four to six animals were analyzed independently. Primer sets used were as follows:

Metallothionein-1 (Mt-1), 5′-ACCCCAACTGCTCCTG(C/T)(T/G)CC-3′ and 5′-AGGTGTACGGCAAGACTCTG-3′ [28];
Zip2, 5′-GATGCATATGACTGCTGAA-3′ and 5′-AAGATCGGCACTG-GACC-3′;
Osteocalcin (OC), 5′-GAGGACCCTCTCTCTGCTCA-3′ and 5′-GTAAACGGTGGTGCCATAGAT-3′;
Nuclear factor of activated T cells c1 (Nfatc1), 5′-CGAGGAAGAA-CACTACAGTT-3′ and 5′-GGTCTCTGTAGGCTTCCA-3′;
Collagen Type 1 α1 (Col1α1), 5′-CAGCTTTGTGGACCTCCGGCT-3′ and 5′-CAGCCGTGCCATTGTGGCAG-3′;
Core-binding factor α-1 (Cbfα1), 5′-CGAGTCAGTGAGTGCTCT-3′ and 5′-GACACGGTGTCACTGCA-3′;
GAPDH, 5′-CCCTCAAGATTGTCAGCAATGC-3′ and 5′-GTCCTCAGTG-TAGCCCAGGAT-3′.

### 2.8. Bone mineral analysis

For mineral analysis, organic matter in the dry femur was destroyed by alternatively heating for 2 days at 450°C and adding concentrated nitric acid which was evaporated to dryness on a hot plate. The resulting ash was solubilized in 6 N hydrochoric acid, then diluted to volume, and the mineral content determined by inductively coupled plasma (ICP) emission spectrophotometry.

### 2.9. Histological preparation of tibiae

Tibiae were disarticulated from femur and foot/ankle by using scissors and blunt dissection and then cleaned. Bisected tibiae were fixed in 10% formalin and soft tissue was removed prior to decalcification in 0.5 M EDTA-HCl, pH 7.4, 4°C for 90 h. Dehydrated samples were *en bloc* stained with 0.5% Basic Fuchsin for 24 h. Paraffin-embedded samples were cut into 10-μm serial sections and mounted on SuperFrost Plus slides (Erie Scientific).

### 2.10. Histomorphometric analysis of tibiae

Sections for image analysis were selected by utilizing anatomical markers and an indexing factor based on the weight of the animal to ensure that comparable areas were analyzed in different animals. Samples were viewed by using a transilluminating Leica MZ 6 stereo microscope to provide digital images resolved at 208.8 pixels/mm using a Cool Snap 1300Y digital camera (RS Photometrics, 1500×1500 resolution). Digital images were analyzed by using Image-Pro Plus (4.5.1.26) software (Media Cybernetics). The central region of the trabecular space was defined as the area of interest (AOI). The AOI for each sample was determined by superimposing a rectangle that extended from the distal most limit of the growth plate to the cortical bone, adjacent to the proximal limit of the diaphysis in order to compensate for differences between animal and bone sizes. The number of trabecular profiles within the AOI was determined by using Image-Pro software and reported as the number of trabeculae per $mm^2$.

### 2.11. Statistical analysis

The effects of dietary Zn concentrations on tissue Zn enzymatic activities were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparisons when appropriate. For sample analysis, $n$=3 to 6 animals per dietary treatment group. Gene expression results were analyzed by using a one-way ANOVA

Table 1
Effect of dietary Zn concentration on body weight gain, genetic expression of selected markers of bone extracellular matrix resorption and mineralization, and activities of MMP-2 and MMP-9 in the femurs of growing rats

| Dietary Zn, μg/g | 2.5 | 5.0 | 7.5 | 15 | 30 |
|---|---|---|---|---|---|
| Weight gain, g | 62±5[a] | 153±5[b] | 185±8[c] | 181±5[c] | 180±8[c] |
| Metallothionein-I, RE * | 1.7 (0.6, 4.8)[ab] | 0.5 (0.2, 1.4)[a] | 1.3 (0.5, 3.2)[ab] | 6.5 (2.9, 14.4)[b] | 1.0 (0.5, 2.1)[ab] |
| Zip2, RE | 9.5 (1.0, 93.7) | 50.9 (5.4, 482.2) | 3.0 (0.3, 26.2) | 1.1 (0.1, 9.2) | 1.0 (0.2, 5.6) |
| MMP-2, RA [1] | 26.22±2.80[a] | 4.14±2.80[b] | 1.58±2.80[b] | 0.96±2.97[b] | 1.00±1.98[b] |
| MMP-9, RA | 8.85±1.00[a] | 2.36±1.00[b] | 1.10±1.00[b] | 0.94±1.06[b] | 1.00±0.71[b] |
| Col1α1, RE | 0.42 (0.25, 0.70) | 0.93 (0.55, 1.57) | 0.77 (0.44, 1.34) | 0.89 (0.51, 1.57) | 1.00 (0.68, 1.48) |
| Osteocalcin, RE | 0.23 (0.14, 0.38)[a] | 0.63 (0.41, 0.98)[ab] | 0.67 (0.43, 1.03)[ab] | 1.74 (1.05, 2.88)[b] | 1.00 (0.69, 1.44)[b] |
| Cbfα1, RE | 0.74 (0.57, 0.96) | 1.02 (0.78, 1.34) | 1.52 (1.11, 2.08) | 0.97 (0.76, 1.23) | 1.00 (0.84, 1.19) |
| Nfatc1, RE | 0.76 (0.49, 1.16) | 0.85 (0.55, 1.30) | 2.28 (1.48, 3.50) | 1.73 (1.07, 2.80) | 1.00 (0.71, 1.40) |

