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Phylogeny and chemistry of biological mineral transport

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Abstract

Three physiologically mineralizing tissues — teeth, cartilage and bone — have critical common elements and important evolutionary relationships. Phylogenetically the most ancient densely mineralized tissue is teeth. In jawless fishes without skeletons, tooth formation included epithelial transport of phosphates, a process echoed later in bone physiology. Cartilage and mineralized cartilage are skeletal elements separate from bone, but with metabolic features common to bone. Cartilage mineralization is coordinated with high expression of tissue nonspecific alkaline phosphatase and PHOSPHO1 to harvest available phosphate esters and support mineralization of collagen secreted locally. Mineralization in true bone results from stochastic nucleation of hydroxyapatite crystals within the cross-linked collagen fibrils. Mineral accumulation in dense collagen is, at least in major part, mediated by amorphous aggregates — often called Posner clusters — of calcium and phosphate that are small enough to diffuse into collagen fibrils. Mineral accumulation in membrane vesicles is widely suggested, but does not correlate with a definitive stage of mineralization. Conversely mineral deposition at non-physiologic sites where calcium and phosphate are adequate has been shown to be regulated in large part by pyrophosphate. All of these elements are present in vertebrate bone metabolism. A key biological element of bone formation is an epithelial-like cellular organization which allows control of phosphate, calcium and pH during mineralization.

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Keywords

Bone mineral; Hydroxyapatite; Osteoblast; Calcium transport; Phosphate transport; Acid transport

1. The first densely mineralized tissue, teeth

To develop a context and perspective for later discussion it is helpful and important to consider evolution and development of teeth. Teeth arise in epithelial tissues and require membrane transport to form calcium-phosphate mineral. This process depends on genes occurring in tooth development and prominent in bone of modern vertebrates, including mammals. The data we present are a consensus view, emphasizing the chemical basis for the appearance of densely mineralized tissue. That said, this is not the place for a comprehensive review of tooth development and we apologize for important contributions not included.

Teeth appeared in early jawless fishes (thelodonts or ostracoderms) as appendages to skin epithelia, at the endoderm-ectoderm interface, preceding the evolution of the mineralized skeleton [1]. The hard matrix of teeth depends on calcium phosphate salts, mainly hydroxyapatite, but phosphate is not present in sea water in concentrations needed for precipitation of calcium salts [2]. Phosphate is accumulated by the cells of these organisms and permits the making of teeth. Tooth formation occurs in epithelia internal to the animal; teeth then are moved to the surface of the integument. This reflects that calcium is abundant in sea water (or plasma), but phosphate is present in sea water at nanomolar concentrations [2]. Phosphate for teeth therefore *must* be concentrated locally by cells. Hence, teeth develop behind epithelia internal to the animal (Fig. 1B–C), later moving to the surface of the integument. Two epithelial layers function in tooth formation: one forms enamel; an epithelial root sheath generates bone-like dentin [3]. Because of the calcium and phosphate mineral components, enamel and dentin require mineral transport and deposition. Mineral transport depends on membrane potential, pH and ion gradients. This is a defining function of epithelia. In most cases, work in the area of tooth development has focused on inductive signals in the dental epithelium [4]. However, disruption of phosphate transport affects dentin mineralization [5], supporting the concept. The major phosphate transporters appear to be sodium-dependent [6], which later will be discussed in presenting bone mineral transport by osteoblast epithelial-like cell layers.

Teeth were retained, or perhaps reappeared, as the skeleton evolved in bony fishes and cartilaginous fishes [7]. The cartilaginous fishes, rays and sharks, go back ~450 million years but employ genes in tooth expression found in the jawless fishes [8]. Modern sharks do not have bone but exhibit well developed teeth that are structurally and evolutionarily homologous to the teeth of air-breathing vertebrates. This co-evolution of teeth that are structurally and genetically similar demonstrates the developmental and functional pressure characterizing the appearance of teeth independent of the bony skeleton. Data include considerable information on growth factors and differentiation of dental epithelium showing that hard tissues are from neural crest cells. These differentiation patterns are conserved in animals, including vertebrates with teeth [1,8].

Shark teeth are composed of dentin of several types, and heavily mineralized enamel [9]. The enamel is generated first, involving amyloid or a closely related ancestral protein. The dentin is not well characterized but contains exclusively type I collagen [10]. All of the higher vertebrates have teeth that retain this pattern (Fig. 1A). In the mammal, there are separate epithelial layers for the ameloblasts and odontoblasts (Fig. 1B–C). We hypothesize that chondrichthyes teeth are closely related to those structures of higher phyla, and that the tooth epithelia form enamel and bone-like dentin layers of teeth in vertebrates, representing a precursor of bone.

In the molecular era, the genetic relationships of bone and teeth are seen [12]. Differences include that only teeth make enamel [13] and only bone is remodeled. Genes expressed in bone and teeth are very similar: tooth or bone stem cells produce bone [14]. Both dentin and bone are composed of type I collagen and express strongly osteocalcin, osteopontin, and alkaline phosphatase [15–18]. Many consider this the consequence of convergent evolution, but identical genes are expressed, so the functional relationship is clear. Specifically there is evidence that some enamel proteins might function in bone [19]. Our experience with cRNA screens for human osteoblasts suggests that, if enamel genes are expressed, they occur only at low levels (not shown). A further important fact is that, while bone-like dentin is composed of proteins and mineral found in bone, teeth do not remodel. Teeth are formed and lost, but once formed they are not significantly modified.

2. Cartilage and mineralized cartilage

This discussion is based on cartilage and its transition to bone *via* mineralized cartilage in mammals; homology in the lower classes of animals is interesting, but too complex and variable for inclusion. However, the discussion here applies to all mammals with little variation.

Developmentally, the skeleton is formed mainly as a cartilage template that is converted to bone. Exceptions are membranous or dermal bones, the skull and clavicles, which are not discussed. Cartilage is a matrix produced by embedded chondrocytes, mainly type II collagen held to an stiff elastic consistency by hydrophilic proteoglycans. Chondrocytes are cells interspersed in this matrix without cell-cell communications in a anisotropic distribution. Unlike bone, cartilage is avascular. Cartilage is expanded by mitosis of chondrocytes, which redistribute within the cartilage [20]; the mechanism for chondrocyte migration is not well studied. In the region of chondrocyte proliferation, type II collagen and collagenase synthesis are documented [21], presumably allowing new matrix to be formed between cells and limited matrix remodeling.

Mineralization of cartilage occurs at points where bone is to be formed. Preceding cartilage mineralization, chondrocytes align in rows (Fig. 2B, C). There is a complex series of events including expression of Indian hedgehog and the PTH-related protein receptor [22]. The cells then expand with gradual elimination of organelles leaving only vesicles in the cells [23]; at this point they, traditionally, are named “hypertrophic”. In the opinion of the authors, mineralizing chondrocytes is a better term; the cells do not make cartilage and are, effectively, pre-apoptotic. The chondrocytes are in the process of matrix mineralization

coordinated with death and degradation. On the other hand, while apoptosis promotes mineralization it might not be essential for mineralization [24]. The apoptotic fate of chondrocytes is currently unclear although macrophages, along with blood vessels invade the mineralizing region and form osteoclasts [25].

