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Matrix vesicle-mediated mineralization in bone

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ABSTRACT : Matrix vesicle-mediated mineralization is orchestrated by ultrastructural and biochemical events that lead to crystal nucleation and growth. Osteoblasts secrete extracellular matrix vesicles equipped with a variety of membrane transporters and enzymes, which are necessary for the initial nucleation and subsequent growth of calcium phosphate crystals. The influx of phosphate ions into the matrix vesicle is a complex process mediated by several enzymes and transporters, such as tissue nonspecific alkaline phosphatase (TNAP), ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), sodium/phosphate co-transporter type III (Pit1). The catalytic activity of ENPP1 generates pyrophosphate (PPi) using extracellular ATPs as a substrate, and the resultant PPi binds to growing hydroxyapatite crystals to prevent crystal overgrowth. However, TNAP hydrolyzes PPi into phosphate ions (PO_4^{3-}), a constituent of calcium phosphates, which are then transported into the matrix vesicle through Pit1. Accumulation of calcium ion (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. Apparently, mineralized nodules – which are globular assemblies of needle-shaped mineral crystals – retain some of those transporters and enzymes. The subsequent growth of mineralized nodules is regulated by surrounding organic compounds, finally leading to collagen mineralization. In this review, ultrastructural and biochemical aspects on matrix vesicle-mediated mineralization will be introduced.

Key Words : matrix vesicle, mineralization, TNAP, ENPP1, mineralized nodule

Introduction

Physiological mineralization can be seen only in bone and teeth, and therefore, appears to be strictly regulated by physicochemical, enzymatic and cellular activities. Mineralized bone matrix is composed of abundant calcium phosphates, type I collagens and non-collagenous organic materials. Bone mineralization is divided into two ultrastructural phases – primary and secondary mineralization. Primary mineralization is biologically orchestrated by osteoblasts and osteoclasts during bone modeling and remodeling ; osteoblasts secrete a large amount of collagen fibrils, non-collagenous proteins, and matrix vesicles, which are extracellular vesicles that trigger mineralization via a variety of membrane

transporters and enzymes. Thus, primary mineralization is achieved by fine-tuning syntheses of organic materials and subsequent apposition of calcium phosphates. However, secondary mineralization is a phenomenon by which bone mineral density would be chronologically elevated after the primary mineralization. It is therefore hypothesized that secondary mineralization is regulated physicochemically, *i.e.*, through crystal maturation, and by the osteocytic network inside the mineralized bone matrix. However, the biological and ultrastructural mechanisms behind secondary mineralization are still veiled.

In bone, primary mineralization is constituted with two distinct phases : matrix vesicle-mediated mineralization and collagen mineralization. During the process of the

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matrix vesicle-mediated mineralization, osteoblasts secrete matrix vesicle on which the membrane transporters and enzymes involved in mineralization are equipped. Discovery of matrix vesicles was a highlighted breakthrough in the research field of biological mineralization¹⁻¹⁰. Until then, it was proposed that alkaline phosphatase may supply mono-phosphate ions by hydrolyzing phosphate substrates, and then, accelerate to form crystalline calcium phosphates, so called, alkaline phosphatase theory¹¹. However, this theory is based on the physicochemical regulation of bone mineralization. In contrast, the theory behind matrix vesicle-mediated mineralization sustains that the mineralization processes are mainly under the biological control of osteoblasts, by mediating the regulation of membrane transporters/enzymes and surrounding extracellular organic materials. Accumulated knowledge of the ultrastructure and biological activities of membrane transporters/enzymes definitely supports the postulation that the cellular mechanisms of matrix vesicle-mediated mineralization is essential for mineralization in bone.

In this review, the ultrastructural and biochemical evidences of matrix vesicle-mediated mineralization is introduced.

1. Ultrastructural evidences of matrix vesicle-mediated mineralization in bone

Bone mineralization initiates inside matrix vesicles. Matrix vesicles are small extracellular vesicles enveloped by a plasma membrane secreted by osteoblasts^{2, 4, 5, 8-10} (Fig. 1). Matrix vesicles equip several membrane transporters and enzymes on their plasma 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 [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$], appears inside the matrix vesicles, growing and eventually breaking through the plasma membrane of matrix vesicles to form mineralized nodules – also known as calcifying globules (Fig. 1). Under transmission electron microscopy (TEM), mineralized nodules are observed as the radially-assembled globular structures of hydroxyapatite crystals featuring small, ribbon-like structure profile approximately 25 nm wide, 10 nm high and 50 nm long^{12, 13}.

