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Review Article

Ultrastructural and biochemical aspects of matrix vesicle-mediated mineralization

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Mineralized nodule

Summary Matrix vesicle-mediated mineralization is an orchestrated sequence of ultrastructural and biochemical events that lead to crystal nucleation and growth. The influx of phosphate ions into the matrix vesicle is mediated by several proteins such as TNAP, ENPP1, Pit1, annexin and so forth. The catalytic activity of ENPP1 generates pyrophosphate (PPi) using extracellular ATPs as a substrate, and the resultant PPi prevents crystal overgrowth. However, TNAP hydrolyzes PPi into phosphate ion monomers, which are then transported into the matrix

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vesicle through Pit1. Accumulation of Ca^{2+} and PO_4^{3-} inside matrix vesicles then induces crystalline nucleation, with calcium phosphate crystals budding off radially, puncturing the matrix vesicle's membrane and finally growing out of it to form mineralized nodules.
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1. Introduction

Bone is a living mineralized tissue composed of calcium phosphates and a variety of organic materials, with collagen being the most abundant. Bone mineralization has two phases: primary and secondary. Primary mineralization is orchestrated by osteoblasts; osteoblasts secret a large amount of collagen fibrils, non-collagenous proteins, and matrix vesicles, which are extracellular vesicles that trigger mineralization *via* membrane transporters and enzymes. The degree of the primary mineralization is controlled by fine-tuning bone formation and mineral apposition rates. In contrast, secondary mineralization is a phenomenon whereby there is a gain in bone mineral density after primary mineralization. It is hypothesized that secondary mineralization is regulated physicochemically, *i.e.*, through crystal maturation, and by the osteocytic network inside the mineralized bone matrix. Still, the mechanisms behind secondary mineralization are relatively untapped.

In bone, primary mineralization can be divided into two phases: first, matrix vesicle-mediated mineralization, and second, collagen mineralization. During the process of the matrix vesicle-mediated mineralization, osteoblasts regulate vesicle synthesis as well as the activity of membrane transporters and enzymes with which matrix vesicles are equipped. Discovery of matrix vesicles was a breakthrough in the field of bone mineralization [1—7]. Long before that, it was proposed that alkaline phosphatase may supply phosphates by hydrolyzing phosphate substrates and then help forming crystalline calcium phosphates [8]. However, this theory is based on the physicochemical regulation of bone mineralization. In contrast, the theory behind matrix vesicle-mediated mineralization sustains that the processes are mainly under the control of osteoblasts through the regulation of membrane transporters/enzymes and surrounding extracellular organic materials. Knowledge of the ultrastructure and biological activities of membrane transporters/enzymes may clarify the cellular mechanisms of matrix vesicle-mediated mineralization and explain, for instance, why mineral appositional rate is higher in regions of accelerated bone turnover.

In this review, we will present the ultrastructural and biochemical aspects of matrix vesicle-mediated mineralization in bone.

2. Ultrastructural aspects of matrix vesicle-mediated mineralization in bone

Matrix vesicles are extracellular vesicles enveloped by a plasma membrane (ranging 30—1000 nm in diameter) secreted by osteoblasts [2] (Fig. 1). Matrix vesicles bear several membrane transporters and enzymes on their membranes and in their interior, providing a nurturing microenvironment for calcium phosphate nucleation and subsequent crystal growth. Mineralization begins when a crystalline calcium phosphate, *i.e.*, hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], appears inside the matrix vesicles, growing and eventually breaking through the vesicle's membrane to form mineralized nodules—also known as calcifying globules (Figs. 1 & 2). Under transmission electron microscopy (TEM), each hydroxyapatite crystal has a small, ribbon-like structure profile approximately 25 nm wide, 10 nm high and 50 nm long [9,10].

2.1. Ultrastructural properties of the matrix vesicles under electron microscopy

Some of these incipient mineral crystals were initially found associated with the inner leaflet of the matrix vesicle mem-