Early mineralization of cartilage [26] is associated with expression of collagen X and matrix metalloproteinase-13 [22]. As the chondrocytes lose organelles vesicles are formed that exhibit high levels of tissue-nonspecific alkaline phosphatase and PHOSPHO1 [27]. PHOSPHO1 is a phosphatase that removes phosphate from phosphocholine and phosphoethanolamine [28] in intact membranes [29]. Phospholipase C uniquely provides this substrate releasing phosphocholine and phosphoethanolamine, although the same molecules are generated by kinases in vertebrate cells. How these pathways influence mineralization is not clear. Indeed, expression of phospholipases in mineralizing cartilage (and osteoblasts) is not well studied, and PHOSPHO1 is recently discovered; its distribution is uncertain, although it clearly is important along with alkaline phosphatase in bone and cartilage [21]. At present we view alkaline phosphatase, organic phosphates, and membrane lipids as sources of phosphate for mineral formation (Fig. 2). Additional sources include Na-dependent phosphate transport in vesicles and osteoblasts, with the pyrophosphatase (ENPP1), producing a high ratio of Pi to PPi [27]. Cartilage is not a vascular tissue and chondrocytes are not connected by tight junctions, so substrates and products of mineralization would diffuse, but perhaps not as effectively as in hierarchical bone, see below.

The morphology of the cartilage-bone transformation has long been studied by light and electron microscopy. From these studies it was concluded include that the cells often lose organelles and nuclei, becoming collections of vesicles in otherwise empty lacunae [24]. The mechanisms by which these transformations occur is unclear. A key issue is how bone mineral deposition occurs in matrix. As reported by standard fixation for electron microscopy (that is, with calcium in solutions) with vacuum processing, hydroxyapatite crystals appear inside vesicles. This was studied by Anderson [34] and later by Glimcher in a comprehensive review of mineralization [35]: while early cartilage mineralization is consistent with retention of large crystals in vesicles, later cartilage mineralization produces smaller, collagen associated crystals that are distinct from vesicle crystals. We hypothesize that the large crystals are, in part, artifacts of sample processing for electron microscopy and offer an alternative suggestions for cartilage mineralization and endochondral bone formation. During mineralization in living tissue and conversion of cartilage to bone, calcium phosphate aggregates (Posner clusters) and amorphous calcium aggregates of 2–5 nm form [36], small enough to diffuse into dense collagen in mineralizing cartilage and in compact bone matrix of osteoblasts [37]. There is, however, convincing characterization of matrix vesicle mineral deposition, including by atomic force microscopy [38]. The problem is that calcium crystals larger than a few nm cannot diffuse into dense collagen, and crystals of ~100 nm cannot transit vesicle membranes, but might contribute to the non-hierarchical cartilage matrix.

Type I collagen in bone is a material into which mineral crystals are added in a defined arrangement; in the type II collagen of cartilage mineral packing is not well understood.

While the issue is controversial, mineral deposition in cartilage might involve a mixture of type II, type X [33], and type I collagen in mineralizing cartilage. In this regard, there is convincing data indicating that chondrocytes, during epiphyseal bone formation, can produce bone proteins including type I collagen, and might trans-differentiate to form bone [32]. That said, a satisfactory overall mechanism for the transition from apatite crystals with matrix vesicles to mineralized cartilage matrix is not available. In any case, mineralization of avascular cartilage reaches ~half the density achieved in bone (Fig. 3), where an epithelial-like transport system allows packing inward of phosphate and proton removal [40]: see Mineralization of True Bone, below. Mineralized cartilage has been studied in detail, pointing, in addition to lower density than bone, to a more variable mineral composition [39].

3. Regulation of mineralization in normally non-mineralized tissues

Orderly mineralization of bone is essential for the development of higher organisms. However, absence of mineralization in soft tissue is equally critical. The calcium and phosphorous in circulation is adequate for mineral formation in the absence of a preventative mechanism [41]. In this context, we are not discussing ectopic bone, but mineralization of tissues that normally do not mineralize. There are many examples of ectopic mineralization of varying severity, sometimes associated with trauma, *e.g.* [42–44]. However, an important mechanism of potentially fatal ectopic mineralization results from inability to form low levels of pyrophosphate (PPi) in normally non-mineralized tissues. We will concentrate on syndromes related to this, which are studied at the molecular level. Pyrophosphate is long recognized as an inhibitor of mineralization, including in bone [45,46], but its importance in non-bone tissues has only recently been established.

Generalized arterial calcification of infancy (GACI) is a rare syndrome in which afflicted patients calcify their hearts, aorta, and large arteries during the third trimester of pregnancy [47,48]. If the fetus survives to term, newborns present before 4 months, half in the first week of life, in respiratory or cardiac distress. Half of affected infants will not survive to 6 months of age despite supportive care. Those that survive undergo a physiologically adaptive response with elevated FGF23 and phosphate wasting [49]. Vascular and cardiac calcifications stabilize or may even regresses [50,51], but stabilization of ectopic calcification is at the expense of a rachitic skeletal phenotype, autosomal recessive hypophosphatemic rickets Type 2 [52,53].

There are two genetic causes of GACI. One is homozygous loss of function mutations in the enzyme ectonucleotide pyrophosphate/phosphodiesterase-1 (ENPP1), seen in 75% of patients [54,55]. Remaining patients with GACI have bi-allelic loss of function mutations in the transporter ABCC6 [56,57], which regulates extracellular ATP. Mutations of ABCC6 cause soft tissue calcification disorders overlapping with GACI, also called pseudoxanthoma elasticum (PXE) [58]. Severely affected PXE presents as GACI. ENPP1 is the only enzyme in humans for synthesis of extracellular pyrophosphate (PPi) [54]. Both ENPP1 and ABCC6 deficiency cause decreased plasma [PPi] [59–61] accounting for the overlapping clinical phenotype.

ENPP1 generates PPi by cleaving NTPs to generate PPi (Fig. 4). Extracellular PPi is never generated unless ENPP1 is present. However, when ENPP1 is present, PPi is produced at a rate of approximately 3.5 molecules per second, an acceleration of 10^{27} fold above the non-catalyzed rate.