Under TEM, incipient mineral crystals were initially found associated with the inner leaflet of the

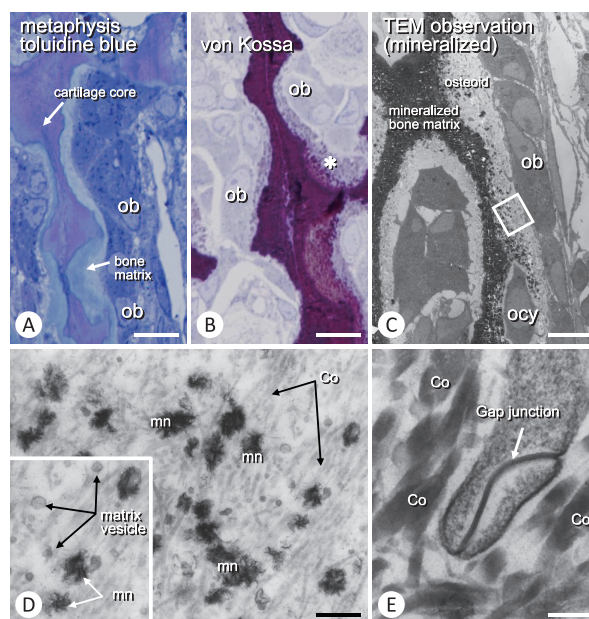


Fig. 1 Ultrastructures of matrix vesicles and mineralized nodules in bone

Panels A and B demonstrate metaphyseal trabeculae visualized with toluidine blue staining (A) and von Kossa staining (B). Metaphyseal primary trabeculae are composed of cartilage cores (purple color) and surrounding bone matrices (blue) (A), and include many mineralized granules in osteoid (an asterisk, B). Panel C is a TEM image of osteoid underlying mature osteoblasts (ob). Panel D is a highly-magnified image of a square in C. Notice many mineralized globular structures referred to as mineralized nodule (mn) in the osteoid. An inset reveals both matrix vesicles and mineralized nodules (mn). Under TEM, the cytoplasmic processes of osteoblasts often show gap junction (E).

Bars, A-C : 10 μm , D : 0.5 μm , E : 0.1 μm

matrix vesicle membranes, and it seems likely that crystal nucleation would start at the specific site. The plasma membranes of matrix vesicles are rich in acidic phospholipids such as phosphatidylserine and phosphatidylinositol, which have high affinity for Ca^{2+} due to phosphate residues. Phosphatidylserine has a particularly high affinity for Ca^{2+} , and assumed to produce a stable calcium phosphate-phospholipid complex associated with the inner leaflet of the vesicle's membrane⁷. The possibility that such complexes may play an important role in crystal nucleation has been pointed out before^{14, 15}. However, the electron diffraction of freeze-substitution and cytochemical calcium detection methods such as κ -pyroantimonate combined with energy-dispersive X-ray spectroscopy showed immature calcium phosphates associated with the vesicles' plasma membranes as non-crystalline structures containing calcium and phosphate¹⁶⁻¹⁸. Thus,

the early phases of calcium phosphate nucleation inside the matrix vesicles may be originally amorphous, thereafter chronologically becoming mature crystalline structures, *i.e.*, hydroxyapatite, in a later stage. Calcium phosphate crystals formed inside matrix vesicles can thereafter grow through the influx of Ca^{2+} and PO_4^{3-} from extracellular fluid, by means of the membrane transporters and enzymes (*discussed later*). Inside the matrix vesicle, “needle-shaped” crystalline calcium phosphates form a stellate assembly, grow in all direction, and then, come out of the vesicles penetrating the plasma membrane to form mineralized nodules, also referred to as calcifying globules⁶.

