RESEARCH ARTICLE

# Zinc deprivation inhibits extracellular matrix calcification through decreased synthesis of matrix proteins in osteoblasts

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**Scope:** Zinc is implicated as an activator for bone formation, however, its influence on bone calcification has not been reported. This study examined how zinc regulates the bone matrix calcification in osteoblasts.

**Methods and Results:** Two osteoblastic MC3T3-E1 cell subclones (SC 4 and SC 24 as high and low osteogenic differentiation, respectively) were cultured in normal osteogenic (OSM), Zinc deficient (Zn–, 1 μM), or adequate (Zn+, 15 μM) media up to 20 days. Cells (SC 4) were also supplemented with (50 μg/mL) or no ascorbic acid (AA) in combination with Zinc treatment. Zn– decreased collagen synthesis and matrix accumulation. Although AA is essential for collagen formation, its supplementation could not compensate for Zinc deficiency-induced detrimental effects on extracellular matrix mineralization. Zn– also decreased the medium and cell layer alkaline phosphatase ALP activity. This decreased ALP activity might cause the decrease of Pi accumulation in response to Zn–, as measured by von Kossa staining. Ca deposition in cell layers, measured by Alizarin red S staining, was also decreased by $Zn^{-}$.

**Conclusion:** Our findings suggest that zinc deprivation inhibits extracellular matrix calcification in osteoblasts by decreasing the synthesis and activity of matrix proteins, type I collagen and ALP, and decreasing Ca and Pi accumulation. Therefore zinc deficiency can be considered as risk factor for poor extracellular matrix calcification.

**Keywords:**
Alkaline phosphatase / Bone calcification / Osteoblasts / Type I collagen / Zinc

## 1 Introduction

Zinc is implicated as an activator for bone formation. However, the action of zinc on bone matrix calcification remains to be clarified. One of the major characteristics of zinc deficiency is retarded skeletal growth, which implies a role for zinc in bone formation. Unlike any other tissues, bone is not only made of cells but contains a large portion of mineralized extracellular matrix (ECM) [1]. Two major processes are involved in bone matrix formation by osteoblasts; (i) ECM maturation characterized by bone matrix protein synthesis, including type I collagen, alkaline phosphatase (ALP), osteopontin (OPN), bone sialoprotein, and osteocalcin (OC) (matrix maturation phase) and their secretion and accumulation in the matrix by the differentiated osteoblasts, and (ii) matrix mineralization by calcium deposition into the fibrillar collagen network (matrix mineralization phase) [2, 3]. Therefore, bone

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**Abbreviations: AA,** ascorbic acid; **ALP,** alkaline phosphatase; **ANOVA,** analysis of variance; **ECM,** extracellular matrix; **HSD,** Tukey's Honestly Significant Difference; **MV,** matrix vesicle; **OC,** osteocalcin; **OPN,** osteopontin; **OSM,** osteogenic medium; **Pi,** inorganic phosphate; **PPi,** inorganic pyrophosphate; **PNP,** para-nitrophenyl phosphate; **TPEN,** N,N,N′,N′-tetrakis-(2-pyridyl-methyl)-ethylenediamine

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extracellular mineralization by differentiated osteoblasts starts late and lasts throughout life. After mitogenic growth of osteoblast precursors, osteoblasts start to produce type I collagen, followed by ALP along with other bone-specific matrix proteins, including OPN, and OC [4]. In bone tissues, ECM communicates with osteoblasts through extracellular signal molecules and their receptors, such as BMP and collagen receptors [5–7]. Therefore, bone matrix is routinely mineralized to form a rigid matrix and ECM calcification is a major part of maintaining healthy bone formation [3, 8, 9].

In bone matrix calcification, two proteins have been a focus of interest: type I collagen and ALP. The former being the major matrix component protein providing structural scaffold to which minerals are formed, and the latter functions by removing the mineralization inhibitor pyrophosphate (PPi) thereby producing the inorganic phosphate (Pi) [8, 10–13]. Type I collagen accounts for 90% of the protein content with only a small amount of noncollagenous bone proteins and proteolytic enzymes comprising the remaining bone ECM [14]. Ascorbic acid (AA) stimulates collagen synthesis through proline hydroxylation, necessitating osteoblast differentiation and matrix mineralization both in vivo and in vitro [15]. ALP is present ubiquitously but is particularly abundant in bone and liver [3]. This enzyme hydrolyzes pyrophosphate (PPi), ATP, and a variety of organic phosphate compounds, and consequently can increase Pi which is a nucleation site of apatite crystal formation in extracellular conditions [4]. Therefore, bone matrix mineralization in bone tissue requires the synthesis and accumulation of a collagen-rich ECM and the activity of ALP. These two environmental circumstances are favorable conditions for bone matrix mineralization [13].