Extracellular [PPi] is ~2.5 μM in patients without loss of function mutations in either ENPP1 or ABCC6. Bi-allelic ENPP1 mutations lead to extracellular PPi < 10% of WT [59,62]. Patients with bi-allelic ABCC6 mutations have PPi ~30% of WT (~0.75–1.0 μM) [61]. Lower limits of 'normal' plasma PPi have not been established, but in chronic kidney disease vascular calcifications occur at plasma [PPi] below 1.5 μM [63]. Patients with heterozygous ENPP1 mutations have plasma PPi ~ half of normal [59,64]. Patients with heterozygous loss of function mutations in ENPP1 are may develop early onset osteoporosis [59], believed to be a consequence of adaptive response as discussed above.

3.1. Modeling of GACI

The best current model is the Enpp1$^{asj/asj}$ mouse [65], progeny of an ENU-mutagenized C57BL/6 mouse. 'Asj' signifies 'age with stiffened joints' due to a V246D point mutation in ENPP1 [65], near the catalytic threonine at AA 238 in mouse ENPP1. This attenuates the enzymatic activity ~75%. Enpp1$^{asj/asj}$ mice have accelerated mortality, pervasive arterial calcifications, and cardiac death on a special diet to accelerate calcification [65,66]. These mice have been used to verify the mechanism by ENPP1 enzyme replacement [67]. To convert the membrane-bound enzyme to a soluble, stable, and bioavailable protein, the signal sequence of Enpp2 was used to release Enpp1 from the membrane surface. The protein was fused to the Fc domain of murine IgG1 to increase the bioavailability and stability. The resulting solubilized Enpp2 has a half-life of ~6–8 h. In initial dosing experiments, there was a dramatic clinical response to ~8–10 mg/kg daily of replacement enzyme. Treated Enpp1$^{asj/asj}$ mice normalized their weights compared to WT, normalized plasma PPi levels, and eliminated vascular and cardiac calcifications [67].

4. Mineralization of true bone

We focus on the air breathing vertebrates, emphasizing mammals. Lower orders are useful in study of specific genetic defects, such as type 1 collagen defects in zebrafish [68], but differences in hard tissue structure and regulation detract from the utility of fish models to inform the study of bone in mammals.

4.1. The bone composite

Over a half century ago it was postulated that the addition of mineral to the bone organic matrix was critical in achieving the necessary mechanical properties [69]. Decades of sophisticated study concluded that bone represents a composite mainly of hydroxyapatite (HA) and collagen [70]. This suggested to many that the mechanism of bone formation to be the stochastic nucleation of HA crystals around the cross linked collagen fibrils. Over time, it has been agreed that in order to achieve the observed and required changes in properties, HA crystallization must occur in association with collagen molecules and not as appendages of collagen fibrils [71,72]. A hierarchical and defined structure of this sort allows the two

major components to share resistance to stress and provide the elastic properties that are present in cortical bone. In human cortical bone, synchrotron X-ray diffraction and scattering revealed the intimacy of the mineral–organic interaction [73]. In bone tissue, the maximum displacement achieved in response to stress forces increases to twice the estimated fracture tolerance of bulk apatite. This indicates a bidirectional relationship between the organic and mineral components of the bone composite [73]. Recent studies using cryo-transmission EM and transmission EM indicate that crystalline structures infiltrate the individual fibrils, even when solution crystallization is suppressed with inhibitors [74]. This was extended *in vitro* to confirm that intra-fibrillar mineralization occurs in the presence of crystallization inhibitors, even though they can be covalently linked to collagen, suppressing inter-fibrillar crystallization but not mineralization and its effects on bone quality [75]. Most importantly, mineralization improves the collagen Young's modulus to values similar to that in quality bone. These observations lead us to conclude that the local environment in the collagen fibril of the osteoid matrix is critical in fostering effective bone mineralization that provides the strength and elasticity of quality bone. Furthermore the intra-fibrillar mineralization may not be the result of stochastic crystallization, but rather result from specific chemistry between calcium-phosphates and the collagen.

4.2. Key elements of bone formation

Osteoblasts perform the key biological functions during bone formation. Osteoblasts form an epithelium-like layer over the osteon allowing the development of an osteogenic environment (Fig. 5). Osteoblasts synthesize Type I collagen in massive quantities, which is heavily crosslinked and produced in lamina with alternating orientation along and orthogonal to the axis of bone growth [18]. Osteoblasts make small quantities of other bone proteins including osteopontin and bone sialoprotein. Osteoblasts convey phosphate and calcium into the osteogenic matrix [76], and remove acid produced by mineral deposition [18,40]. The mechanism linking mineral transport to dense mineral formation remains controversial; calcium phosphate complexes (Posner complexes) diffuse to the site of dense mineralization. A small fraction of apatite in bone is substituted by carbonate or ionized phosphates [77–79], but hydroxyapatite constitutes the vast majority of bone mineral.

4.3. The tight epithelium-like cell layer of bone

A comprehensive understanding of bone remodeling requires that one appreciate that skeletal formation is locally bounded by a tight epithelium-like layer that has operational homology to dental epithelium, Fig. 1. The osteon surface layer of epithelioid osteoblasts displays functional and histological characteristics of a tight cell layer with regulated permeability. Living bone is impermeable to most anions. Dead bone is generally diffusely permeable to these ions, [79], (Fig. 5A). The rest of the time osteoblasts form a tight cell layer excluding small molecules including anionic fluorescent indicators such as tetracycline. The impermeability is related to tight junctions in the surface cells, which have long been known [81]. Primary osteoblasts form epithelial layers with significant trans-epithelial resistance of ≈150 Ohm/cm^2 [82]. In addition to tight junctions, gap junctions have long been recognized [83], which mostly are connexin45 and connexin43 based, and link both surface osteoblasts including with calcium signaling, along with deeper layers of

osteocytes [84,85]. During matrix synthesis tetracyclines and other fluorescent anions are rapidly taken up and deposited in the osteogenic compartment. This activity is short lived but is responsible for a widely used experimental and clinical assay of bone growth [79], (Fig. 5A). It is also clear that bone regulates the water content of matrix [86], which, again, reflects isolation of the matrix from general extracellular space. The mechanism for regulation of bone matrix water is not established.

The osteoblast cell layer supports transport of phosphate [76] and calcium (discussed below) into the osteogenic compartment and the removal out of that same compartment of acid produced during sustained mineralization [40]. As previously reviewed, the osteon is a bone forming unit that occurs between the osteoblast epithelial-like layer and the cement lines [87]. This compartment is morphologically complex but possible to identify in rapidly fixed semi-thin sections and in culture [88]. At the macroscopic level this barrier has been used for decades to evaluate bone growth; a range of calcium-binding fluorescent anions are taken up by new bone specifically in narrow layers only during bone synthesis. These are useful as probes for live-cell microscopic studies *in vitro*, and have long been used to mark bone synthesis *in vivo* [89]. Similar tetracycline deposition occurs in teeth, and highlights the known dependence of tooth development on the dental epithelium for mineralization [90].