According to observations of the osteoid in bone derived from the quick frozen-freeze substitution technique with electron energy loss spectroscopy or EELS, which enables elemental mapping at the molecular level, Ca^{2+} was evenly distributed in the proteoglycan-rich, peripheral region of matrix vesicles, while PO_4^{3-} was detected predominantly in organic materials such as collagen fibrils¹⁹. Therefore, it seems likely that, in non-mineralized sites, the extracellular meshwork of organic substances limits the production of hydroxyapatite and inhibits precipitation of mineralized 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 take place, since PO_4^{3-} is not abundant in the periphery of matrix vesicles. There seems some molecular and biochemical mechanism providing an adequate microenvironment for the development of crystalline calcium phosphates in the matrix vesicles and subsequent mineralized nodules in bone.

2. Ultrastructural and biochemical function of enzymes and membrane transporters for matrix vesicle-mediated mineralization

One may wonder how Ca^{2+} and PO_4^{3-} would be transported and accumulated into matrix vesicles and mineralized nodules. As described above in this review, abundant Ca^{2+} was evenly distributed in the peripheral region of matrix vesicles, while PO_4^{3-} was predominantly associated with organic materials such as collagen fibrils¹⁹. Therefore, the biological actions of membrane transporters and enzymes to produce PO_4^{3-} , rather than Ca^{2+} , and to warrant the influx of PO_4^{3-} into the matrix vesicles

appear to be necessary. Several enzymes and membrane transporters found in the matrix vesicles are involved in mineralization. Among these enzymes and transporters, tissue nonspecific alkaline phosphatase (TNAP), ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), ankylosis (ANK), phosphoethanolamine/phosphocholine phosphatase (PHOSPHO1) and sodium/phosphate cotransporter type III (Pit1) appear to play a pivotal role in phosphate transport on matrix vesicle-mediated mineralization (Fig. 2).

i) Tissue nonspecific alkaline phosphatase (TNAP)

One of the most important enzymes that initiate mineralization in bone must be TNAP, a glycosylphosphatidylinositol (GPI) anchor enzyme associated with cell membrane (Fig. 2). TNAP can hydrolyze various phosphate esters, especially pyrophosphate (PPi), and is responsible for the production of inorganic phosphate: therefore, many believe it is a potent inducer of mineralization. In bone, TNAP activity was detected on osteoblasts and matrix vesicles^{20, 21}. Interestingly, the distribution of TNAP on cell membranes is not uniform in osteoblasts, which are polar cells with distinct basolateral and secretory (osteoidal) domains^{8, 9}. 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 domain²². Consistently, using specific antiserum to TNAP²³, we could observe relatively intense immunoreactivity and enzymatic activity for TNAP on preosteoblasts and on basolateral cell membranes of mature osteoblasts²⁴. Thus, in bone, the membranes featuring an intense activity of TNAP are not identical to those that serve as the site of matrix vesicle formation.

ii) Ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (ENPP 1)

Ecto-nucleotide pyrophosphatase/phosphodiesterase (ENPP) 1 is a member of the ENPP family of proteins. ENPP1 is composed of two N-terminal somatomedin B (SMB)-like domains, a catalytic domain and nuclease-like domain. ENPP1 participates in different biological processes through distinct sets of domains: the catalytic and nuclease-like domains for bone mineralization and the SMB-like domains for insulin signaling, respectively. Crystalline structure analysis of ENPP1 implies that the nucleotides are accommodated in a pocket formed by an insertion loop in the catalytic domain of ENPP1, explaining the preference of ENPP1 for an