* Body weight gain is expressed as mean±S.E.. Gene expression is given in RE units indicating relative expression, with the 30 μg Zn/g group serving as the baseline (=1) and with the data representing the geometric means (minus 1 S.E., plus 1 S.E.). MMP-2 and MMP-9 activities are expressed in RA units indicating relative activity±S.E., also using the 30 μg Zn/g group serving as the baseline (=1). Means in the same row without the same superscript differ significantly.

with analysis run included as a blocking factor. For all statistical analyses, a value of $P<.05$ was considered statistically significant. All statistical analyses were done by SAS/Stat Version 9.1 (SAS Institute, Cary, NC).

## 3. Results

### 3.1. The effect of dietary Zn on growth, femur Zn concentration and metaepiphyseal Mt-1 and Zip2 mRNA expression

As expected, the Zn-deficient diets significantly affected rat growth, with substantially impaired weight gain in the most Zn-deficient group, and a more moderate reduction in growth in the group fed 5 μg Zn/g diet, with maximal growth achieved at 7.5 μg Zn/g diet and above (Table 1). Femur Zn concentrations revealed a different pattern of response to dietary Zn during growth. Femur Zn increased significantly ($P<.05$) from ~1 μmol/g dry weight with 2.5 or 5 μg Zn/g diet, up to 4 μmol/g dry weight, for animals fed 30 μg Zn/g diet, respectively (Fig. 1). Following a linear increase between 5 and 15 μg Zn/g diet, femur Zn increased only slightly between 15 and 30 μg Zn/g diet, suggesting that the physiological bone Zn requirement during growth was nearly, but not fully, met with 15 μg Zn/g diet.

Femur metallothionein-1 (Mt-1) and the $Zn^{2+}$ solute carrier Slc39a2 (Zip 2) were used as Zn-specific indicators of cellular Zn status in metaphyseal tissue. Femoral expression of Mt-1 was elevated at 15 ($P<.05$) but, otherwise, did not differ significantly between 5 and 30 μg Zn/g diet (Table 1). Expression of Zip 2 tended to be inversely related to dietary Zn between 5 and 15 μg Zn/g (Table 1). Although expression levels for both messages varied considerably within groups, the overall results are consistent with a metabolic transition involving changes in metaphyseal Zn concentrations.

### 3.2. The effect of dietary Zn on femur metaepiphyseal extracts of alkaline phosphatase, CAII and TRAP activity

Biochemical analyses of Zn-dependent enzymes involved in normal osteogenic activities were used to assess the effects of Zn status on key elements of endochondral bone formation. Colorimetrically determined ALP activity increased incrementally with each increase in dietary Zn from (mean±S.E.M.) 16±10 to 61±11 μmol PNPP/mg protein (Fig. 2). The end points were statistically different ($P<.05$), representing a nearly fourfold increase in ALP activity when dietary Zn increased from 2.5–30 μg Zn/g.

TRAP enzyme activity was also measured by using femur growth plate extracts. Like ALP, analysis of extracted TRAP showed a fourfold increase (3.7±1.5 to 15.7±1.5 μmol PNPP/mg of protein) in activity as dietary Zn increased from 2.5 to 30 μg Zn/g diet, respectively (Fig. 3A).

CAII activity was also determined from femur epiphyseal growth plate extracts for use as an indicator of osteoclastic ECM mineral resorption potential. Overall, CAII activity ranged from 13±2 to 25±2 mU/mg of protein (Fig. 3B), with an average activity level of 21 mU/mg protein, between 2.5 and 15 μg Zn/g diet. Treatment with 30 μg Zn/g diet resulted in a 43–51% decrease in CAII activity, when compared to that in animals fed either 15 or 2.5 μg Zn/g, respectively. This significant ($P<.05$) decrease in activity of the osteoclastic enzyme CAII demonstrates a remarkable contrast to the increases

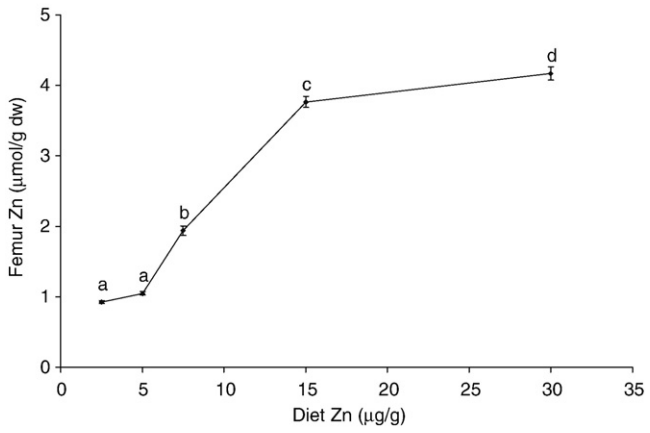


Fig. 1. Effect of dietary Zn concentration on femur Zn concentrations, expressed per dry weight (dw) of bone. Male weanling rats were fed 2.5, 5, 7.5, 15 and 30 μg Zn/g diet for 21 days. The plotted data represent the mean±S.E.M. for each dietary treatment group. For statistical differences between treatments, marked by different letters, $P<.05$ was used.