4.4. pH and transport

There is limited data on the pH of mineralizing bone matrix, but studies on the chemistry and structure of calcium phosphate precipitation provides solid support for the importance of pH in bone mineralization. Over 60 years ago a monograph characterizing the chemistry of bone mineral as dynamic and uniquely pH sensitive was published [91]. This assertion was confirmed by showing that the calcium phosphate solubility increased dramatically as pH went from 7.4 to 6.8 [92]. It was further noted that even in buffered solutions, as precipitation proceeded, the pH decreased, indicating that protons were generated by precipitation of hydroxyapatite. Finally, it was observed that soluble collagen promoted precipitation and the pH change, while gelatin did not. Later it was shown that under conditions when pH was held constant at a slightly alkaline value (for example 7.4) that HPO_4^{-2} was the dominant phosphate species (> 60%) entering the apatite and that each phosphate entering the crystal generated two protons [93]. This allows the formulation of a balanced equation for a solution of calcium phosphate becoming apatite crystals:

$$6HPO_4^{2-} + 2H_2O + 10Ca^{2+} \leftrightarrow Ca_{10}(PO_4)_6(OH)_2 + 8H^+ \quad (1)$$

Thus predicting that bone mineralization will produce substantial protons, in the osteon, that must be removed.

In a direct study of pH in healing bone, Chakkalakal et al. [94] found that in early healing, tissue pH is lower than serum pH, consistent with accumulation of acidic metabolites. Subsequently, pH increased to alkaline levels with rapid mineralization, in keeping with removal of acid produced. The results of these studies support observations suggesting that the pH of tissue plays a regulatory role in mineralization of bone. Considering *in vitro* studies where hydroxyapatite precipitation generates protons ([92] and Eq. (1)) the observed

alkalinization is consistent with high activity of Na^+/H^+ exchangers in active osteoblasts [40,95].

4.5. Calcium transport into bone

Bone and teeth constitute a major reservoir of calcium. Specific calcium transport mechanisms are largely undefined in osteoblasts, but it appears that calcium is not actively transported. In other sites including the gut and kidney, para-cellular transport is of key importance, and regulated [96]. Calcium transport involves transient receptor potential vanilloid 6 (Trpv6) calcium channels mediating calcium transfer across the intestinal apical membrane [97], but Trpv6 is expressed only at low levels in osteoblasts [98], so this mechanism is unimportant in bone. Osteoblasts express several claudins [82] that might be involved in similar transport in bone, but calcium paracellular transport in osteoblasts is not specifically studied. These include claudins 2 and 12 [82], critical for paracellular calcium absorption in enterocytes and renal tubular cells [99]. Significant active calcium transport by osteoblasts is unlikely: Osteoblasts have Na^+/Ca^{2+} exchangers that are *negatively* regulated by dihydroxyvitamin D and parathyroid hormone [100]. Osteoblasts express a calmodulin-activated plasma membrane Ca^{2+}-ATPase, but not in quantities expected for mineralization transport of calcium [101,102]. Osteoblasts and osteocytes contain the calcium binding protein calbindin, which is upregulated by dihydroxyvitamin D [103]. In teeth, calcium transport is independent of calbindin [104], suggesting independence of bulk calcium transport from an intracellular calcium pool. On the other hand, sodium-calcium exchangers are expressed in quantities consistent with transport at the osteoblast-matrix (apical) membrane [105], so it cannot be excluded that some calcium transcytosis occurs in osteoblasts.

4.6. Phosphate transport to form bone mineral

Phosphate metabolism is a universal aspect of eukaryotic biology but, especially in vertebrates, is important in bone and teeth. It has been studied extensively, but it is complex, and major conceptual and experimental gaps remain. One major issue the contribution of organic phosphate esters to the transcellular movement of phosphate for collagen mineralization. Massive osteoblast expression of alkaline phosphatase was reported 100 years ago, as phosphate ester hydrolysis [106] by the orthophosphoric-monoester phosphohydrolase, now generally called alkaline phosphatase, reflecting to the enzyme's alkaline pH optimum [107], in contrast to the osteoclast's acid phosphatase. Twenty-five years later hypophosphatasia was designated an inborn error of metabolism with severe skeletal consequences [108]. Subsequently hypophosphatasia has been studied in illuminating detail, contributing to our understanding of collagen mineralization [109]. The bone isoform of the enzyme is an unusual ecto-enzyme, that is, attached (at least mostly) to the matrix side of the osteoblast apical surface [110]. This supports the hypothesis that bone alkaline phosphatase isoenzyme (also called tissue nonspecific alkaline phosphatase or liver/bone/kidney alkaline phosphatase – transcribed from the same gene) supplies phosphate for bone mineral. It is clear that phosphate is trafficked into the osteogenic matrix by a transcellular pathway and is presented for mineralization extracellularly as summarized below and illustrated in Fig. 6. The alkaline phosphatase degrades pyrophosphate, which accumulates in its absence [46], and all organic phosphates as well. One key organic

phosphate that accumulates in its absence is pyridoxal phosphate [111,112], although it is not known whether pyridoxal phosphate is a major part of organic phosphate transmitted by osteoblasts.

4.7. Phosphate transport overall and in bone

Phosphate is transported in major quantities in the gut and in the kidney, as well as in bone. In all cases described to date, phosphate movement *into* cells is mediated by sodium-phosphate co-transporters, a subject not specific to bone and reviewed recently [113]. Phosphate entry in osteoblasts is demonstrated to occur by sodium-dependent neutral phosphate transport [76]. Neutral phosphate transport in osteoblasts is, in part, regulated by the sodium hydrogen exchange regulator factor-1 (NHERF1) [95]. Known sodium-linked transport mechanisms in apical membranes of epithelial cells are SLC34 and SLC20 [114]. SLC34 isoforms are found in osteogenic cells and during tooth mineralization; this transporter is specific for the phosphate di-anion and might to lessen acid load during mineralization [115]. Neutral phosphate transporters SLC20A1 and A2 are active in bone [76]. Selective genetic suppression of sodium-dependent phosphate transport produces strong phenotypes with the absence of SLC34A2 is an embryonic lethal condition in mice [116]. Knockdown or mutation of SLC34A1 [117] or A3 [118] in mice are survivable, with disorders of mineral metabolism, the portion due to osteoblast effects being unknown.

4.8. The distribution of phosphate after uptake in osteoblasts

It was conclusively demonstrated that phosphate entering bone in the presence of sodium is rapidly, and essentially quantitatively, converted into organic phosphates. Specifically, by ^{32}P analysis, 87% of phosphate entering osteoblasts is found in the organic fraction after 5 min [119,120] (Fig. 6).