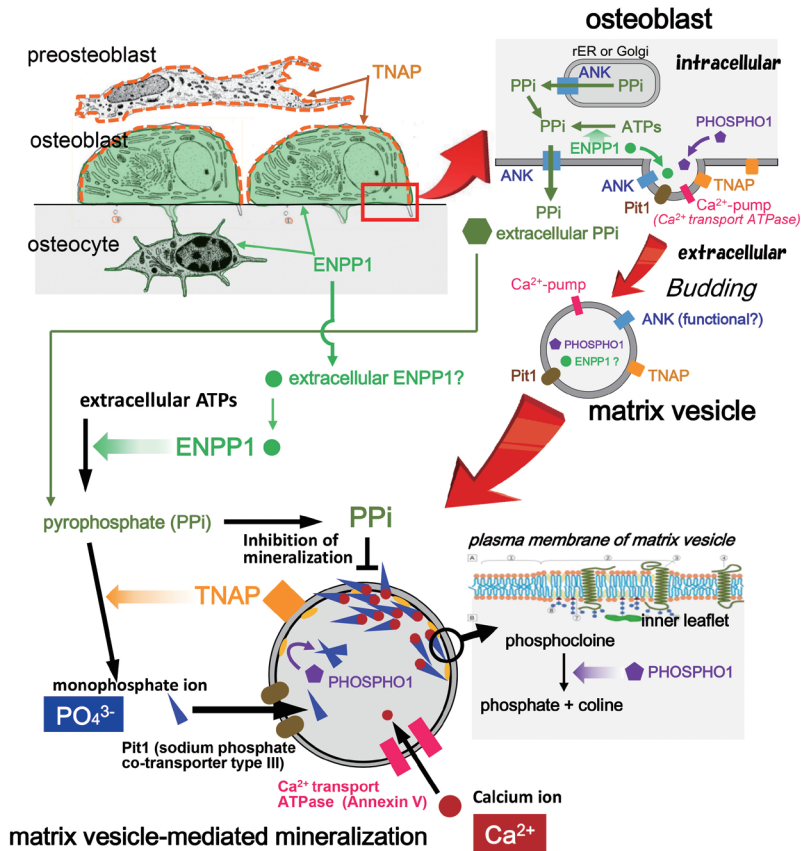


Fig. 2 A schematic design of matrix vesicle-mediated mineralization in bone. Matrix vesicles provide adequate micro-circumstance for initiation of mineralization. Membrane transporters and enzymes including TNAP, ENPP1, ANK and sodium phosphate co-transporter type III (Pit1) equipped on matrix vesicles play a pivotal role in Ca²⁺ and PO₄³⁻ 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. In addition, PHOSPHO1 hydrolyzes phosphocoline into colines and phosphates that are constituents of calcium phosphates inside the matrix vesicles. Thereafter, amorphous calcium phosphates develop hydroxyapatite to form needle-shaped mineral crystals (Illustrated by the author).

ATP substrate²⁵⁾. In bone mineralization, its catalytic activity generates pyrophosphate (PPi), presumably using authentic ATPs in extracellular fluid, and the resultant PPi inhibits mineralization by binding to nascent hydroxyapatite crystals, thereby preventing overgrowth of these crystals²⁶⁻²⁸⁾ (Fig. 2). In human infants, severe ENPP1 deficient states were recently linked to a syndrome of spontaneous infantile arterial and periarticular mineralization^{29, 30)}. They suggested that PPi physiologically generated by ENPP1 activity of certain active cells, *e.g.*, vascular smooth muscle cells and chondrocytes, potently suppresses pathological mineralization. In our own observation, ENPP1 was mainly localized in mature osteoblasts and osteocytes, while TNAP was seen on preosteoblasts and mature osteoblasts. It is interesting that ENPP1, an inhibitory enzyme for mineralization, is localized on cells in the close proximity of matrix vesicles and mineralized nodules, whereas TNAP-activity can be seen relatively distant from matrix vesicles.

iii) Ankylosis (ANK)

Ankylosis (ANK) is a non-enzymatic plasma-membrane pyrophosphate (PPi) channel (Fig. 2). ANK is encoded

by the mouse progressive ankylosis (*Ank*) gene, appears to function as a multiple-pass, transmembrane PPi-channelling protein, allowing PPi to pass through the plasma membrane from the cytoplasm to the outside of the cell^{31, 32)}. ANK-mediated regulation of PPi levels as a possible mechanism regulating tissue mineralization and susceptibility to arthritis³¹⁾. *Ank* mRNA has been shown to be expressed in many tissues in adult mice including heart, brain, liver, spleen, lung, muscle and kidney, as well as articular cartilage of joints in shoulder, elbow, wrist and digits. PPi and its derivatives are potent inhibitors mineralization both *in vivo* and *in vitro*. In order to avoid pathological mineralization, many cells may channel intracellular PPi into extracellular fluid³¹⁾. However, the infants carrying *Ank* mutation caused a three-to-five fold decrease in extracellular level of PPi in contrast to stimulatory effect on intracellular PPi. Thus, the *Ank* gene appears to regulate both intra- and extracellular levels of an important inhibitor of hydroxyapatite crystal formation³¹⁾. Local elaboration of PPi provides a natural inhibitor of hydroxyapatite deposition, blocking unpreferable mineralization in articular cartilage and other tissues. With loss of ANK activity, however, extracellular PPi levels attenuate, intracellular PPi levels