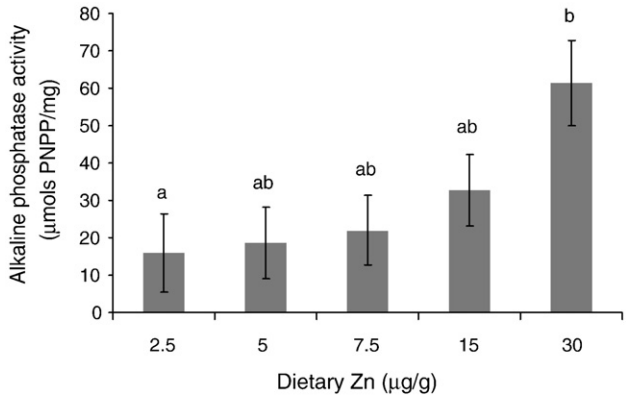


Fig. 2. Effect of dietary Zn on alkaline phosphatase enzyme activity. Alkaline phosphatase enzyme activity was determined by spectrophotometric measurement of p-nitrophenol formation at 405 nm, from 45-day-old rat proximal femur epiphyseal extracts, after treatment with 2.5, 5, 7.5, 15 and 30 μg Zn/g diet for 21 days. The plotted data represent the mean±S.E.M. for each dietary treatment group. For statistical differences between treatments, marked by different letters, $P<.05$ was used.

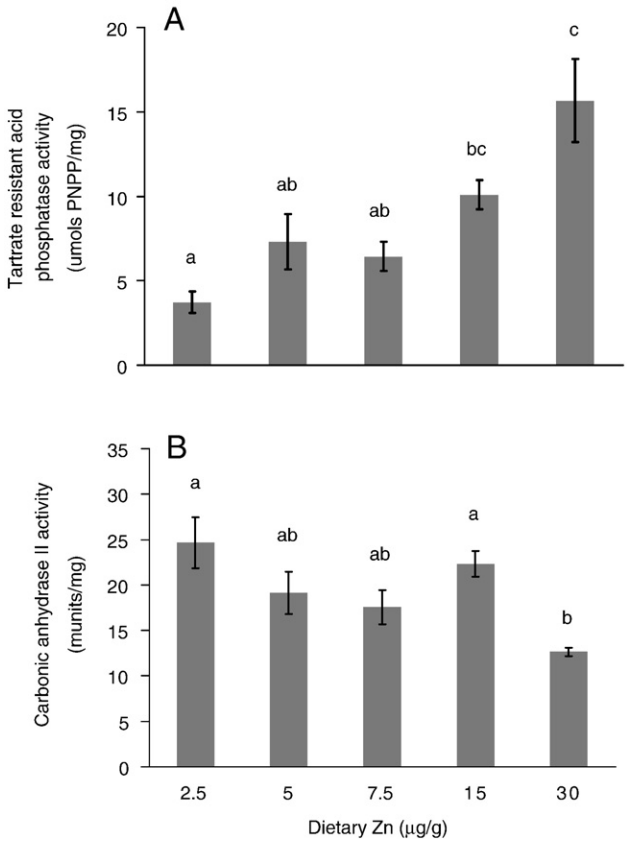


Fig. 3. Dietary Zn modulates indicators of osteoclast resorption and maturation in proximal femur epiphyses. The effect of dietary Zn on TRAP enzyme activity (A) and CAII enzyme activity (B) was determined as described in the methods section from rat proximal femur epiphyseal extracts, after treatment with 2.5, 5, 7.5, 15 and 30 μg Zn/g diet for 21 days. The plotted data represent the mean±S.E.M. for each dietary treatment group. Statistical differences in enzyme activities between treatments marked with different letters were defined by $P<.05$.

in both alkaline and TRAP activities, as dietary Zn increased from deficient to adequate.

### 3.3. Femur metaepiphyseal growth plate Type IV gelatinase activities

Matrix metalloproteinase (MMP) 2 and 9 were assessed by substrate zymography to evaluate the impact of dietary Zn on Zn-dependent factors associated with organic matrix remodeling. The activities of MMP-2 and MMP-9 were strongly elevated in femur extracts from rats fed 2.5 μg Zn/g diet but were substantially reduced in femur extracts of rats fed at least 5 μg Zn/g diet (Table 1). Thus, active MMP-2 and MMP-9 levels were both elevated by Zn deficiency during growth.

### 3.4. Histomorphometric analysis of tibial metaepiphyses

To assess how diet-related differences in ECM Zn-dependent enzyme activities impact bone formation, alterations in epiphyseal plate migration and cartilage proliferation were determined by histological analysis of tibias. The extent and distribution of metaphyseal trabecularization was noticeably different between animals fed Zn-deficient and Zn-adequate diets (Fig. 4). Trabecular bone formation was primarily localized along the distal epiphyseal, cartilage-bone junction in severely and marginally Zn-deficient rats. Metaepiphyseal trabecularization extended distally, when at least 7.5 μg/g dietary Zn was present in the diet. In addition, the number of fine structures stained immediately distal to the mineralization-front appeared to increase in rats fed 30 μg Zn/g diet. Overall, these results indicated differences in the density, distribution and, possibly, architectural quality of tibial trabeculae related to nutritional Zn availability during growth.