Zinc can be considered as a potential mediator of these two bone matrix proteins for bone mineralization since retarded skeletal development is associated with zinc deficiency [16–18]. Zinc is a cofactor of ALP, and the inactivation of ALP can be caused by the dissociation of this active zinc. It is reported that Pi can increase ALP activity through inhibiting the zinc dissociation, and therefore ALP activity in osteoblast can be regulated by balancing zinc and Pi levels, rather than by modulating the absolute amount of zinc within the enzyme [19]. Zinc was also found to induce the sodium-dependent vitamin C transporter (SVCT2) in osteoblastic cells, which implies zinc involvement in collagen synthesis and action [20]. In our previous study, we clarified that zinc deficiency decreases the transcription of osteoblast differentiation genes via downregulation of the bone-specific transcription factor Runx2 in MC3T3-E1 cells, and therefore decreasing and retarding osteoblast differentiation [21].

In this study, we investigated whether zinc deficiency inhibits ECM calcification and how zinc modulates matrix calcification in osteoblastic MC3T3-E1 cells. To clarify this mechanism, we assessed zinc regulation of type I collagen synthesis and ALP activity. We used two types of MC3T3-E1 cell subclones (subclone 4, SC 4, high osteoblast differentiation and subclone 24, SC 24, poor osteoblast differentiation), and treated with and without the collagen synthesis cofactor ascorbic acid (AA). Our study goal was eventually to understand how zinc modulates ECM biomineralization in osteoblasts.

## 2 Materials and methods

### 2.1 Cell culture and zinc depletion

Two different subclones of mouse origin osteoblastic MC3T3-E1 cells were used; subclone 4 (ATCC, CRL-2593, high osteoblast differentiation and mineralization) and subclone 24 (ATCC, CRL-2595, poor osteoblast differention and mineralization). Both subclones were seeded at a density of $1 \times 10^5$ cells/mL and maintained in regular growth medium (α-MEM with 10% FBS, 1 mM sodium pyruvate, and 1% penicillin) in a humidified atmosphere of 5% $CO_2$ at 37°C. At 100% confluency, cells were switched to differentiation media consisting of regular growth media plus 3 mM Pi and 50 μg/mL AA. Zn depletion was done by adding zinc chelator (*N,N,N′,N′*-tetrakis-(2-pyridylmethyl)–ethylenediamine [TPEN], 4 μM) and the designated zinc levels for Zn deficiency (Zn−, 1 μM) and Zn adequacy (Zn+,15 μM) were adjusted by externally adding $ZnCl_2$. Cells cultured in osteogenic differentiation medium without any Zn and TPEN treatment were used as normal osteogenic control (osteogenic medium, OSM). Cells were cultured up to 20 days as appropriate during osteoblast differentiation and mineralization phases.

### 2.2 Alizarin red S staining

Alizarin red S stain was used to monitor the mineralization of ECM by Ca accumulation. Cells were fixed with 2% paraformaldehyde and stained with 40 mM Alizarin red S (pH 4.2). The culture plates were photographed under a light microscope and mineralized nodules were shown as dark red center and light red peripheral area.

### 2.3 von Kossa staining

von Kossa staining was used to assess the accumulation of extracellular Pi which normally coprecipitates Ca ions. Cell cultures were treated with 5% silver nitrate solution incubated under UV light for 1 h at room temperature. Culture plates were photographed under a light microscope and mineralized nodules were shown as thick dark brown stripes resembling cuboidal cells.

 

### 2.4 Collagen staining and measurement

The amount of collagen in cell layers was assessed by Van Gieson staining. The cells were fixed with 2% formaldehyde and stained with Van Gieson reagent (acid fuchsin in picric acid). The concentration of cellular and medium collagen was measured using the Picrosirius red method. Cells were hydrolyzed with 0.5 M acetic acid and were allowed to dry at 37°C for 24 h. After drying, 100 μL of 0.1% Picrosirius red dye in picric acid was added to each well and incubated for 1 h at 37°C. The samples and standards were washed with 10 mM HCl to remove the unbound dye. Finally, the bound collagen was dissolved by adding 200 μL of 0.1 M NaOH for 5 min and absorbance was read at 540 nm. Cellular collagen concentration was normalized against total protein concentration.

### 2.5 ALP staining and enzyme activity assay

ALP staining and enzyme activity assay were performed as described previously [21]. Cellular ALP activity was normalized against total protein concentration measured using a BCA protein assay kit (Pierce, Rockford, IL, USA). The activity of ALP was expressed as nmol PNP/min/mL (media) or nmol PNP/min/mg protein (cellular).

### 2.6 RT-PCRs

Osteoblastic bone marker gene expression was measured by reverse-transcriptase PCR. Total RNA was extracted using Trizol Reagent (Gibco BRL, USA). For first-strand cDNA synthesis, 100 ng of RNA from each sample was reverse transcribed using 20 U of AMV reverse transcriptase and Oligo-p (dT) 1× random primers (Roche Diagnostics, USA). The resulting cDNAs were PCR-amplified by using a mixture of the corresponding forward and reverse primers (ALP [Fw: GCT GAT CAT TCC CAG GTT TT, Rv: CTG GGC CTG GTA GTT GTT GT], OC [Fw: AAG CAG GAG GGC AAT AAG GT, Rv: CTA AAC GGT GGT GCC ATA GAT], OPN [Fw: TGC ACC CAG ATC CTA TAG CC, Rv: TTT GTA GGC GGT CTT CAA GC], β-actin [Fw: TGG ACT TCG AGC AAG AGA TG, Rv: ATC TCC TTC TGC ATC CTG TCG]). The PCR conditions were 95°C for 2 min and then 28 cycles at 95°C for 30 s, 54°C for 45 s, and 72°C for 1 min and a final extension at 72°C for 5 min. The PCR products were separated on 1.8% agarose gel and band intensity was measured and normalized against the housekeeping gene intensity.