4.9. Export of phosphate from cells

The delivery of phosphate *from* cells is much less fully understood (Fig. 6). Only one transporter is known to deliver phosphate from cells on the side opposite sodium-dependent phosphate uptake, that being, in kidney, the xenotropic and polytropic retrovirus receptor 1 (XPR1 or SLC53A1), believed to liberate free phosphate [122,123], although Na^+ dependency of transport, possibly related to secondary factors, is reported [124]. Sodium dependency of transport by a closely related protein, GLVR1, was reported but would not favor Pi release into the mineralizing matrix [125]. Deletion of XPR1 in the renal proximal tubule causes hypophosphatemic rickets [126]. In the intestine, although transport into the body is well known, the mechanism phosphate export is not studied, but is hypothesized to use XPR1 [113].

In osteoblasts specific phosphate export mechanisms into the osteon matrix are not described (Fig. 6). Expression libraries from osteoblasts show XPR1 is present only at low levels (not shown). Furthermore, when two homologs of XPR1 were genetically eliminated in zebrafish, bone formation was not impaired [127]. On the other hand, organic phosphates transported to the apical membrane are degraded to produce free phosphate [112,128] by the osteoblast external membrane-bound alkaline phosphatase [110]. Among the interesting and yet not fully studied possibilities include that, in hypophosphatasia, pyridoxal phosphate

accumulates in serum [111], suggesting that pyridoxal phosphate is a substrate for the alkaline phosphatase ectoenzyme and might contribute directly to phosphate transport in osteoblasts. This has only been studied indirectly [129]. Pyridoxal kinase is one of several mechanisms to mediate rapid conversion of osteoblast intracellular phosphate [76,114] to organic forms [119]. Other substrates for alkaline phosphatase include pyrophosphate, which is a second barrier to mineral formation in hypophosphatasia [46]. This is particularly, if still hypothetically (Figs. 4, 6), important in bone because osteoblasts express highly the pyrophosphate transport-regulating protein ANKH [121], thus, potentially, capturing, by pyrophosphate export, the large amount of pyrophosphate produced during collagen synthesis in osteoblasts. However, pyrophophosphate exported must be completely degraded to avoid preventing hydroxyapatite formation. The role of pyrophosphate inhibiting mineralization discussed in “Regulation of mineralization in normally non mineralized tissues”, above. Free phosphate and calcium near the apical membrane of osteoblasts produce small calcium phosphate aggregates that are consistent with the small hydroxyapatite crystals that develop in dense mineral within type I collagen subjacent to the cells [35]; (see Fig. 5).

4.10. Initialization of mineralization

No-one disputes that hydroxyapatite is the dominant bone mineral. Similarly, the organic matrix is overwhelmingly Type I collagen. Controversy occurs when considering the transformation from soluble mineral components and collagen gel to the bone composite, Earlier in this review we present a speculation on the formation of the hydroxyapatite-collagen composite that is catalyzed by Type I collagen substrate and strongly promoted by pH. Here we present our understanding of several alternate pathways through early mineralization.

There are many parallels between bone mineralization and cartilage mineralization, including matrix vesicles [130] at mineralization sites, and high levels of alkaline phosphatase and PHOSPHO1 [131,132]. In cartilage the majority of the mineral is located in the organic matrix, not matrix vesicles [35]. However, the mineral density and the material properties of cartilage never reach those of bone (see Fig. 3). We propose that this is primarily because cartilage is not mineralized behind an epithelial-like cell layer (osteoblasts) controlling the local environment [133]. Thus, deposition of mineral in cartilage is driven by exceeding the mineral solubility product, and not influenced by fine pH control and a uniform dense Type I collagen substrate.

4.11. Intra-vesicular and intra-cellular crystallization

A confounding thread of speculation follows observations of crystals in vacuoles in cartilage [34] and bone [134]. This hypothesis is that mineral formation occurs within osteoblasts or osteoblast matrix vesicles [130]. These crystals are proposed to be transferred by unknown mechanisms to form, or catalyze, the formation of dense and smaller hydroxyapatite crystals in cross-linked collagen fibrils. Indeed, it has been hypothesized that there is *no direct transport of mineral into bone matrix* and mineralization occurs by the formation of amorphous crystals in ~0.2 to 0.3 μm cell vesicles that are released into the extracellular matrix, where they can accrete more mineral [135]. This is often called the “*crystal*

nucleation hypothesis." This hypothesis is attractive for its simplicity, but it supposes the spontaneous and coordinated occurrence of complex events and in the opinion of the authors needs significant modification. Specifically, it does not include phosphate (or calcium) transport into the matrix or account for export of acid produced in formation of hydroxyapatite [40]. It does not account for the isolation of bone matrix from the general extracellular fluid [79], or for why the activity of alkaline phosphatase resides on the matrix side of the osteoblast cell membrane. As discussed above, calcium transcytosis is believed to be a minor part of calcium transport for bone formation, so calcium is less available for intra-cellular or intravesicular crystal formation. It should be noted that for crystal formation there is *always* crystal nucleation. We assert that kilograms of mineral in the human skeleton are accreted mainly by regulated transport of calcium, phosphate, and removal of acid released from formation of hydroxyapatite. Further in this regard, Landis et al. pointed out that extracellular vesicles are not uniformly present during mineralization [136]. We know of no proposal defining how the crystalline structures are transferred out of the extracellular vesicles into crosslinked collagen fibrils to assemble the bone composite. Finally, bone mineralization is exquisitely controlled, and yields an atomistically integrated composite with physical properties distinct from separate collagen and hydroxyapatite components [73].

4.12. The fate of osteoblast membrane vesicles

In the mineralized matrix the only membrane bilayer structures are associated with osteocytes, indeed vesicles are excluded once dense cartilage develops and the only exceptions are canaliculi connecting osteocytes and osteoblasts (Fig. 5D–E). This raises an important question as to where vesicles produced by mineralizing osteoblasts are destined. Since there are no macrophages in bone matrix, the vesicles are not degraded by phagocytic cells; other possibilities include that the vesicles are taken back up by osteoblasts, or that the membranes are completely degraded. While vesicles bear the alkaline phosphatase ectoenzyme, and the phosphates from the membranes are removed by PHOSPHO1, and this phosphate probably contributes to initial mineralization, there are no precedents for membrane bilayer dissolution, although some reports point to penetration and release of crystals from vesicles [137]. For formation of vesicles, there are also multiple hypotheses including ectosomes from vesicle fusion and exosomes from multi-vesicular bodies [80]. We hypothesize that membrane vesicles in developing bone matrix fuse back to osteoblasts, since bone matrix does not contain significant membrane lipid, bone matrix is not patrolled by macrophages, and matrix vesicles are excluded from dense type I collagen (Fig. 5F). Autophagy in osteoblasts [138,139] is in keeping with recycling of membrane vesicles.