Digital quantization of tibial trabecular density indicated a steady increase from 15±2 to 23±4 profiles/mm$^2$ as dietary Zn increased from 2.5 to 30 μg Zn/g diet (Fig. 4), the difference becoming significant ($P<.05$) between rats fed the highest and lowest concentrations of dietary Zn. This shows the importance of Zn for development of trabecular bone. Furthermore, the tendency for trabecular bone to increase further between 15 and 30 μg Zn/g diet suggests that 15 μg Zn/g diet may be suboptimal for bone development in growing male rats.

### 3.5. RT-PCR analyses of metaepiphyseal Type 1 α1 collagen and osteocalcin expression

The effects of Zn status on ECM formation were investigated by comparing the relative expression of Col1α1 and osteocalcin (OC) from total metaepiphyseal RNA of rats from the various dietary groups. Severe Zn deficiency tended to depress expression of Col1α1 and significantly depressed expression of OC, relative to the expression levels observed with 30 μg Zn/g diet (Table 1). The expression of OC appeared to be more sensitive than Col1α1 to marginal Zn deficiency.

### 3.6. Cbfα1 and Nfatc1 expression

Evidence of sensitivity to Zn nutrition by osteoblasts and osteoclasts was sought by analyzing the impact of Zn status on the relative gene expression of the respective key transcriptional regulators of differentiation for each cell type. RT-PCR analysis of Cbfα1 (Runx2) showed no significant effect of dietary Zn (Table 1).

Similar analysis of Nfatc1 found no significant effect of dietary Zn (Table 1). Together, these results suggest that dietary Zn availability does not limit the expression of either transcription factor, during growth.

## 4. Discussion

Numerous studies have shown evidence of the important relationship between adequate Zn nutriture and bone health. Low dietary levels and plasma concentrations of Zn were associated with increased osteopenia, osteoporosis and fracture risk in men [29,30]. Inverse associations between Zn intake and bone loss in postmenopausal women [31] and bone mass in premenopausal women [32] have also been reported, and Zn with other trace elements positively affected bone mineral density in a placebo-controlled trial [33]. In addition, urinary Zn excretion is higher in osteoporotic versus nonosteoporotic individuals [34–36]. These studies highlight the importance of understanding the nutrition requirements for maintaining an optimal balance between bone accumulation and remodeling in order to develop simple and effective diet-based strategies which favor accretion and restrict pathogenic loss of skeletal tissue in humans [32]. A number of studies have used rats to investigate the effects of Zn on various aspects of bone quality [3,37,38]. In the present investigation, histomorphometric markers, as well as biochemical and molecular elements associated specifically with osteoblast and osteoclast ECM formation, resorption and differentiation were used to characterize the effects of Zn on bone metabolism in rats. Notably, this study marks the first demonstration of a specific biochemical mechanism, involving CAII, by which Zn regulates bone resorption, in rats. In addition, this study shows that, in contrast to the Zn requirement of 15 μg Zn/g determined by break-point analysis to maximize bone Zn concentrations [9,39,40], 30 μg Zn/g is needed for the abatement of elevated CAII activity.

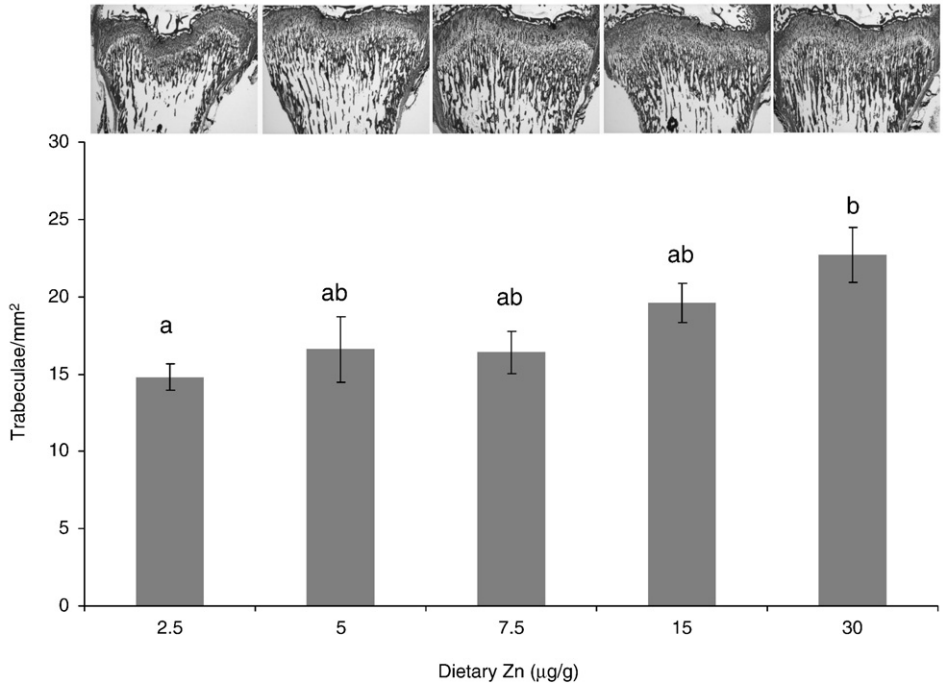


Fig. 4. Histomorphometry of trabecular bone formation. Proximal tibia sections (A) stained with Basic Fuchsin demonstrate increased trabecular bone formation extending from the distal epiphyseal, cartilage-bone junction to the distal metaphysis region of male weanling rats fed diets containing 2.5, 5, 7.5,15 and 30 μg Zn/g for 24 days. Digitally quantified trabeculae in rat tibial sections (B) are represented as the mean±S.E.M. of five or six animals. Different letters indicate significant differences ($P<.05$).