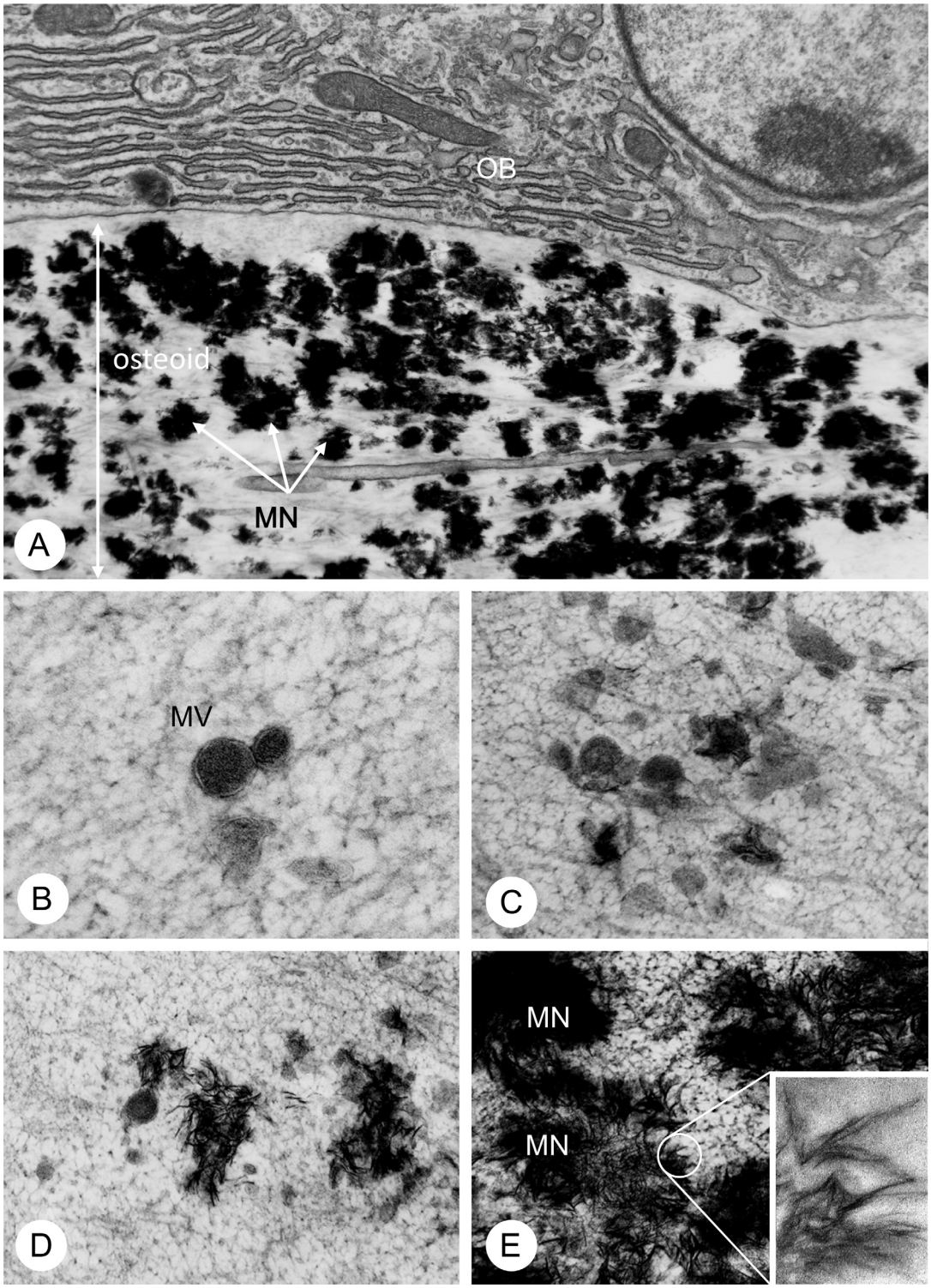


Figure 1 TEM observation of matrix vesicles and mineralized nodules in osteoid.
(A) TEM image of ultratrsucture of osteoid underlying mature osteoblasts (OB). Notice many mineralized globular structures referred to as mineralized nodule (MN) in the osteoid. (B—E) TEM images of matrix vesicles (MV) (B), appearance of mineral crystals in the matrix vesicles (C), exposure of mineral crystals out of the matrix vesicles (D) and mineralized nodule (MN) formation (E). An inset demonstrate a highly-magnified image of needle-shape of mineral crystals.
Panel A is derived from Ref. [71] (Amizuka and Ozawa), while panels B—F are modified from Ref. [72] (Amizuka et al.).

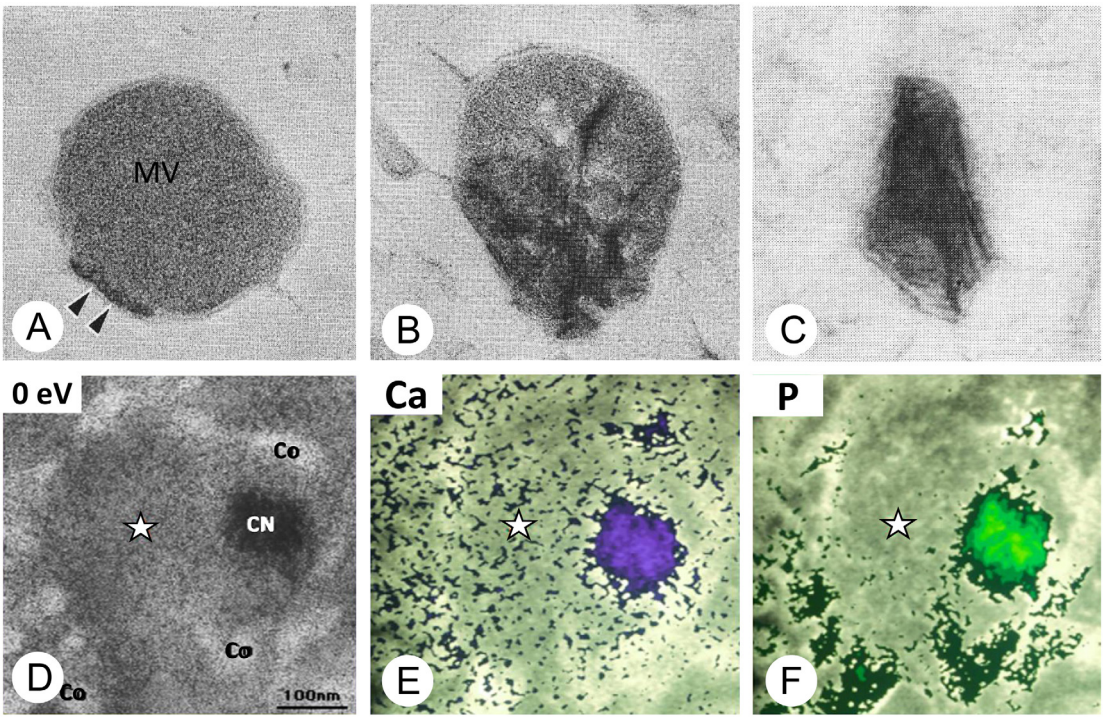


Figure 2 Highly-magnified images of matrix vesicles and elemental mapping of calcium and phosphate ions.
(A—C) TEM images of matrix vesicles (MV). (A) amorphous electron-dense structure (double arrows, black) are shown to be associated with plasma membrane of the matrix vesicle. (B) The grown mineral crystals (black structures) are seen inside the matrix vesicles. (C) Mineral crystals are getting out of the matrix vesicles. (D—F) TEM image (D) and elemental mapping of calcium (Ca, panel E) and phosphorus (P, panel F) assessed by electron energy loss spectroscopy. Note that calcium (Ca) was evenly distributed in the peripheral region of matrix vesicles, while phosphorus (P) is predominant in collagen fibrils.
The images of A—C are modified from Ref. [73] (Ozawa et al.), and D—F are from Ref. [17] (Hoshi et al.).