rise, and unregulated mineralization begins in the joints and other tissues.

iv) PHOSPHO1

PHOSPHO1 is an enzyme highly expressed in mineralizing cells, *e.g.*, in bone and cartilage, with systematic name phosphoethanolamine phosphohydrolase^{33, 34} (Fig. 2). This enzyme is implicated in bone and cartilage formation, and thought to function inside cells and matrix vesicles, in order to catalyze phosphocoline, a constituent of plasma membrane, dividing into choline and phosphates. Roberts *et al.* have reported that PHOSPHO1 is restrictedly localized to mineralizing regions (matrix vesicles) of bone and growth plate, and that PHOSPHO1 plays a role in the initiation of matrix vesicle-mediated mineralization³⁵. In contrast to extracellular function of ENPP1 and TNAP, PHOSHO1 thus, appear to serve for initial mineralization inside matrix vesicles.

3. Formation and development of mineralized nodules

Mineralized nodule is a globular assembly of numerous needle-shaped mineral crystals that has been exposed to extracellular environment from matrix vesicles (Fig. 3). 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^{36, 37}. Osteopontin is localized in the periphery of mineralized nodules, where it might act as a blocker of excessive mineralization³⁸. Since other organic materials can combine with osteopontin³⁹, they can form the so-called “crystal ghosts”⁴⁰⁻⁴². Among these materials, osteocalcin is known for containing γ -carboxyglutamic acid and for their ability to bind to mineral crystals⁴³⁻⁴⁵. 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^{43, 46}. In our observations, the administration of warfarin resulted in the dispersion of numerous fragments of needle-shaped crystal minerals throughout the osteoid⁴⁷. Recently, γ -carboxylase-deficient mice revealed the same abnormality with disassembled, scattered crystal minerals in bone⁴⁸. Therefore, osteocalcin may play an important role in the globular assembly of needle-shaped mineral crystals, probably binding together the organic components of the crystal sheath.

4. Ultrastructure of collagen mineralization

After the onset of matrix vesicle-mediated mineralization, mineralized nodules would contact the surrounding collagen fibrils. The collagen mineralization begins at the point of contact with mineralized nodules. There are at