### 2.7 Western blotting

Cells were harvested and lysed with lysis buffer (150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 25 mM Tris-HCl, pH 7.6, supplemented with 1% protease inhibitor). Equal amounts of protein (100 μg) were resolved in SDS-PAGE under reducing conditions. Proteins were transferred to polyvinylidene fluoride membrane at 12 V for 30 min. Blots were blocked for 2 h at room temperature with 5% skimmed milk in PBS/0.1% Tween-20 (PBS-T) and incubated with primary antibodies overnight at 4°C. The blots were incubated with secondary antibodies conjugated to horseradish peroxidase. The blots were visualized with enhanced chemiluminescence (Super Pico Detection Reagent, Pierce) and quantified using the ChemiDoc Gel Quantification System (Bio-Rad, Hercules, CA, USA).

### 2.8 Statistical analysis

Statistical analysis of the data was performed using SPSS software (version 17.0). A two-way analysis of variance (ANOVA) was used to evaluate the effect of zinc, ascorbate, or the interaction between zinc and ascorbate. Within the ascorbate treatment groups (without AA or with AA), data were analyzed by one-way ANOVA, followed by Tukey's Honestly Significant Difference (HSD) test as post hoc comparison. Differences between the without AA and with AA groups within a time point were analyzed by *t*-test. Results were expressed as the mean ± SEM and differences in mean values are considered to be statistically significant at $p < 0.05$.

## 3 Results

### 3.1 Zinc deficiency decreases Ca deposits in ECM

The image of Alizarin red S stain and its corresponding extract showed that zinc deficiency (Zn−) decreased extracellular Ca deposition in both MC3T3-E1 SC 4 and SC 24 at 15 days (Fig. 1B, upper panel for microscopic images and lower panel for cell dish images). The decreased Ca deposits by Zn− are not related to cell number as shown by the morphology using phase-contrast microscopy (Fig. 1A). There was a notable formation of Ca deposits by zinc treatment from 5 days to 15 days. Also, the Ca deposition in ECM formed by the SC 4 cells resembled a fibrillar formation in which Ca deposits in matrix are associated within the collagen fibrils, in contrast to the Alizarin staining of SC 24, which shows a diffuse granular appearance of Ca deposits. The dissolved Alizarin extracted from the staining plates also confirmed the Ca staining pattern (Fig. 1B).

To assess Pi accumulation in the ECM by zinc treatment, von Kossa staining (Fig. 1C) was used to visualize Pi accumulation by coprecipitation with calcium ions [3, 10]. Consistent with the pattern of Alizarin staining, Zn− decreased phosphate accumulation in both subclones. The difference in mineral morphology of the two subclones was

 