branes, and it seems plausible that crystal nucleation would begin at that specific site. Matrix vesicle membranes are rich in acidic phospholipids such as phosphatidylserine and phosphatidylinositol, which have high affinity for Ca^{2+}. The affinity of phosphatidylserine for Ca^{2+} is particularly high and can produce a stable calcium phosphate—phospholipid complex associated with the inner leaflet of the vesicle's membrane [7] (Fig. 2A). The possibility that such complexes may play an important role in crystal nucleation has been pointed out before [11,12]. However, the early phases of calcium phosphate nucleation inside the matrix vesicles may be rather amorphous [13], but gradually become crystalline (hydroxyapatite) in later stages. Calcium phosphate crystals formed inside matrix vesicles can grow through the addition of Ca^{2+} and PO_4^{3-}, which enter the vesicles through the action of membrane transporters and enzymes. Inside the matrix vesicle, ''needle-shaped'' crystalline calcium phosphates form a stellate assembly, bud off radially from the vesicle's interior, and then, rip through the plasma membrane to form mineralized nodules, also referred to as calcifying globules [6] (Figs. 1E and 2D). While the needle-shaped crystals are identified as crystalline structures by electron diffraction, freeze-substitution and cytochemical calcium detection methods such as κ-pyroantimonate combined with energy-dispersive X-ray spectroscopy may show them as non-crystalline structures containing calcium and phosphate [14—16].

According to observations of the osteoid in bone derived from the quick frozen-freeze substitution technique with electron energy loss spectroscopy, which enables elemental mapping at the molecular level, calcium was evenly distributed in the proteoglycan-rich, peripheral region of matrix vesicles, whereas phosphate was detected predominantly in collagen fibrils [17] (Fig. 2D—F). Therefore, it seems feasible that, in non-calcified sites, the extracellular meshwork of organic substances limits the production of hydroxyapatite and inhibits precipitation of calcified crystals by controlling the spatial distribution of Ca^{2+} and PO_4^{3-}, even if the extracellular fluid is supersaturated with those ions. In addition, a biological mechanism of PO_4^{3-} supplementation and subsequent transport into matrix vesicles must be in place, since PO_4^{3-} is not abundant in the periphery of matrix vesicles. Later in the text, we will discuss about the biological actions of membrane transporters and enzymes to produce PO_4^{3-} and to warrant the influx of Ca^{2+} and PO_4^{3-} into the matrix vesicles.

2.2. Mineralized nodules developed from matrix vesicles

Matrix vesicles and developing mineralized nodules can be observed in the osteoid below mature osteoblasts (Fig. 1A). It seems that the osteoid provides an adequate microenvironment for the development of mineralized nodules in

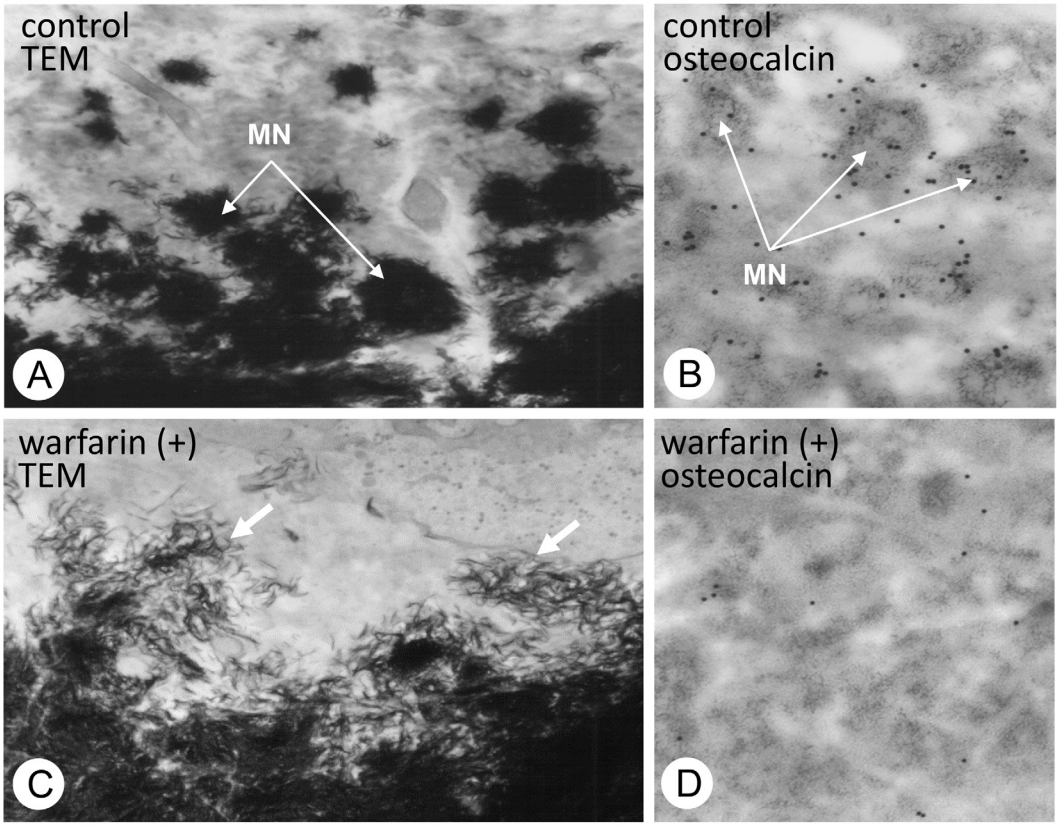


Figure 3 TEM observation on mineralized nodules of normal rats and warfarin-treated rats.
Panels A and C demonstrate the ultrastructure of mineralized nodules in the osteoid of normal and warfarin-administered rat bones. In control group, globular mineralized nodules (MN) are shown to be composed of many needle-shaped mineral crystals (A). However, the warfarin-administered osteoid shows dispersed mineral crystals throughout the osteoid (C). (B and D) Immunoelectron microscopy for osteocalcin localization. Osteocalcin immunoreactivity (black particles) can be seen on the mineralized nodules (MN, grey colored globular structures) of the control osteoid (B), while little immunoreactivity for osteocalcin is seen in the warfarin-administered osteoid (D).
The images are derived from Ref. [29] (Amizuka et al.).

bone. Even though the vesicle's membrane is ripped during the process of crystal growth, the mineralized nodules might retain the membrane-associated enzymes and transporters, which will be mentioned later in this text.