Endochondral bone formation is characterized by osteoclastic resorption of hypertrophic cartilage prior to bone matrix deposition and subsequent remodeling by osteoblasts and osteoclasts, respectively, as the epiphyseal plate advances longitudinally during growth. In long bones of rats fed less than 1 ppm Zn, this process was disrupted as indicated by significantly decreased cancellous and trabecular bone [3,7]. Arguably, in this and other studies, interpretation of results obtained from dietary Zn treatments of less than 5 ppm, Zn may not be straight forward because of difficulty distinguishing between the influence of anorexic [41], general metabolic [42,43] and tissue- or cell-specific adaptations caused by such conditions. When pair-feeding was used, the effects of Zn deficiency on bone were attributed to reduced osteoblast modeling activity only [3,38] or in combination with reduced osteoclast numbers [7]. In the present study, stepwise increases in dietary Zn produced incremental differences in metaphyseal trabecularization, consistent with previous comparisons between severely deficient and adequate groups [1,4–7]. Here, graded levels of dietary Zn ranged from severely deficient (2.5 μg/g) to adequate (30 μg/g), and normal growth was maintained by 7.5 μg Zn/g diet. Notably, the results from histomorphometric and other analyses were similar among the lower dietary Zn groups in this study. Therefore, interpretation of these results was based on the assumption that one or more common Zn-responsive factors were responsible. The conclusions from these results are discussed in terms of possible mechanisms to explain the roles of nutritional Zn in bone metabolism.

Resorption and remodeling of ECM mineral and organic phases by osteoclasts are prerequisites for bone formation. Zn reduction of osteoclast-dependent mineral resorption by 39% to 47% has been shown by using cell culture models [13,44]. CAII and MMP-9 are primarily expressed by osteoclasts [45–50] while MMP-2 expression occurs in osteoblasts [51]. CAII is indispensable for the catalytic hydrolysis of $CO_2$ to generate $HCO_3^-$ and $H^+$ for solubilizing the ECM inorganic phase of bone ECM [52–58]. MMP-9 and MMP-2 substrate specificities include native and solubilized Type 1 collagen [50,59] and are therefore useful as indicators of ECM organic remodeling potential. During bone formation, MMP-9 expressing osteoclasts were localized at the cartilage-bone junction and trabeculae, adjacent to the growth plate in mice [60]. Studies of MMP-9-deficient mice have revealed major roles for MMP-9 in the solubilization and neovascularization of hypertrophic cartilage, in addition to osteoclast recruitment during bone formation [60,61]. In vivo chondrosarcoma vascularization studies demonstrated an angiogenic role for MMP-2, as well [62]. In the present investigation, Zn deficiency was associated with elevated CAII enzyme activity, and active MMP-2 and MMP-9, with attenuation of these factors by higher dietary Zn. Importantly, these findings demonstrate that osteoclastic resorption and remodeling activities are up-regulated by limited Zn availability. Whether underlying changes in message, protein or expression of tissue specific inhibitors are involved remains to be determined. Nonetheless, the inverse relationships between Zn-dependent bone ECM resorption activities and Zn status are counterintuitive. In vitro studies have shown that the osteoclast phenotype is characterized by reduced resorption activity and elevated TRAP expression when Zn is sufficient [13]. In our study, concurrent reduction of CAII activity as TRAP enzyme activity increased indicates a similar effect of Zn on osteoclast phenotype in vivo. An inverse relationship between CAII and TRAP enzyme activities was also observed with Zn depletion of rats previously fed a Zn-adequate diet (30 μg/g) (unpublished data). Overall, the increased Zn-dependent bone resorption activities during Zn deficiency are inconsistent with a cofactor depletion mechanism and therefore require an alternate explanation. Changes in the expression ratios of CAII and TRAP are characteristic of mononuclear osteoclast precursors and mature multinucleated osteoclasts [47,48].

The results of this experiment could therefore indicate that Zn modulates osteoclast maturation or function. Nfatc1, an autoregulatory transcription factor induced by receptor activator of nuclear factor-κB ligand, is critical for the terminal maturation of osteoclasts and regulates gene transcription of CAII and TRAP [63]. However, the affects of severe and marginal Zn deficiencies on Nfatc1 expression

were minimal in this experiment. Thus, no clear indication that Zn controls NFAT gene expression was evident. This conclusion does not exclude the possibility that Zn status may modify the expression of some NFAT-inducible genes, however.

Because bone mass is ultimately determined by the balance between ECM matrix resorption and synthesis [64], the effects of Zn status on indicators of osteoblast differentiation, maturation and matrix deposition were also investigated. Col1α1 is highly expressed by differentiated osteoblasts [65] and functions as an organic scaffold for mineral deposition [66,67]. Similarly, ALP and OC are primarily expressed by post-proliferating and terminally mature osteoblasts [68–71], respectively, and regulate mineralization of the ECM [72–74]. Expression of these genes occurs largely under the influence of Cbfα1 [75,76], the master determinant for osteoblast differentiation and function [77,78]. In this investigation, dietary Zn did not clearly affect either Cbfα1 or Col1α1 expression. Therefore, the influence of Zn status on ALP and OC levels seen in the metaepiphysis here and in plasma elsewhere [38] seems likely due to modification of their expression, independently of Cbfα1. Collectively, these results indicate that suboptimal Zn status limits expression of the mineralizing phenotype in osteoblast [75,76] while favoring the expression of the resorptive phenotype by osteoclast, during bone formation in growing rats.