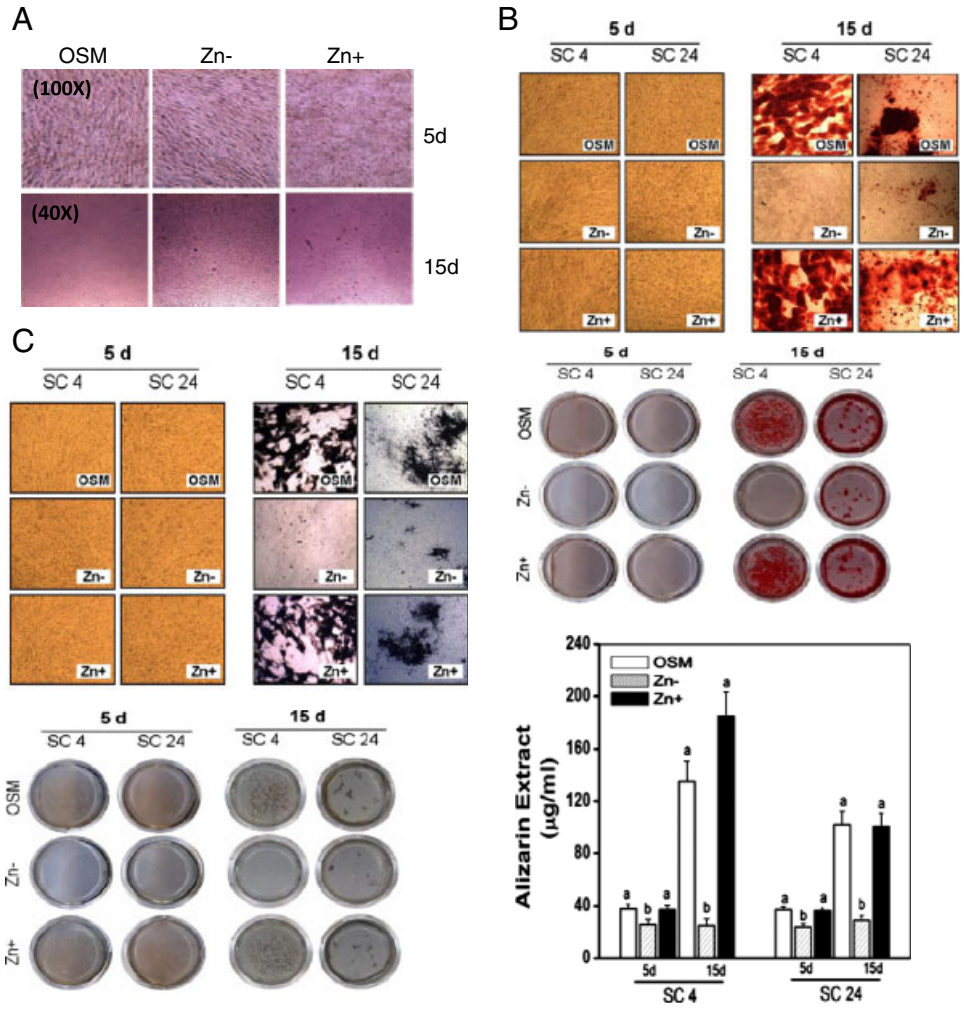


**Figure 1.** The influence of zinc on ECM Ca deposition on osteoblastic MC3T3-E1 cells. MC3T3-E1 cell subclone 4 (SC 4, high osteoblast differentiation) and subclone 24 (SC 24, poor osteoblast differentiation) were cultured in OSM (normal osteogenic media), Zn− (1 μM) and Zn+ (15 μM) medium. (A) Cell morphology under Zn treatment in SC4. (B) ECM Ca deposits for matrix mineralization was measured by staining with Alizarin red S and shown by light microscopy and culture dish images (representative of $n = 8$). Quantitative analysis of matrix Ca deposits, measured by solubilizing the Alizarin red S dye, yielded the same pattern, with a significant increase in Zn+ in both subclones (mean ± SEM. $n = 6$, Tukey's HSD test, one-way ANOVA within time factor in each subclone, $p < 0.05$). (C) ECM inorganic phosphorus (Pi) accumulation in relation to zinc treatment. The accumulation of Pi as phosphate ion in the ECM was measured by von Kossa staining and showed the same pattern as Alizarin staining (representative images of $n = 8$).

also observed, and the pattern is very similar with that found with Alizarin staining. Microscopic images revealed a finer fibrillar form of the mineralized nodules for the phosphate precipitate that are equally distributed within the collagen fibrils of SC 4. However, a more concentrated (not evenly distributed) and granular form of mineralized nodules was exhibited by the less-mineralizing osteoblast SC 24.

### 3.2 Zinc deficiency decreased collagen synthesis and matrix accumulation

We next assessed the distribution of collagen within the ECM by Van Gieson staining. The detrimental effect on matrix collagen accumulation by zinc deficiency (Zn−) was observed and this pattern was more prominent in SC 4 (highly mineralizing clone) and with the presence of AA, with increased study duration (Fig. 2A). Since only SC 4 showed a prominent zinc effect on collagen accumulation in ECM, we cultured only SC 4 for collagen concentration measurement and protein expression. As compared with Zn+, Zn− decreased both cellular collagen synthesis (Fig. 2B) and collagen secretion to ECM (Fig. 2C) with or without AA supplementation. Even with AA treatment (+AA groups in Fig 2B and C), the collagen concentration in Zn− remained significantly decreased compared with the level in the OSM control or Zn+ ($p < 0.001$), which implies that even in the presence of AA as cofactor for collagen synthesis, the detrimental effect of zinc deficiency on the synthesis and secretion of the collagen is not mitigated. There was, however, a zinc-independent AA effect on medium collagen concentration (Fig. 2C, $p < 0.001$) by two-way ANOVA. An interaction effect of zinc and AA was observed, except on medium collagen concentration at 10 days.

We also measured the cellular synthesis of procollagen type I by Western blot, which is the precursor of the type I collagen and being secreted as type I collagen. The intensity of procollagen type I protein expression was decreased by Zn− in both AA supplemented and unsupplemented medium (Fig. 2D, upper two panels). Zn− could not compensate cellular procollagen type I protein synthesis even with AA addition at 5 days and at 15 days, and the same lack of effect was found for the cellular and medium collagen concentrations.