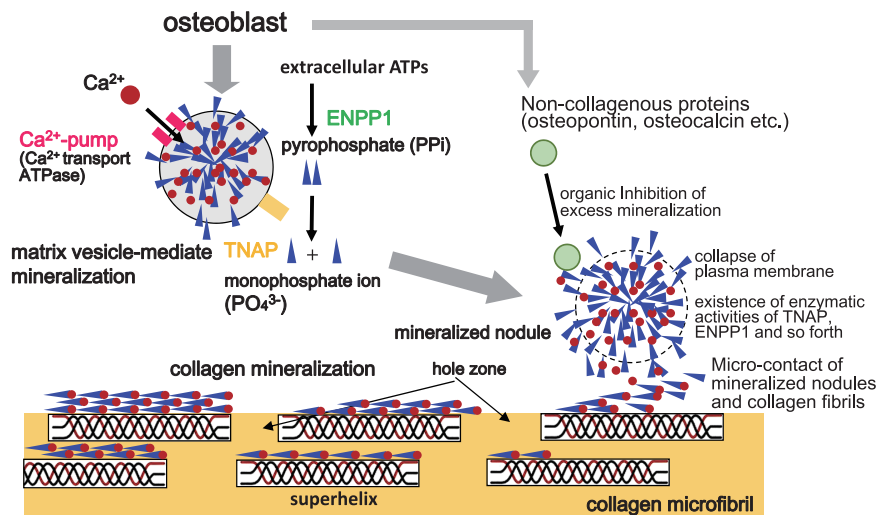


Fig. 3 A schematic design of collagen mineralization

Many mineral crystals penetrate the vesicles' membranes to form a globular assembly of numerous mineral crystals, *i.e.*, a mineralized nodule. Even though the plasma membrane of matrix vesicles collapse, the enzymatic activities still remains to deposit Ca^{2+} and PO_4^{3-} onto mineralized nodules. When mineralized nodules make contact with surrounding collagen fibrils, mineral crystals from the mineralized nodule extend along the superhelix of collagen fibrils (Illustrated by the author).

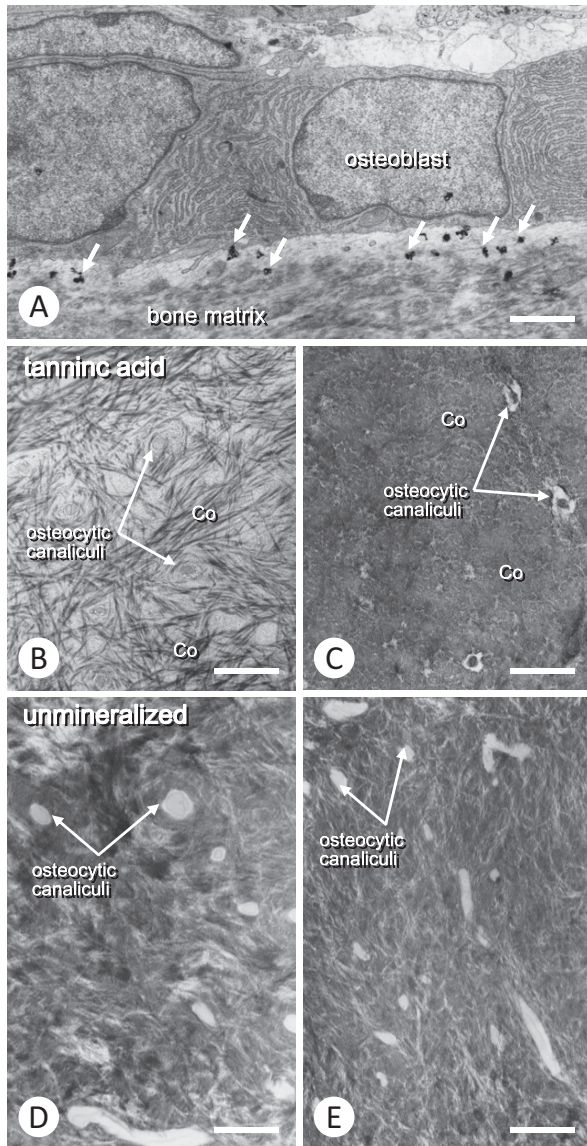


Fig. 4 TEM images of the collagen distribution in bone
 A panel A is an autoradiography of ^3H -proline, indicative of collagen synthesis in metaphyseal primary trabeculae. See many silver grains of ^3H -proline (white arrows) in the region of secretory surfaces of mature osteoblasts (A). Panel B shows a TEM image of tannic acid-stained bone matrix of immature bone (primary trabeculae), including many fine collagen fibrils (black, Co). In contrast, panel C displays tannic acid-stained bone matrix of mature bone (cortical bone), featuring densely-packed collagen fibrils (Co). Panels D and E demonstrate TEM images of mineral crystals of immature (D) and mature (E) bones. See much densely-deposited mineral crystals in E, rather than in D.
 Bars, A : 3 μm , B-E : 0.5 μm

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 (Fig. 3). 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 stimulated⁴⁹). Still, collagen mineralization based on the process of removal of small proteoglycans may need further investigation. On the other hand, TEM observations demonstrated that mineralization spread out from the contact point of mineralized nodules towards the periphery of collagen fibrils⁵⁰). 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 superhelix of collagen fibrils, which indicates that mineral crystals are deposited on the superhelix, which serves as a scaffold for collagen mineralization. After micro-contact of mineralized nodules, collagen fibrils will be completely mineralized as time goes (Fig. 4).

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.

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.

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