Overall, this study provides evidence of physiological Zn regulation of bone homeostasis, apart from its role as a cofactor in ALP and other Zn-dependent enzymes. Additionally, concomitant changes in metaphyseal ECM resorption and mineralization potentials demonstrate the importance of adequate Zn nutriture for establishing a physiological environment in bone, in which the rates of osteogenic activity exceed those of resorption. These findings may therefore have direct application in reducing the pathophysiological resorption and suboptimal modeling that are characteristic of postmenopausal osteoporosis. Further studies to reveal the mechanisms of action by which Zn modulates bone formation and remodeling are needed in order to fully understand the nutritional Zn requirement for optimal bone health during all developmental stages.

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## References

[1] Follis RH, Day RG, McCollum EV. Histological studies of the tissues of rats fed a diet extremely low in zinc. J Nutr 1941;22:223–37.
[2] O'Dell BL, Newberne PM, Savage JE. Significance of dietary zinc for the growing chicken. J Nutr 1958;65:503–18.
[3] Rossi L, Migliaccio S, Corsi A, Marzia M, Bianco P, Teti A, et al. Reduced growth and skeletal changes in zinc-deficient growing rats are due to impaired growth plate activity and inanition. J Nutr 2001;131:1142–6.
[4] Zhang Y, Cheng Y, Hong Y, Li S. Zinc deficiency on pathological changes of femur epiphyseal growth plate in rats. Wei Sheng Yan Jiu 2003;32:16–9.
[5] da Cunha Ferreira RM, Rodriguez Gonzalez JI, Monreal Marquiegui I, Villa Elizaga I. Changes in the fetal tibial growth plate secondary to maternal zinc deficiency in the rat: a histological and histochemical study. Teratology 1991;44:441–51.
[6] Suwarnasarn A, Wallwork JC, Lykken GI, Low FN, Sandstead HH. Epiphyseal plate development in the zinc-deficient rat. J Nutr 1982;112:1320–8.
[7] Eberle J, Schmidmayer S, Erben RG, Stangassinger M, Roth HP. Skeletal effects of zinc deficiency in growing rats. J Trace Elem Med Biol 1999;13:21–6.
[8] Oner G, Bhaumick B, Bala RM. Effect of zinc deficiency on serum somatomedin levels and skeletal growth in young rats. Endocrinology 1984;114:1860–3.
[9] Hunt JR, Johnson LK. Dietary protein, as egg albumen: effects on bone composition, zinc bioavailability and zinc requirements of rats, assessed by a modified broken-line model. J Nutr 1992;122:161–9.
[10] Ovesen J, Moller-Madsen B, Thomsen JS, Danscher G, Mosekilde L. The positive effects of zinc on skeletal strength in growing rats. Bone 2001;29:565–70.
[11] Becker WM, Hoekstra WG. Effect of vitamin D on 65Zn absorption, distribution and turnover in rats. J Nutr 1966;90:301–9.
[12] Hurley LS, Gowan J, Milhaud G. Calcium metabolism in manganese-deficient and zinc-deficient rats. Proc Soc Exp Biol Med 1969;130:856–60.
[13] Holloway WR, Collier FM, Herbst RE, Hodge JM, Nicholson GC. Osteoblast-mediated effects of zinc on isolated rat osteoclasts: inhibition of bone resorption and enhancement of osteoclast number. Bone 1996;19:137–42.
[14] Vallee BL, Falchuk KH. The biochemical basis of zinc physiology. Physiol Rev 1993;73:79–118.
[15] Wilhelm SM, Collier IE, Marmer BL, Eisen AZ, Grant GA, Goldberg GI. SV40-transformed human lung fibroblasts secrete a 92-kDa type IV collagenase which is identical to that secreted by normal human macrophages. J Biol Chem 1989;264:17213–21.
[16] Collier IE, Wilhelm SM, Eisen AZ, Marmer BL, Grant GA, Seltzer JL, et al. H-ras oncogene-transformed human bronchial epithelial cells (TBE-1) secrete a single metalloprotease capable of degrading basement membrane collagen. J Biol Chem 1988;263:6579–87.
[17] Pocker Y, Sarkanen S. Carbonic anhydrase: structure catalytic versatility, and inhibition. Adv Enzymol Relat Areas Mol Biol 1978;47:149–274.
[18] Susi FR, Goldhaber P, Jennings JM. Histochemical and biochemical study of acid phosphatase in resorbing bone in culture. Am J Physiol 1966;211:959–62.