Mineralized nodule is a globular assembly of numerous needle-shaped mineral crystals that has been exposed to extracellular environment (Fig. 1E). It seems likely that the growth of mineralized nodules is regulated by a large number of extracellular organic materials in the osteoid. Among them, osteopontin is especially suited to the task of regulating mineralization, because it effectively inhibits apatite formation and growth [18,19]. Osteopontin is localized in the periphery of mineralized nodules, where it might act as a blocker of excessive mineralization [20]. Since other organic materials can combine with osteopontin [21], they can form the so-called ''crystal ghosts'' [22—24]. Among these materials, osteocalcin is known for containing γ-carboxyglutamic acid and for their ability to bind to mineral crystals [25—27]. Warfarin, which is an inhibitor for γ-carboxylation of glutamine residues, induces an embryopathy consisting of nasal hypoplasia, stippled epiphyses and distal extremity hypoplasia when given to women in the first trimester of pregnancy [25,28]. In our observations, the administration of warfarin resulted in the dispersion of numerous fragments of needle-shaped crystal minerals throughout the osteoid [29] (Fig. 3). Recently, γ-carboxylase-deficient mice revealed the same abnormality with disassembled, scattered crystal minerals in bone [30]. Therefore, osteocalcin may play an important role in the globular assembly of needle-shaped mineral crystals, probably binding the organic components of the crystal sheath together.

3. Enzymes and membrane transporter are necessary for matrix vesicle-mediated mineralization in bone

Several enzymes and proteins found in the matrix vesicles are involved in the metabolism of proteoglycans and pyrophosphate. Among these enzymes and membrane transporters, tissue nonspecific alkaline phosphatase (TNAP), ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), and others, *e.g.*, annexin and ankylin protein (ANK) and sodium/phosphate co-transporter type III (Pit1) appear to a play pivotal role in matrix vesicle-mediated mineralization.

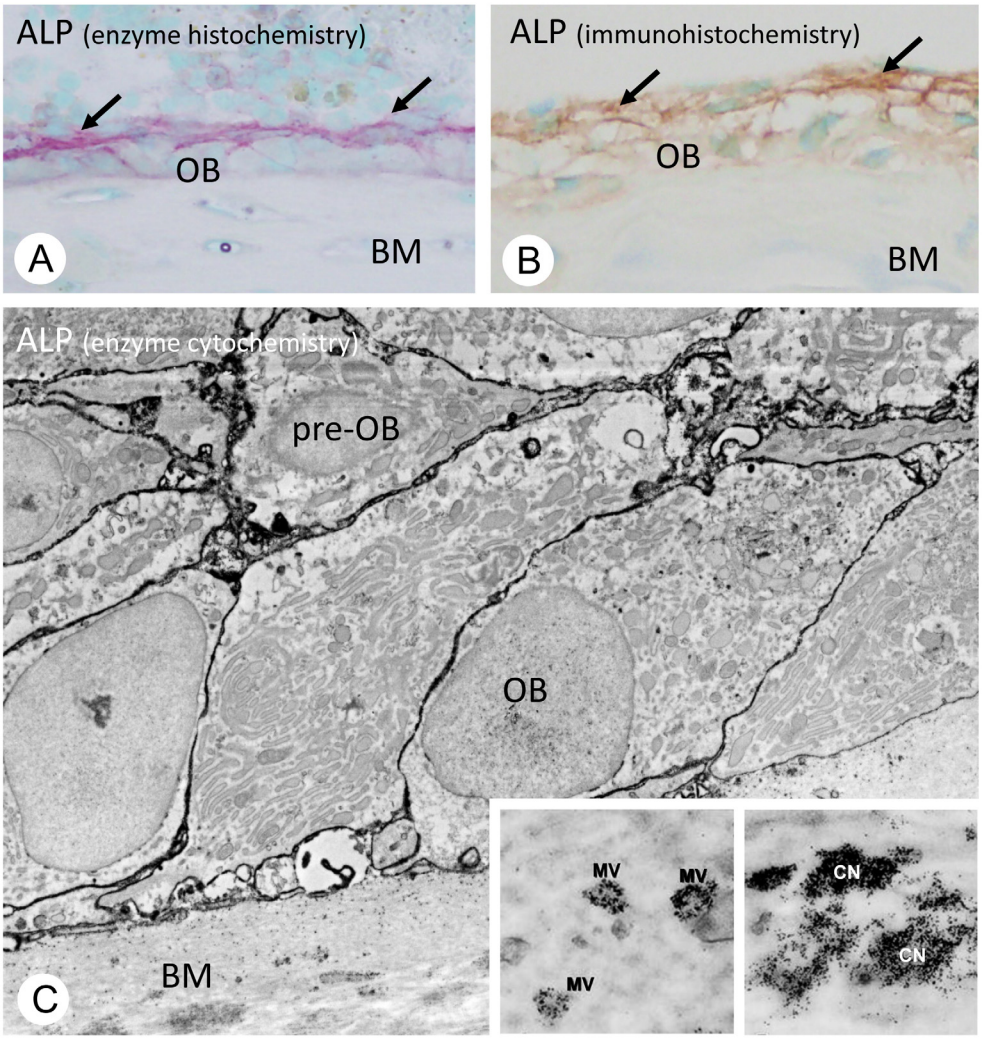


Figure 4 Histochemical localization of alkaline phosphatase in bone.
(A and B) Enzyme histochemistry of alkaline phosphatase (ALPase, red color) and tissue nonspecific ALPase (TNAP, brown color) immunohistochemistry. Both histochemical technique consistently reveal an intense enzymatic activity of ALPase and immunoreactivity of TNAP in the regions of perosteoblasts (arrows, in A and B), rather than mature osteoblast (OB) located on bone matrix (BM). (C) TEM image of ALPase enzyme cytochemistry. Note the ALPase activity (black) can be seen on cell membranes of preosteoblasts (pre-OB) and mature osteoblasts (OB) on the bone surfaces. Insets demonstrate the ALPase enzyme activity on matrix vesicle (MV) and mineralized nodule referred to as calcified nodules (black, CN).
Panels A and B are derived from Ref. [72] (Amizuka et al.), while panel C is from Ref. [71] (Amizuka and Ozawa).