 

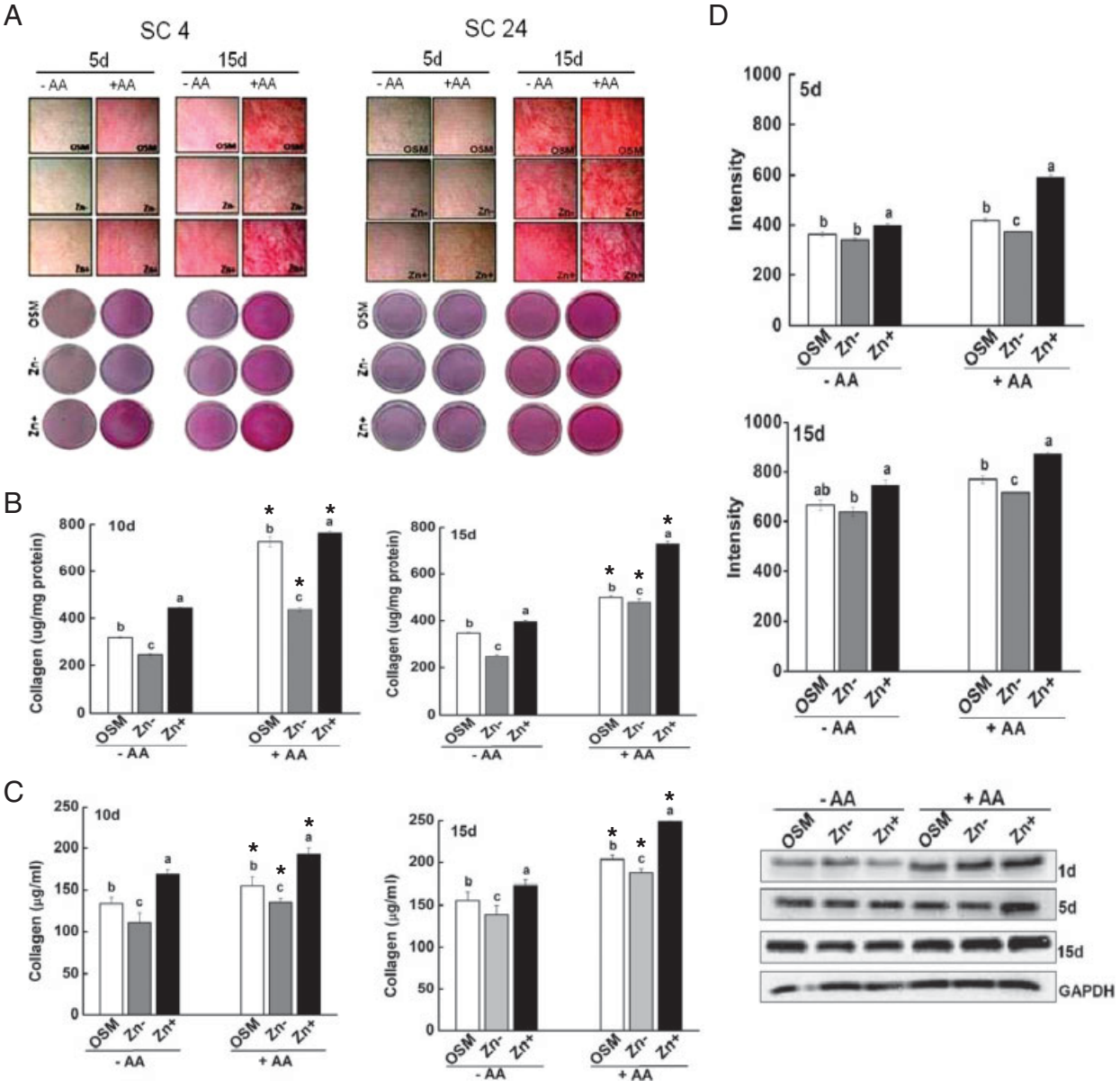


**Figure 2.** ECM accumulation (A), cellular synthesis (B), secretion to the medium (C), and cellular protein expression (D) of collagen by zinc deficiency in MC3T3-E1 cells. (A) MC3T3-E1 cells subclone 4 (SC 4) and subclone 24 (SC 24) were cultured and ECM collagen accumulation was measured by Van Gieson staining. Both light microscopy (upper panel, 100 ×) and cell culture images (lower panel) are shown. (B and C) Cellular and medium collagen concentrations in MC3T3-E1 cell culture (SC 4) were measured by the Picrosirius red method and shown in relation to Zn deficiency (1 μM, Zn−) and Zn adequacy (15 μM, Zn+) and the normal osteogenic differentiation media (OSM) control, without and with (50 μg/mL) AA. Different superscripts mean significant differences within AA treatments (mean ± SEM, $n = 4$ for cell and $n = 8$ for medium, Tukey's HSD test, one-way ANOVA, $p < 0.05$). Asterisks indicate significant differences between AA treatment by zinc ($n = 8$, $p < 0.001$) by *t*-test. Two-way ANOVA analysis for zinc and AA interaction: $p < 0.001$ for all AA, zinc, and AA × zinc interaction in cell at 10 days and 15 days (A), and medium collagen concentration at 15 days (B, right panel). *p*-Value of 0.844 for AA × zinc interaction for medium collagen concentration at 10 days (B, left panel). (D) Zn sufficiency (Zn+) upregulated cellular procollagen type I protein expression, regardless of the addition of AA, whereas Zn deficiency (Zn−) could not compensate this protein synthesis even under the condition of AA addition (AA+) in both days 5 and 15 (upper panel) as measured by Western blotting (mean ± SEM; $n = 3$, duplicated measurement, Tukey's HSD test within AA treatment, one-way ANOVA, $p < 0.05$). Two-way ANOVA analysis was carried out at $p < 0.001$ for AA, zinc, and AA × zinc interaction at day 5 and for AA and zinc at day 15, AA × zinc interaction at day 15 is nonsignificant, $p = 0.364$.