Apical membrane vesicles (Fig. 5C–D) are proposed to participate in the production of new mineral. However the importance of this could be judged better if their mechanism of formation was understood, budding verses multi-vesicular-body exocytosis. The enzymes for phosphate production, PHOSPHO1 and alkaline phosphatase, are present, although the amount of phosphate associated with vesicles is by itself insufficient for the mass of dense mineral formation in bone. Osteogenesis is known to require membrane transport of phosphate in, and of acid out, by an epithelial (or epithelioid) osteoblast cell layer [40,140]. Alkaline phosphatase and PHOSPHO1 can support initial mineralization, for which there is

a large literature [141]. However, the connection of this to collagen-bound hydroxyapatite, and the development from initial mineral to dense hydroxy-apatite collagen layers, is not worked out. Aside from initial mineralization and matrix vesicles, bulk phosphate transport through the osteoblast surface layer, and removal of acid produced by hydroxyapatite deposition, is at a further step critical to the formation of dense mineral in bone [18].

Osteoblasts also shed vesicles from their basolateral surface (Fig. 5B) [142]. These may be generated from either plasma membrane budding (ectosomes) or fusion of multi-vesicular bodies with the plasma membranes. While the fate and function of these vesicles is also not established, potential roles in RNA and protein transport to modify differentiation of developing new osteoblasts and related cells have been described [142]. Signaling by cell surface glycosaminoglycans promoting osteoblast formation is described [143].

5. Reprise: support of mineralization by the surface layer of bone forming cells

The osteoblast surface cells are an epithelial-like layer that isolates the forming bone matrix from surrounding extracellular fluid (Fig. 5A) [79]. As shown in Eq. (1), formation of hydroxyapatite from the soluble calcium and phosphate ions at physiologic pH generates protons. The number of protons depends upon the phosphate species that participates in crystallization. The removal of these protons is accomplished in the same layer of surface osteoblasts that define the mineralization frontier. Along their basolateral border these cells express massive sodium−hydrogen exchange capacity that can transport acid out of the osteon [40,144]. This transport capacity is under the control of the sodium-hydrogen exchange regulator-1 [95], which is expressed on surface epithelial-like osteoblasts, but not significantly on the deeper osteocytes derived from these cells in bone formation. Actual H^+ transport is a mixture of NHE1 and NHE6 activity, a likely reason for survivability of NHE1 knockout [145]. NHE activity removes H^+ from the osteoblast using the Na^+ gradient maintained in all mammalian cells. Antibody localization of NHE-1 in the basolateral membrane of osteoblasts [144] is consistent with this, although how NHE activity is regulated is unclear.

It is not clear how protons from mineral formation enter through the osteoblast apical membrane [18]. In expression libraries of bone-producing osteoblasts the levels of mRNA for the chloride-proton anti-porters, ClC-3 and ClC-5 are elevated to unprecedented levels, and the proteins are, surprisingly, histologically associated with the apical membranes of osteogenic osteoblasts [140]. Further, surface membranes from mineralizing osteoblasts in culture, reconstituted into membrane vesicles, show that chloride-dependent proton transport is present, but is blocked if Cl^- inside vesicles is replaced by gluconate. These same preparations do not exhibit ATP-dependent acidification are therefore not detectably contaminated by endosome, lysosome or secretory vesicle membrane, the usual location of ClC-3 and 5 [40]. However endosome and secretory vesicle membranes cycle through the plasma membrane and it is known that ClC-3 cell surface isoforms depend on an N-terminal retention signal [146].

6. Conclusion

Physiologic mineralization of tissues is essential in mammals. Non-physiological mineralization is disease producing, often fatal. Although complex, key aspects of mineralization are conserved in phylogeny and are important to consider in medicine. Included are the occurrence of mineral behind an epithelial barrier and selective transport of Ca^{2+}, phosphate, and H^{+}. Important gaps in understanding of mineralization remain, but we are confident the phylogeny and ontology of mineralization are constrained by physics, chemistry [147], reflect ancestral mechanisms and parallel transport mechanisms in other organs.

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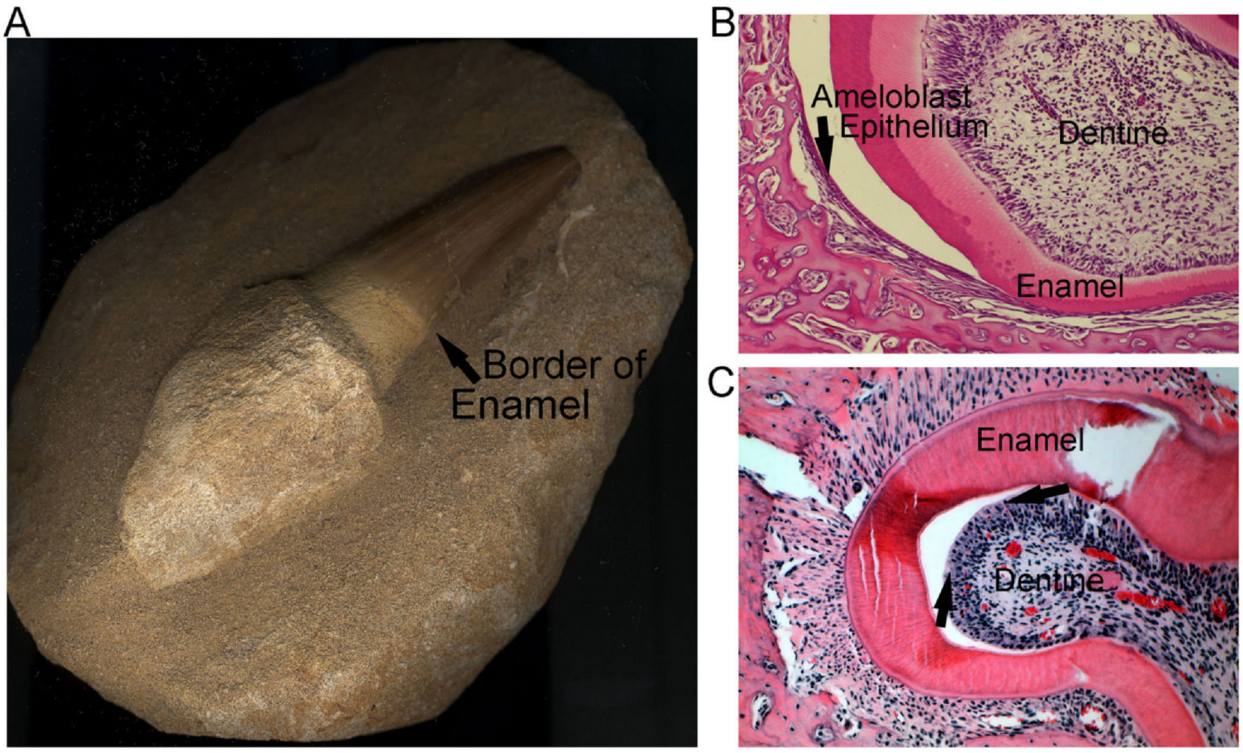

Fig. 1.