3.1. Biological function of tissue nonspecific alkaline phosphatase (TNAP) in matrix vesicle-mediated mineralization

One of the most important enzymes to initiate mineralization in bone is TNAP, a glycosylphosphatidylinositol anchor enzyme associated with cell membranes (Fig. 4). TNAP can hydrolyze various phosphate esters, especially pyrophosphate (PPi), and is responsible for the production of inorganic phosphate, *i.e.*, TNAP serve as pyrophosphatase to generate PO_4^{3-} monomer. The resultant PO_4^{3-} is transported into the matrix vesicles by means of sodium/phosphate co-transporter type III (Pit1). Therefore, many believe TNAP is a potent inducer of mineralization.

In bone, TNAP activity has been detected on osteoblasts and matrix vesicles [31,32]. Interestingly, the distribution of TNAP on the cell membrane is not uniform in osteoblasts, which reflect the cell polarity with distinct basolateral and secretory (osteoidal) domains (Fig. 4). It has been reported that the plasma membrane Ca^{2+} transport ATPase was restricted to the osteoidal domain of the osteoblastic cell membrane, while TNAP was predominantly present on the basolateral domain [33]. Consistently, using specific antiserum to TNAP [34], we could observe relatively intense immunoreactivity and enzymatic activity for TNAP on preosteoblasts and on the basolateral aspect of mature osteoblasts' membranes [35]. Thus, in bone, the membranes featuring intense activity of TNAP are not identical to those

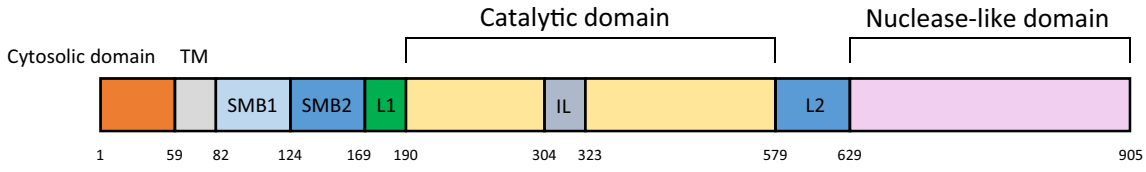


Figure 5 Domain organization of mouse ENPP1. Modified from Ref. [44] (Kato et al.).

that serve as sites for matrix vesicle formation, for matrix vesicles derive from the secretory (osteoidal) cell membrane of osteoblasts—which show weak TNAP enzymatic activity. While the actual reason for that difference is still a matter of debate, it could be that high levels of TNAP activity in the matrix vesicles would induce mineral crystals overgrowth within the osteoid.

Mice homogyzous for *Tnap* gene depletion have been generated [36,37] and mimic severe hypophosphatasia, indicating that TNAP is likely involved in mineralization. While TNAP might act as a pyrophosphatase [38], other pyrophosphatases may exist. *Tnap*$^{-/-}$ mice are born with intact bones, but gradually develop growth retardation and other skeletal deformities. Although the absence of endogenous TNAP activity did not result in complete lack of mineral uptake in skeletal tissues [37], *Tnap*$^{-/-}$ mice revealed a severe disturbance of the growth plate, suggesting abnormal endochondral ossification [39]. It was shown that depletion of *Tnap* gene results not only in hypomineralization of the skeleton, but also in a severe disorder of mineral crystal alignment in growing long bones with a disordered bone matrix architecture [40]. Murshed et al. have reported that transgenic mice expressing *Tnap* in the dermis showed extracellular mineralization consisting of hydroxyapatite crystals [41]. Given the evidence, it seems that TNAP activity is essential for mineralization in bone, but it is still unknown why the cell membranes with intense TNAP activity in bone are not identical to those forming matrix vesicles.

Recently, basic research on TNAP provided a promising tool for clinicians. The FDA approved asfotase alfa (Strensiq) in 2015 for the treatment of hypophosphatasia caused by a rare hereditary mutation in the alkaline phosphatase gene. Patients with either perinatal or infant onset of the disease who were treated with asfotase alfa showed improvement in overall survival: 97% patients receiving the drug were alive at age 1 year compared with 42% of control patients selected from a natural history study group. Patients with juvenile-onset hypophosphatasia also experienced improved growth and bone health compared with patients in a natural history database [42,43].

3.2. Biological action of ecto-nucleotide pyrophosphatase/phosphodiesterase 1, ENPP 1 in bone mineralization

ENPP 1 is a member of the ENPP family of proteins, which are conserved in vertebrates and hydrolyze pyrophosphate or phosphodiester bonds in various extracellular compounds such as nucleotides and phospholipids. ENPP1 is composed of two N-terminal somatomedin B-like domains (SMB1 and SMB2), a catalytic domain and nuclease-like domain (Fig. 5). ENPP1 participates in different biological processes through distinct sets of domains: the catalytic and nuclease-like domains act in bone mineralization, while the SMB-like domains for insulin signaling. The crystalline structure analysis of ENPP1 suggests that the nucleotides are able to be accommodated in a pocket formed by an insertion loop in the catalytic domain, explaining why ATPs are ENPP1's preferred substrate [44].