 

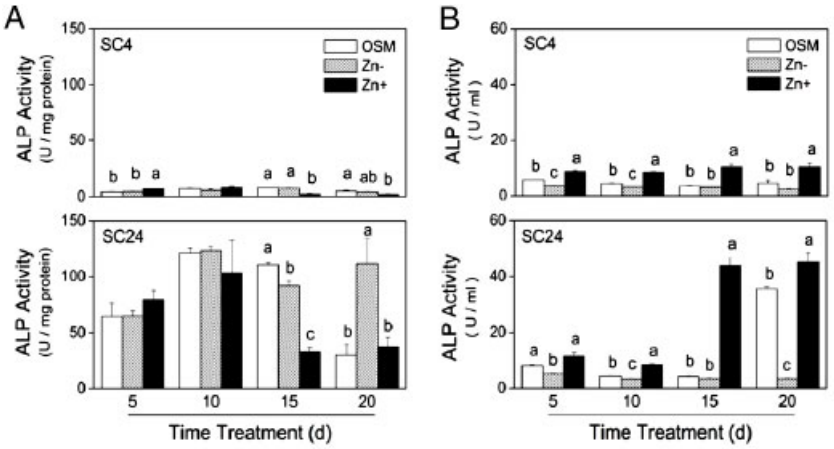


**Figure 3.** ALP activity of cellular (A), extracellular (B) and matrix cell layers (C) in relation to zinc treatment in osteoblastic MC3T3-E1 cell subclones. Both subclone 4 (SC 4, high osteoblast differentiation) and subclone 24 (SC 24, low osteoblast differentiation) were cultured up to 20 days. Values are expressed as Unit (U) (nmol *p*-nitrophenol/min/mg protein of cell lysate and per milliliter of medium). Different superscripts indicate significant difference within time point (mean ± SEM, $n = 4$, Tukey's HSD test, one-way ANOVA, $p < 0.05$). OSM (normal osteogenic differentiation media), Zn− (1 μM), and Zn+ (15 μM).

### 3.3 Zinc deficiency decreased ECM ALP activity

Cellular ALP activity did not show any consistent pattern (Fig. 3A), and this could be due to the immediate secretion of ALP into the media, since ALP is a secretory protein. ALP activity in media was decreased by Zn−, but increased by Zn+ in both SC 4 and 24 subclones (Fig. 3B). Staining for ALP activity in cell matrix also showed that Zn− decreased ALP activity in cell layers (Fig. 3C).

Characteristically, MC3T3-E1 SC 24 cells (poor osteoblast differentiation) showed a higher transcription of ALP than SC 4 cells (high osteoblast differentiation), in general [22], and in our experiment we confirmed that SC 24 cells showed higher ALP activity, compared with SC 4 (Fig. 3A and C).

### 3.4 Expression of noncollagenous bone matrix proteins by zinc

The expressions of osteogenic proteins including ALP, OPN, and OC which are associated with matrix maturation and calcification were also assessed. Expression of ALP as a mineralization-initiating protein and other matrix proteins for maturation and mineralization (OPN and OC) were measured at 15 days in SC 4. Both gene transcripts (Fig. 4A) and protein (Fig. 4B) expression of ALP and OPN were markedly decreased by zinc deficiency, whereas OC expression was not greatly affected at day 15.

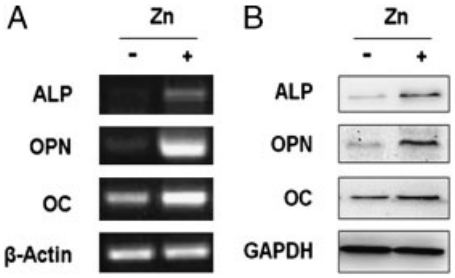


**Figure 4.** ALP and osteoblast-specific gene mRNA (A) and protein (B) in relation to zinc treatment in MC3T3-E1 cells. MC3T3-E1 cells subclone 4 (SC 4, high osteoblast differentiation) were cultured in Zn− (1 μM) or Zn+ (15 μM) for 15 days. mRNA and protein expression were measured by RT-PCR and Western blotting, respectively (representative image of $n = 2 \sim 3$. ALP, alkaline phosphatase; OPN, osteopontin; and OC, osteocalcin).