Key features of teeth in higher vertebrates. Unpublished photographs; the mosasaur tooth, (A), was a kind gift of Dr. Mary Sorrells, Pittsburgh PA. Developing mouse teeth in (B,C) are unpublished sections from [11].

A. A fossil tooth from a mosasaur, a Cretaceous period marine dinosaur-related reptile, ~100–66 million years ago. Note that the enamel, even in the fossil, is clearly seen above the arrow. The tooth, from root to tip, is 5 cm long.

B. Developing tooth from a wild type C57Bl/6 mouse showing the ameloblast layer (arrow); this epithelial layer is difficult to see in intact sections; where the tooth is separated by an artifact of cutting it is clearly seen. Section 500 μm across.

C. Developing tooth from a wild type C57Bl/6 mouse showing the odontoblast layer (example between the two arrows) at a site where the overlapping enamel is separated during cutting of the specimen. The section is tangential, making the odontoblast layer appear more than one cell layer thick. Section 500 μm across.

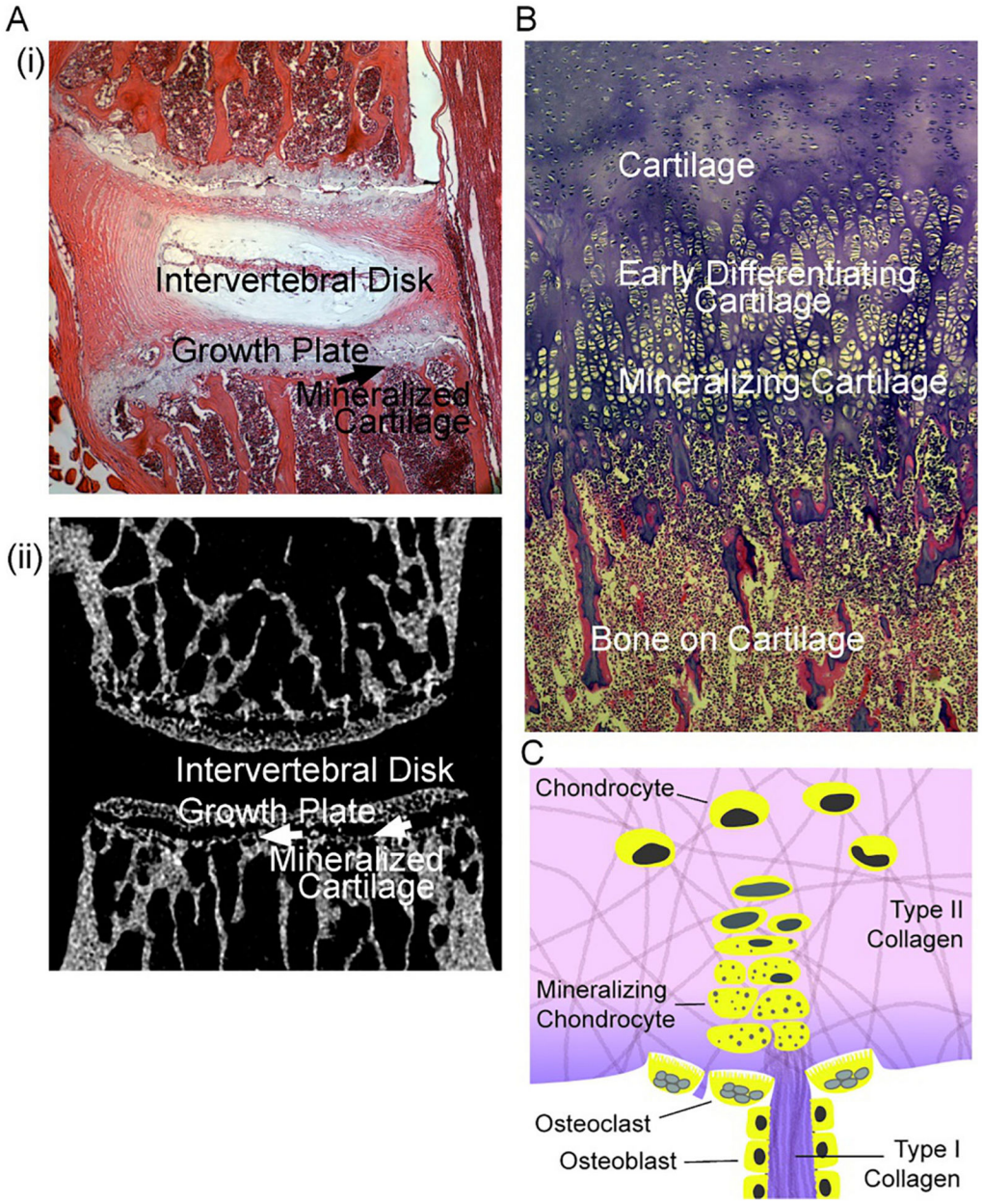

Fig. 2.

Characteristics of mineralizing cartilage. Mouse bone section photographs are unpublished frames of wild type mice [11]; the section of term human bone is an unpublished frame from [30].

A. Left. (i) At the top, a section of mouse vertebrae L2 and L3, showing the disk and adjacent growth plates. (ii) At the bottom, a similar section by micro computed tomography showing the mineralized cartilage at the transition of growth plates to bone, and the adjacent trabecular bone. Each section is 2 mm across.

B. Right. Morphology of cartilage to bone transition in a human term fetus, with cartilage and its transition to bone labeled. This is a section of rib at the transition to bone; in the rib, a broad section of cartilage is involved; narrower regions of growing cartilage occur in

vertebral growth plates as in (A) or diagrammed in (C). The region “mineralizing cartilage” is often called “hypertrophic”, see text. The section is 1 mm across and ~2 mm vertically. D. Diagram of typical structure of a growth plate. Shown are cartilage from the growing plate (top) composed of chondrocytes in mainly type II collagen, and “hypertrophic” chondrocytes that in part lose nuclei and retain vesicles [24] with mineral producing enzymes [31]. At the transition to mineralized cartilage, some type I collagen is present, with trans-differentiation of chondrocytes [32]. Mineralized cartilage (darker color) is degraded mainly by chondroclasts (osteoclasts) and osteoblasts form on this matrix. The authors hypothesize that calcium-phosphate complexes are important in mineralizing collagen, including type I collagen produced in mineralizing cartilage or associated osteoblast-like cells [33], although in early mineralization mineral in vesicles might be important; see text.