In bone mineralization, ENPP1's catalytic activity generates PPi — presumably using extracellular ATPs — and the resultant PPi inhibits mineralization by binding to incipient hydroxyapatite crystals and preventing their overgrowth [45—47] (Fig. 6). In our observations, ENPP1 immunoreactivity was seen throughout the cytoplasm and on the secretory (osteoidal) pole of mature osteoblasts, as well as in osteocytes (*data not shown*). The localization of ENPP1 predominantly on the osteoidal surface of osteoblasts may suggest that it inhibits the overgrowth of mineral crystals. The histological evidence that TNAP is not intensely localized on the osteoidal surface appears to be consistent with the distribution of ENPP1 on osteoblasts.

Severe ENPP1 deficiency was recently shown to be related to a syndrome of spontaneous infantile arterial and periarticular mineralization [48,49]. They suggested that the PPi generated by ENPP1 activity in vascular smooth muscle cells and chondrocytes disrupts the growth of hydroxyapatite crystals. In addition, the linkage of genetic ENPP1 dysfunction to infantile arterial mineralization suggests abnormal PPi metabolism seems to be an important regulatory factor or inducer of osteoblastic differentiation in vascular smooth muscle cell.

Mice with *Enpp1* gene depletion (*Enpp1*$^{-/-}$ mice), also known as tiptoe walking (*ttw*/*ttw*) mice, develop progressive ankylosing intervertebral and peripheral joint hyperostosis and articular cartilage mineralization [50—54]. Mackenzie et al. [54] have recently reported on the details of the skeletal deformities seen in *Enpp1*$^{-/-}$ mice: mineralization in arteries and in the kidney, ectopic formation of cartilage in the joints and spine, despite the reduced circulating calcium and phosphate levels. Also, there was hypermineralization of the talocrural joint and hypomineralization of the femur and tibia with reduced trabecular number, trabecular bone volume and trabecular/cortical thickness. Consistent with the marked increased in fibroblast growth factor (FGF) 23 mRNA, circulating FGF23 was significantly elevated in *Enpp1*$^{-/-}$ mice [54].

3.3. Putative function of ankylosis protein (ANK) in pyrophosphate transport in cells

ENPP1 can be found not only on the cell surface but also in cytoplasmic regions, generating PPi in the both regions of the cell (Fig. 6). Ankylosis protein, ANK, which is encoded

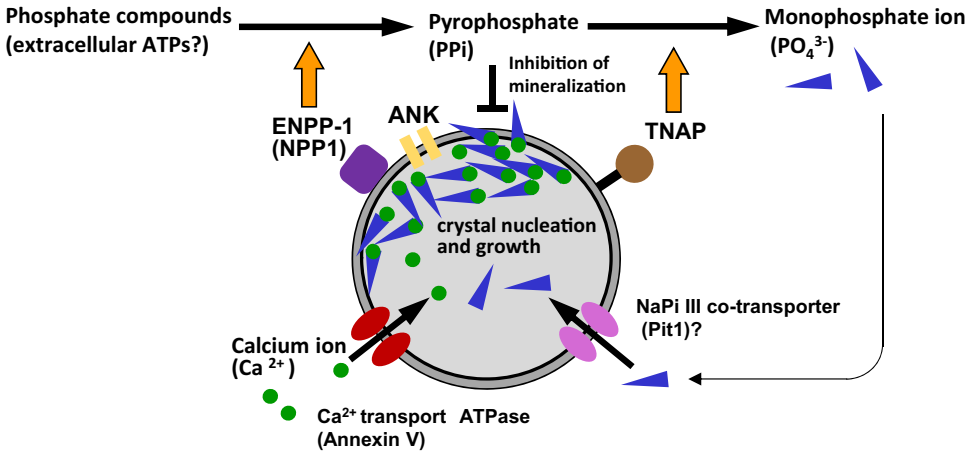


Figure 6 Schematic design of matrix vesicle-mediated mineralization.
Matrix vesicles provide adequate micro-circumstance for initiation of mineralization. Membrane transporters and enzymes including TNAP, ENPP1, annexins, ANK and Pit1 equipped on matrix vesicles play a pivotal role in Ca^{2+} and PO_4^{3-} transport into the vesicles. Phosphatidylserine and so forth in the plasma membrane has a high affinity to produce a stable calcium phosphate—phospholipid complex associated with the inner leaflet of the vesicle's membrane. Thereafter, amorphous calcium phosphates develop hydroxyapatite to form needle-shaped mineral crystals. Many mineral crystals penetrate the vesicles' membranes to form a globular assembly of numerous mineral crystals, *i.e.*, mineralized nodules.

by the mouse progressive ankylosis (*Ank*) gene, appears to function as a multiple-pass, transmembrane PPi-channelling protein, allowing PPi molecules to pass through the plasma membrane from the cytoplasm to the outside of the cell [55,56]. In mice, *Ank* mRNA is expressed in several tissues including the heart, brain, liver, spleen, lung, muscle and kidney, as well as in bone and cartilage such as the articular cartilage in the shoulder, elbow, wrist and digits. Since PPi and its derivatives are naturally potent mineralization inhibitors both *in vivo* and *in vitro*, ANK-mediated regulation of PPi levels provides a room for inhibition of excessive mineralization in several tissues [55]. Considering that infants carrying *Ank* mutations display a three- to five-fold decrease in extracellular PPi, the *Ank* gene appears to regulate both intra- and extracellular levels of an important inhibitor of hydroxyapatite crystal formation [55]. Thus, local PPi production naturally inhibits hydroxyapatite deposition, blocking undesirable mineralization in articular cartilage and other tissues. With loss of ANK activity, however, extracellular PPi levels attenuate, intracellular PPi levels rise, and unregulated mineralization takes place.

3.4. Ca^{2+} transport through annexins

Matrix metalloproteinase (MMP)-3 [57], TNAP [5,16,17,32,57—60], annexins [61], phospholipase A2, carbonic anhydrase II and lactate dehydrogenase [62] are some of the relevant enzymes and proteins involved in matrix vesicle-mediated mineralization.