## 4 Discussion

Bone matrix routinely mineralizes to form a rigid impermeable matrix [23]. In osteoblastic MC3T3-E1 cell

 

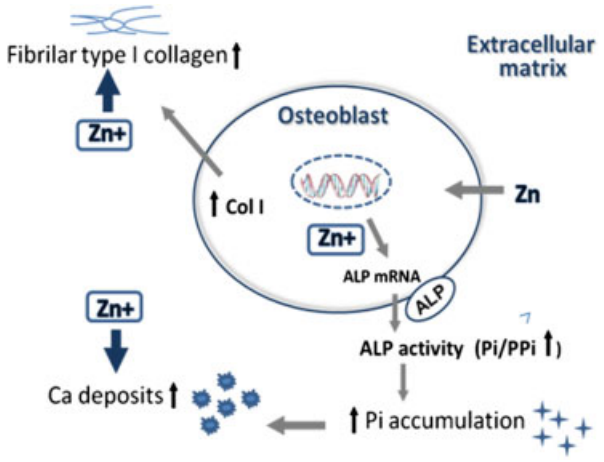


**Figure 5.** The expected scheme for zinc-stimulated extracellular matrix calcification in osteoblastic MC3T3-E1 cells. Extracellular matrix (ECM) calcification in bone tissues is achieved mainly by Ca and inorganic phosphorus (Pi) accumulation into the meshed matrix protein, type I collagen. The suggested role for zinc in ECM calcification from this study are: (1) Zinc can up-regulate the gene and protein expression of ALP, increase its synthesis and secretion into the ECM, and enhance its activity to produce Pi from pyrophosphate (PPi) and other organic phosphate compounds. The increased ALP activity thereby can increase the ratio of Pi/PPi, by removing the mineralization inhibitor, PPi. This increased ratio of Pi/PPi can induce the condition favorable for Pi accumulation in the cell matrix. The increased accumulation of Pi acts as nucleation site for Ca deposits in the ECM thus increasing matrix calcification. (2) Zinc can up-regulate type 1 collagen protein synthesis and secretion into the ECM thus providing a structural scaffold to which Ca and P deposits are accumulated in the cell matrix. The up-regulation of these two major matrix proteins, type I collagen and ALP, by zinc can result in a concomitant increase of Ca deposits in the ECM as well as presumably up-regulation of mineralization-associated proteins such as osteopontin and osteocalcin.

culture, the stages of proliferation, differentiation, and mineralization are analogous to the respective stages of in vivo bone formation, namely, preosteoblast recruitment, osteoblast precursor differentiation to postmitotic matrix-producing osteoblasts, and osteoid mineralization by terminally differentiated osteoblasts [24]. We have previously reported that zinc deficiency retards osteogenesis transiently through delayed Runx2 expression [21]. Although Runx2 is a transcriptional activator of osteoblast differentiation, its mere presence alone is not sufficient for osteoblast differentiation as demonstrated by similar levels of Runx2 expression in mineralizing and nonmineralizing subclones of MC3T3-E1 [22], indicating that other factors must be required for the induction of the osteoblast phenotype.

In this study, we investigated the effect of zinc deficiency on extracellular biomineralization using two subclones of murine osteoblastic MC3T3-E1 cells with low and high differentiation potential. In bone tissue, osteoblasts are located on the surface of the bone tissue, actively producing matrix which is not yet calcified (osteoid tissue), and the ECM of this osteoid region eventually calcifies as an osteoid-like matrix (dense collagen). The interaction between the osteoblasts and the ECM maintains the optimal environment for bone matrix mineralization [4, 25]. Our findings demonstrate that zinc deficiency decreased ECM biomineralization by reducing the synthesis and activity of type I collagen and ALP. Our proposed scheme for zinc action on ECM biomineralization is illustrated on Fig. 5.

ALP, a homodimeric metalloenzyme that contains zinc as cofactor, catalyzes the hydrolysis of phosphomonoester (such as pyrophosphate, PPi) with the release of Pi [8]. ALP is inactivated when the zinc at the active site of the enzyme dissociates [19]. In this study, we confirmed that zinc increased cellular ALP transcript and protein levels, and also medium and ECM ALP activity. PPi, one of the most abundant substrates of ALP, inhibits mineralization by binding to mineral crystals, such as Ca or P. This is partly the reason why the presence of PPi in the extracellular fluid prevents mineralization of soft tissues [8, 11, 12, 26]. ALP hydrolysis of PPi to Pi increases the Pi/PPi ratio in the ECM, and mineralization in bone is tightly linked to this ratio [11, 27]. Therefore, ALP regulates this Pi/PPi homeostasis by degrading the ubiquitous mineralization inhibitor PPi [27], thus providing the extracellular Pi pool which largely determines the rate of hydroxyapatite crystal formation within the collagen fibrils in bone tissue [12]. Inactivation of ALP in serum also prevents the calcification of collagen [28] which further underlines its significant role in biomineralization. In bone matrix, mineralization is initiated inside extracellular organelles called matrix vesicles (MVs). These vesicles are originally synthesized, budded, and pinched-off from the outer plasma membranes of osteoblasts and secreted into the ECM. MVs contain high concentrations of $Ca^{2+}$, Pi, and ALP [4, 29]. On reaching the matrix, initiation of the first mineral crystals within MVs is augmented by the activity of vesicular ALP which produces Pi for nucleation of the crystal growth. In this study, we showed that zinc increased ECM phosphate accumulation, measured by von Kossa dye which combines with phosphate ions in the matrix. This increased Pi accumulation in matrix would be possibly through zinc-stimulated ALP activity, which increased Pi production from PPi in the matrix.