[19] Miller SC. The rapid appearance of acid phosphatase activity at the developing ruffled border of parathyroid hormone activated medullary bone osteoclasts. Calcif Tissue Int 1985;37:526–9.
[20] Minkin C. Bone acid phosphatase: tartrate-resistant acid phosphatase as a marker of osteoclast function. Calcif Tissue Int 1982;34:285–90.
[21] Reeves PG. AIN-93 purified diets for the study of trace element metabolism in rodents. In: Watson RR, editor. Trace Elements in Laboratory Rodents. Boca Raton, FL: CRC Press, Inc.; 1996. p. 3–37.
[22] Eeckhout Y, Delaisse JM, Vaes G. Direct extraction and assay of bone tissue collagenase and its relation to parathyroid-hormone-induced bone resorption. Biochem J 1986;239:793–6.
[23] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[24] Lau KH, Onishi T, Wergedal JE, Singer FR, Baylink DJ. Characterization and assay of tartrate-resistant acid phosphatase activity in serum: potential use to assess bone resorption. Clin Chem 1987;33:458–62.
[25] Bessey OA, Lowry OH, Brock MJ. A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. J Biol Chem 1946; 164:321–9.
[26] Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, et al. Current protocols in molecular biology. , New York, NY: John Wiley & Sons; 1995.
[27] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.
[28] Oda N, Sogawa CA, Sogawa N, Onodera K, Furuta H, Yamamoto T. Metallothionein expression and localization in rat bone tissue after cadmium injection. Toxicol Lett 2001;123:143–50.
[29] Hyun TH, Barrett-Connor E, Milne DB. Zinc intakes and plasma concentrations in men with osteoporosis: the Rancho Bernardo Study. Am J Clin Nutr 2004;80: 715–21.
[30] Elmstahl S, Gullberg B, Janzon L, Johnell O, Elmstahl B. Increased incidence of fractures in middle-aged and elderly men with low intakes of phosphorus and zinc. Osteoporos Int 1998;8:333–40.
[31] Freudenheim JL, Johnson NE, Smith EL. Relationships between usual nutrient intake and bone-mineral content of women 35–65 years of age: longitudinal and cross-sectional analysis. Am J Clin Nutr 1986;44:863–76.
[32] Strause L, Saltman P, Smith KT, Bracker M, Andon MB. Spinal bone loss in postmenopausal women supplemented with calcium and trace minerals. J Nutr 1994;124:1060–4.
[33] Saltman PD, Strause LG. The role of trace minerals in osteoporosis. J Am Coll Nutr 1993;12:384–9.
[34] Herzberg M, Lusky A, Blonder J, Frenkel Y. The effect of estrogen replacement therapy on zinc in serum and urine. Obstet Gynecol 1996;87:1035–40.
[35] Herzberg M, Foldes J, Steinberg R, Menczel J. Zinc excretion in osteoporotic women. J Bone Miner Res 1990;5:251–7.
[36] Relea P, Revilla M, Ripoll E, Arribas I, Villa LF, Rico H. Zinc, biochemical markers of nutrition, and type I osteoporosis. Age Ageing 1995;24:303–7.
[37] Roughead ZK, Lukaski HC. Inadequate copper intake reduces serum insulin-like growth factor-I and bone strength in growing rats fed graded amounts of copper and zinc. J Nutr 2003;133:442–8.
[38] Hosea HJ, Taylor CG, Wood T, Mollard R, Weiler HA. Zinc-deficient rats have more limited bone recovery during repletion than diet-restricted rats. Exp Biol Med (Maywood) 2004;229:303–11.
[39] Pallauf J, Kirchgessner M. Zinkgehalte in knochen und ganzkorper wachsender ratten bei unterschiedlicher zinkversorgung. Z Tierphysiol, Tierernahrg u Futtermittelkde 1972;30:193–202.
[40] Momcilovic B, Reeves PG, Blake MJ. Idiorrhythmic dose-rate variability in dietary zinc intake generates a different response pattern of zinc metabolism than conventional dose-response feeding. Br J Nutr 1997;78:173–91.
[41] Essatara MB, Levine AS, Morley JE, McClain CJ. Zinc deficiency and anorexia in rats: normal feeding patterns and stress induced feeding. Physiol Behav 1984;32:469–74.
[42] Evans SA, Overton JM, Alshingiti A, Levenson CW. Regulation of metabolic rate and substrate utilization by zinc deficiency. Metabolism 2004;53:727–32.