Among these molecules, annexins are Ca^{2+}- and lipid-binding proteins involved in Ca^{2+} transport and serving as ion channels in the matrix vesicles (Fig. 6). Three annexins were identified in the matrix vesicles: annexin A2, A5 and A6 [63—66]. In the first phase of matrix vesicle-mediated mineralization, mineral complexes appears on the inner leaflet of the matrix vesicle's membrane. The affinity of phophatidylserine for Ca^{2+} is quite strong in the inner leaflet of the matrix vesicle membrane, which is enriched with anionic lipids [67,68]. The annexin A5 shows Ca^{2+}-dependent phosphatidylserine-binding properties and may play a central role in mineralization. However, *Annexin A5*$^{-/-}$ mice did not demonstrate abnormal skeletal development; thus, other annexins could replace the biological functions of annexin A5 in knockout mice. Thus, further studies seem to be necessary to clarify the precise role of annexins during bone mineralization.

4. Ultrastructure of collagen mineralization

After the onset of matrix vesicle-mediated mineralization, mineralized nodules would contact the surrounding collagen fibrils. The collagen fibrils mineralization begins at the point of contact with mineralized nodules (Fig. 7). There are at least two theories explaining collagen mineralization: one is the hole zone theory, and another is the one supporting that mineralization takes place along the superhelix of collagen fibrils, which are arranged in parallel, but shifted at certain intervals. According to the hole zone theory, during the non-mineralizing phase, the gaps within the collagen fibrils are occupied by small proteoglycans such as decorin and biglycan. However, after elimination of these proteoglycans, extracellular Ca^{2+} and PO_4^{3-} fill in the gap to generate calcium phosphate nuclei and mineralize the collagen fibrils. Thus, the initial mineralization begins in the collagen fibrils' ''holes''.

However, while decorin/biglycan-double knockout mice revealed osteopenia as a result of impaired GAG-linking to decorin and biglycan core proteins, mineralization was not stimulated [69]. Still, collagen mineralization based on the process of removal of small proteoglycans may need further

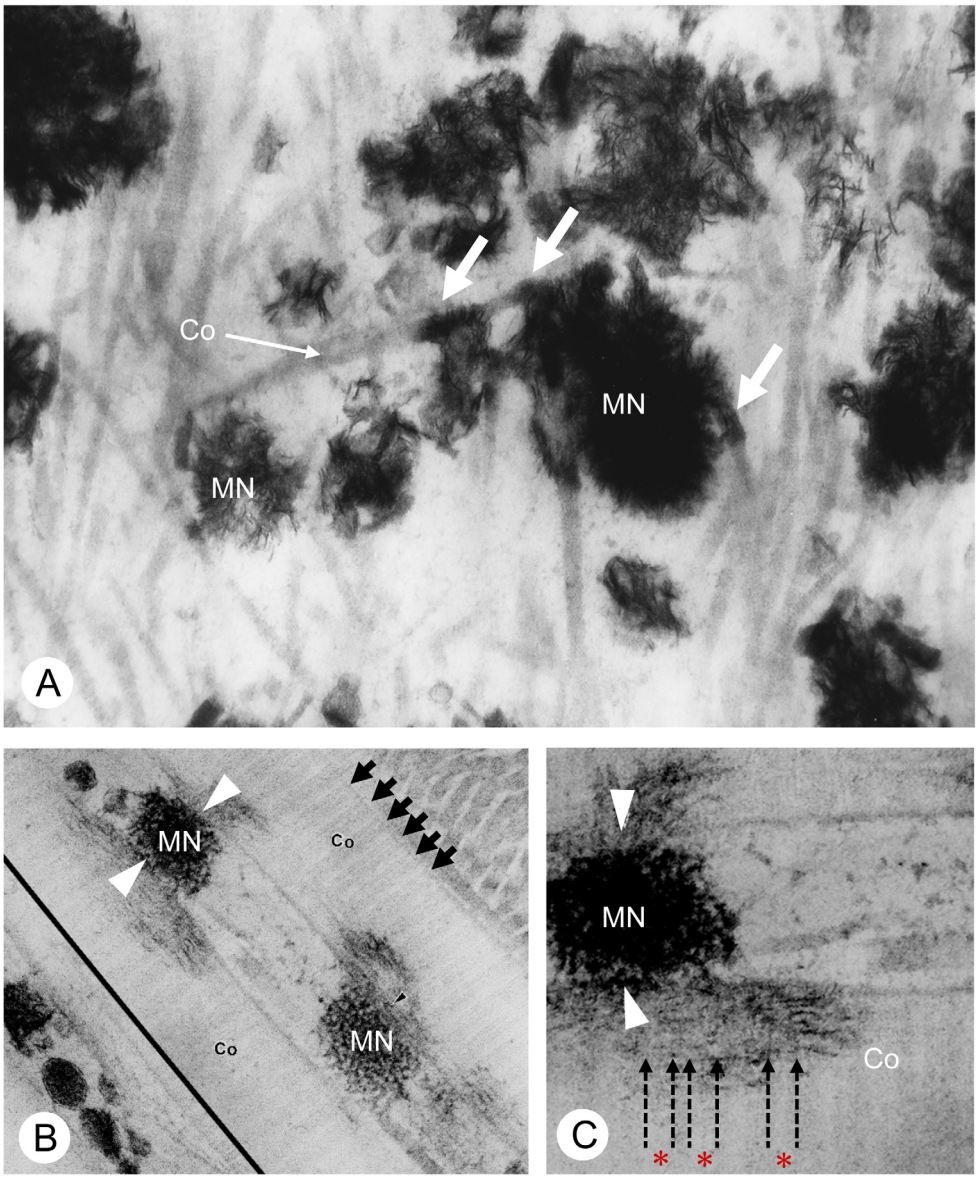


Figure 7 TEM images of the contact between collagen fibrils and mineralized nodules.
(A) In osteoid, several mineralized nodules (MN) are shown to make contact with surrounding collagen fibrils (Co) (arrows). (B) In other region, mineralized nodules spread out minerals to neighboring collagen fibrils. Thus, collagen mineralization seems to be associated with mineralized nodules. In contrast, however, collagen striation (short arrows) do not show any mineral deposition. (C) At a higher magnification, laddering structures of mineral deposition are parallel to the longitudinal axis of the collagen fibrils, and the length of mineral crystal seems to be identical to that of superhelix.
Panel A is from Ref. [74] (Amizuka et al.), and panels B and C are modified from Ref. [73] (Ozawa et al.).

investigation. On the other hand, TEM observations demonstrated that mineralization spread out from the contact point of mineralized nodules towards the periphery of collagen fibrils (Fig. 7B and C). This finding suggests that collagen mineralization is association with mineralized nodules. At a higher magnification, the spicules of calcium phosphate crystals are seen on the fibrillar structures identical to the superheix of collagen fibrils, which indicates that mineral crystals are deposited on the superhelix, which serves as a scaffold for collagen mineralization.