Consistent with the ALP results in this study, zinc also stimulated the synthesis and matrix accumulation of type I collagen. The formation of the collagenous ECM contributes not only to the matrix structural role, but also to the shutdown of proliferation which signals the onset of synthesis of osteoblast-specific proteins for osteoblast differentiation [14, 30]. Disruption of collagen matrix synthesis and accumulation, either through the absence of AA or by using collagen synthesis inhibitors, can block osteoblast marker synthesis [31]. Along with its stimulatory effect on osteoblast differentiation, collagen is critical for bone matrix mineralization since mineralization occurs along collagen fibrils

 

in bone matrix [28]. Our findings demonstrate that zinc stimulated the synthesis and secretion of type I collagen to the ECM where calcification occurs and these results were further confirmed by staining of the accumulated collagen in ECM.

Also, zinc increased the levels of secreted and synthesized collagen in cultures both with and without AA supplementation. This result implies two important points. First, zinc may increase collagen production even in cultures lacking AA which is a cofactor for collagen synthesis. Second, even in cultures supplemented with an essential cofactor, the presence of AA could not compensate for the detrimental effect of zinc deficiency on collagen matrix production. The expression of type I procollagen protein, which is synthesized by osteoblasts and eventually being secreted as a form of type I collagen outside the cells, was not significantly affected by zinc treatment in this study. It is unlikely that the slight increase in collagen protein expression can significantly elevate collagen matrix production in general. Thus, it might be more appropriate to infer that the action of zinc on collagen matrix production might not be to increase the cellular protein expression per se, but rather to increase the subsequent hydroxylation and fibril assembly and turnover of type I collagen, after secretion into the ECM which eventually results in the formation of a stable ECM. Hydroxylation is essential in the processing of procollagen to a properly folded and stable collagen triple helix in the matrix. Hydroxylated procollagen can readily assume a triple-helical conformation that is secreted much faster than nonhelical collagen [31]. Nonhydroxylated procollagen can be denatured easily and this denatured procollagen cannot be processed to collagen [32]. From our results of zinc-stimulated collagen synthesis in cells, media and matrix accumulation, it could be considered that zinc is involved in the hydroxylation of procollagen to be secreted to the ECM.

The bone matrix protein OPN is downregulated by zinc deficiency both at the mRNA and at the protein levels but osteoclacin levels did not change. Downregulation of OPN might be coupled with a decrease in matrix collagen resulting in diminished ALP activity. There are numerous studies indicating the dependency of the induction of ALP and other osteoblast-related genes including OPN and OC on the collagen content of the ECM. Although the mechanism of cell–matrix interaction remains obscure, one possible hypothesis explaining this is that the formation of a properly laid-out collagenous ECM might generate a matrix-associated signal that allows the subsequent induction of osteoblast-related genes and their proteins [15]. So far, what is clear is that a collagen-rich matrix plays an integral part in regulating the subsequent induction of bone marker proteins in osteoblast differentiation and mineralization. Although the mechanisms by which zinc affects bone formation remain to be defined, accumulating evidence indicates that zinc might mediate both in the osteoblast commitment and in the osteoblast phenotype expression stages. For example, in osteoblastic MC3T3-E1 cells, zinc deficiency has been shown to downregulate the gene and protein expressions of Runx2 [21], the bone-specific transcription factor controlling osteoblastic differentiation. Moreover, mice deficient in the zinc transporter Slc39a/Zip13 showed apparent skeletal and connective tissue disease possibly in part due to BMP/TGF-β signaling [33]. Zinc was also shown to increase mineralization in MC3T3-E1 osteoblastic cells through intra and extracellular zinc movement involving metallothionein and zinc transporters [34]; however, the specific zinc action involved in the development of an efficient ECM has not yet been clarified.

## 5 Concluding remarks

In conclusion, our findings showed that zinc deficiency decreases bone matrix mineralization by decreasing the synthesis of two major matrix proteins, type I collagen, and ALP. The decrease in ALP can be coupled with a decrease in the rate of removal of PPi, a ubiquitous mineral inhibitor. The presence of this inhibitor of mineralization, in turn, can decrease the rate of Ca mineral deposition within the collagenous ECM. We also suggest that zinc deficiency may also impair the generation of matrix-associated signals that mediate osteoblast–ECM interactions for bone mineralization. Our findings provide an insight into the role of zinc in ECM calcification and further detailed investigations for how zinc mediates the biomineralization in bone tissue are required.

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*The authors have declared no conflict of interest.*

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