[43] Kennedy KJ, Rains TM, Shay NF. Zinc deficiency changes preferred macronutrient intake in subpopulations of Sprague–Dawley outbred rats and reduces hepatic pyruvate kinase gene expression. J Nutr 1998;128:43–9.
[44] Moonga BS, Dempster DW. Zinc is a potent inhibitor of osteoclastic bone resorption in vitro. J Bone Miner Res 1995;10:453–7.
[45] Tezuka K, Nemoto K, Tezuka Y, Sato T, Ikeda Y, Kobori M, et al. Identification of matrix metalloproteinase 9 in rabbit osteoclasts. J Biol Chem 1994;269:15006–9.
[46] Reponen P, Sahlberg C, Munaut C, Thesleff I, Tryggvason K. High expression of 92-kD type IV collagenase (gelatinase B) in the osteoclast lineage during mouse development. J Cell Biol 1994;124:1091–102.
[47] Huang WH, Lau AT, Daniels LL, Fujii H, Seydel U, Wood DJ, et al. Detection of estrogen receptor alpha, carbonic anhydrase II and tartrate-resistant acid phosphatase mRNAs in putative mononuclear osteoclast precursor cells of neonatal rats by fluorescence in situ hybridization. J Mol Endocrinol 1998;20:211–9.
[48] Takeshita S, Kaji K, Kudo A. Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts. J Bone Miner Res 2000;15:1477–88.
[49] Matsuo K, Galson DL, Zhao C, Peng L, Laplace C, Wang KZ, et al. Nuclear factor of activated T-cells (NFAT) rescues osteoclastogenesis in precursors lacking c-Fos. J Biol Chem 2004;279:26475–80.
[50] Okada Y, Naka K, Kawamura K, Matsumoto T, Nakanishi I, Fujimoto N, et al. Localization of matrix metalloproteinase 9 (92-kilodalton gelatinase/Type IV collagenase=gelatinase B) in osteoclasts: implications for bone resorption. Lab Invest 1995;72:311–22.
[51] Kusano K, Miyaura C, Inada M, Tamura T, Ito A, Nagase H, et al. Regulation of matrix metalloproteinases (MMP-2, -3, -9, and -13) by interleukin-1 and interleukin-6 in mouse calvaria: association of MMP induction with bone resorption. Endocrinology 1998;139:1338–45.
[52] Sly WS, Hewett-Emmett D, Whyte MP, Yu YS, Tashian RE. Carbonic anhydrase II deficiency identified as the primary defect in the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification. Proc Natl Acad Sci U S A 1983;80:2752–6.
[53] Waite LC. Carbonic anhydrase inhibitors, parathyroid hormone and calcium metabolism. Endocrinology 1972;91:1160–5.
[54] Gay CV, Mueller WJ. Carbonic anhydrase and osteoclasts: localization by labeled inhibitor autoradiography. Science 1974;183:432–4.
[55] Zaidi M, Moonga B, Moss DW, MacIntyre I. Inhibition of osteoclastic acid phosphatase abolishes bone resorption. Biochem Biophys Res Commun 1989;159:68–71.
[56] Roth DE, Venta PJ, Tashian RE, Sly WS. Molecular basis of human carbonic anhydrase II deficiency. Proc Natl Acad Sci U S A 1992;89:1804–8.
[57] Lehenkari P, Hentunen TA, Laitala-Leinonen T, Tuukkanen J, Vaananen HK. Carbonic anhydrase II plays a major role in osteoclast differentiation and bone resorption by effecting the steady state intracellular pH and Ca2+. Exp Cell Res 1998;242:128–37.
[58] Schlesinger PH, Mattsson JP, Blair HC. Osteoclastic acid transport: mechanism and implications for physiological and pharmacological regulation. Miner Electrolyte Metab 1994;20:31–9.
[59] Aimes RT, Quigley JP. Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific 3/4- and 1/4-length fragments. J Biol Chem 1995;270:5872–6.
[60] Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, et al. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. Cell 1998;93:411–22.
[61] Engsig MT, Chen QJ, Vu TH, Pedersen AC, Therkidsen B, Lund LR, et al. Matrix metalloproteinase 9 and vascular endothelial growth factor are essential for osteoclast recruitment into developing long bones. J Cell Biol 2000;151:879–89.
[62] Fang J, Shing Y, Wiederschain D, Yan L, Butterfield C, Jackson G, et al. Matrix metalloproteinase-2 is required for the switch to the angiogenic phenotype in a tumor model. Proc Natl Acad Sci U S A 2000;97:3884–9.
[63] Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, et al. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. Dev Cell 2002;3:889–901.
[64] Raisz LG, Kream BE. Regulation of bone formation. N Engl J Med 1983;309:83–9.
[65] Quarles LD, Yohay DA, Lever LW, Caton R, Wenstrup RJ. Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: an in vitro model of osteoblast development. J Bone Miner Res 1992;7:683–92.
[66] Gehron-Robey P. Principles of Bone Biology. , San Diego, CA: Academic Press; 1996.
[67] Byers PH. Brittle bones–fragile molecules: disorders of collagen gene structure and expression. Trends Genet 1990;6:293–300.
[68] Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, et al. Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J Cell Physiol 1990;143:420–30.
[69] Stein GS, Lian JB, Owen TA. Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. FASEB J 1990;4:3111–23.
[70] Weinreb M, Shinar D, Rodan GA. Different pattern of alkaline phosphatase, osteopontin, and osteocalcin expression in developing rat bone visualized by in situ hybridization. J Bone Miner Res 1990;5:831–42.
[71] McCabe LR, Kockx M, Lian J, Stein J, Stein G. Selective expression of fos- and jun-related genes during osteoblast proliferation and differentiation. Exp Cell Res 1995;218:255–62.
[72] Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, et al. Increased bone formation in osteocalcin-deficient mice. Nature 1996;382:448–52.
[73] Fedde KN, Blair L, Silverstein J, Coburn SP, Ryan LM, Weinstein RS, et al. Alkaline phosphatase knock-out mice recapitulate the metabolic and skeletal defects of infantile hypophosphatasia. J Bone Miner Res 1999;14:2015–26.
[74] Anderson HC, Sipe JB, Hessle L, Dhanyamraju R, Atti E, Camacho NP, et al. Impaired calcification around matrix vesicles of growth plate and bone in alkaline phosphatase-deficient mice. Am J Pathol 2004;164:841–7.
[75] Ducy P, Starbuck M, Priemel M, Shen J, Pinero G, Geoffroy V, et al. A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. Genes Dev 1999;13:1025–36.
[76] Ducy P. Cbfa1: a molecular switch in osteoblast biology. Dev Dyn 2000;219:461–71.
[77] Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, et al. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 1997;89:755–64.
[78] Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, et al. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 1997;89:765–71.