We also examined mineralization on osteogenic disorder shionogi (ODS) rats, which carry a hereditary defect in ascorbic acid synthesis [70]. Fragile, fibrillar collagenous structures without evident striation were found in ODS rat bones, which may be a result of misassembling of the triple helices of collagenous α-chains secondary to ascorbic acid deprivation. However, that seemed to bear no effect on mineralization: fine needle-shaped mineral crystals extended from mineralized nodules, and were apparently bound to collagenous fibrillar structures (Fig. 8).

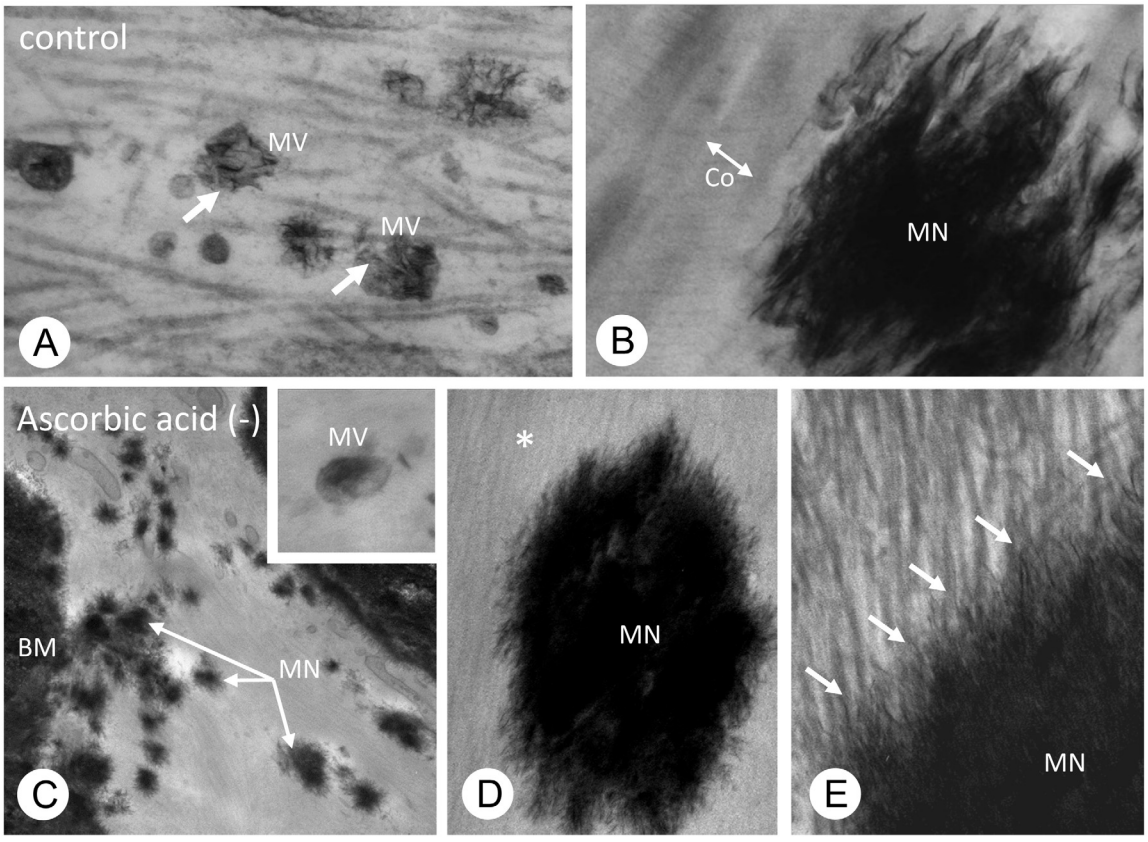


Figure 8 Matrix vesicles and collagen mineralization in ascorbic acid insufficient circumstance.
In a normal state, TEM observations demonstrate numerous matrix vesicles (A) and mineralized nodules (B). At a higher magnification, mineral crystals extending from the mineralized nodules ran along the collagen fibrils (B). In an insufficient circumstance of ascorbic acid, which is necessary for collagen synthesis, TEM observations verified the presence of matrix vesicles and mineralized nodules (C). At a higher magnification, however, collagen fibrils are shown be very fine, but, the fine mineral crystals from the mineralized nodule extended along fine fibrillar structures of collagen fibrils (D and E).
All the images are modified from Ref. [70] (Hasegawa et al.).

Taken together, these two postulations (the hole zone theory and mineralization on the superhelix of collagen fibrils) are both without proper foundations, and therefore, need further scientific scrutiny.

5. Concluding remarks

Matrix vesicle-mediated mineralization causes a series of orchestrated ultrastructural and biochemical events in bone. To achieve proper mineralization, a variety of membrane transporters and enzymes are put at work in matrix vesicles. Of particular importance is the influx of phosphate ions into matrix vesicles, which involves a complex interplay among ENPP1, ANK, TNAP and Pit1. Mineralized nodules, the globular assembly of needle-shaped mineral crystals, are derived from matrix vesicles and may retain some activity of those transporters and enzymes. However, crystal growth is likely regulated by surrounding organic materials prior to subsequent collagen mineralization.

Conflict of interest

None declared.

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