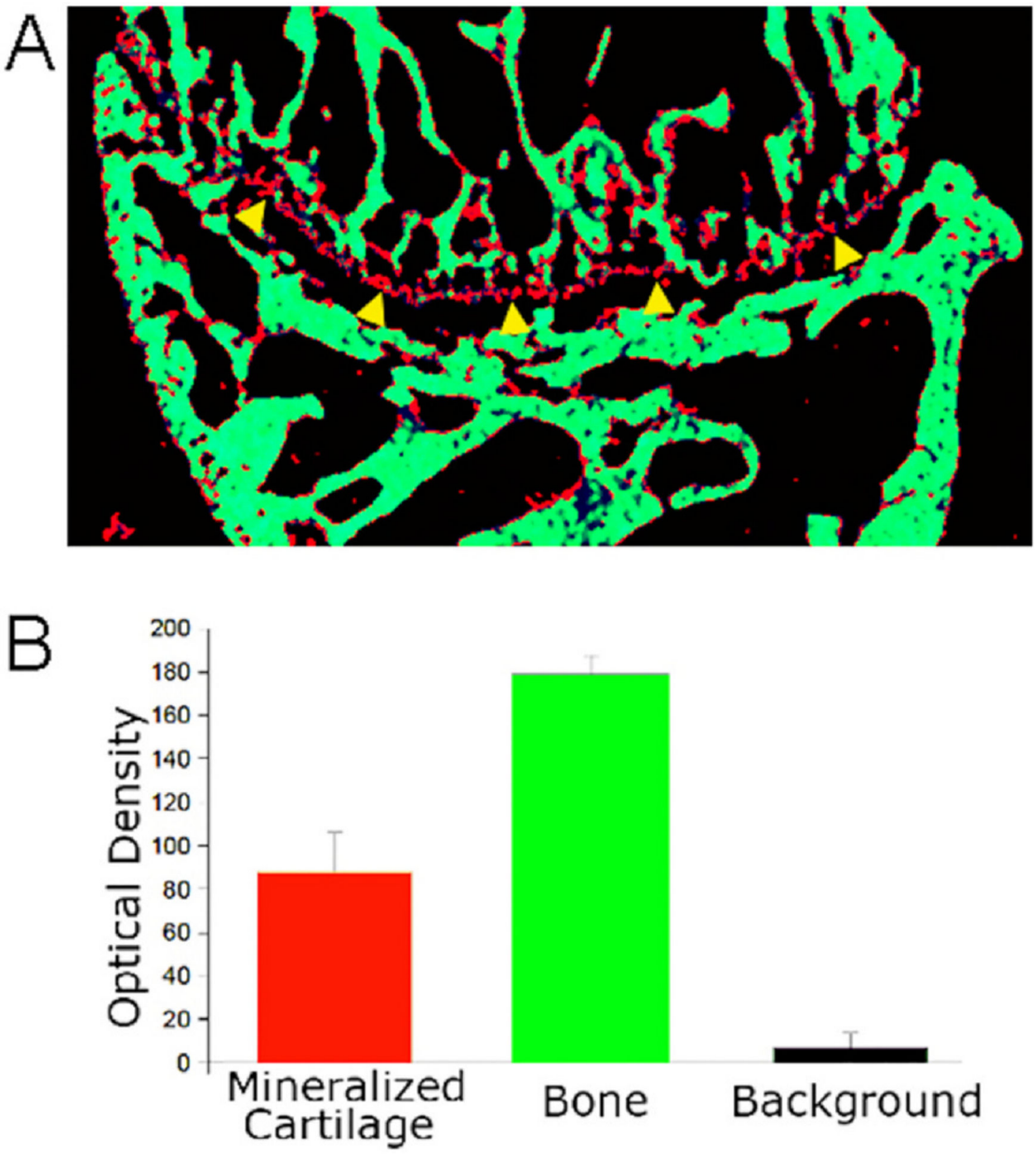

Fig. 3.

Comparison of Cartilage and Bone Mineral Density. Unpublished photographs from [11]; mouse sections and density are of wild type mice, as in Fig. 1.

A. Micro-computed tomography of a mouse femur tibial end. The pixels are color coded for density of < 10, black, 10–100, red, and > 100, green. Note the mineralized cartilage is seen as a red line (marked by yellow arrowheads at its bottom; compare with Fig. 2A, lower panel).

B. Density of mineralized cartilage, bone, and background from micro computed tomography as in (A). $N = 4$, mean ± SD. The cartilage has approximately half the density of bone; the difference is significant, $p < 0.001$. This difference in cartilage and bone mineral density reflects, in major part, that cartilage is not vascular, and in addition to having

a lower mineral density than bone, cartilage has more variable mineral composition [39], see text.

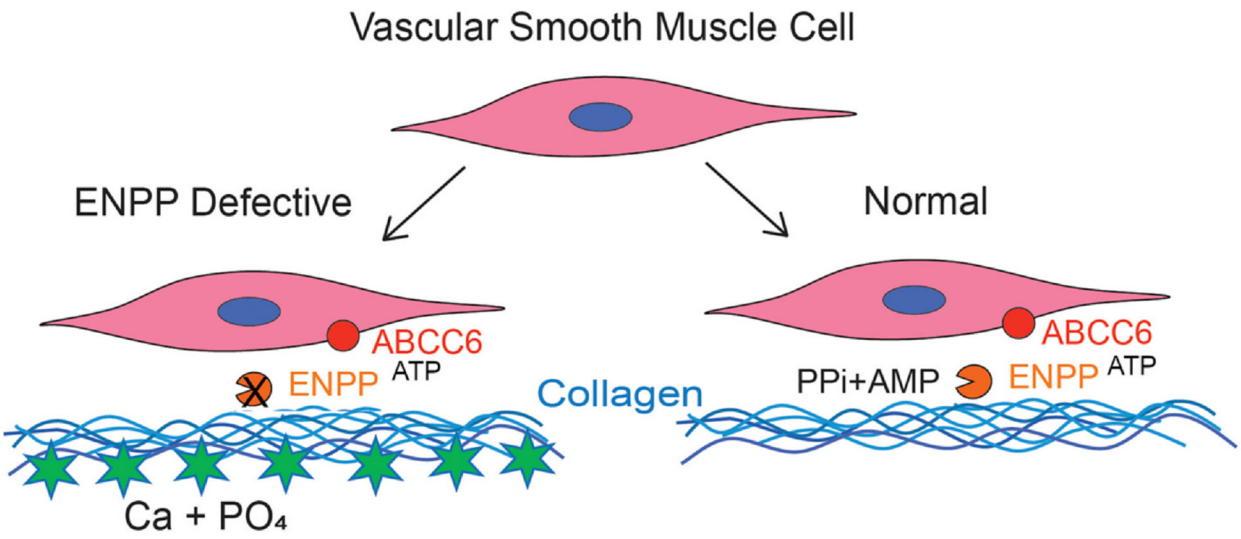

Fig. 4.

Mineral formation in normally non-mineralized tissues occurs when pyrophosphate cannot be produced.

The model shows arterial tissue including type I collagen and arterial smooth muscle cells. Normally, the transporter ABCC6 produces small amounts of extracellular ATP, converted to pyrophosphate and AMP by ENPP1 (right). If ENPP1 or ABCC6 are absent (left) calcium and phosphorus in the serum are adequate to cause extracellular calcification.

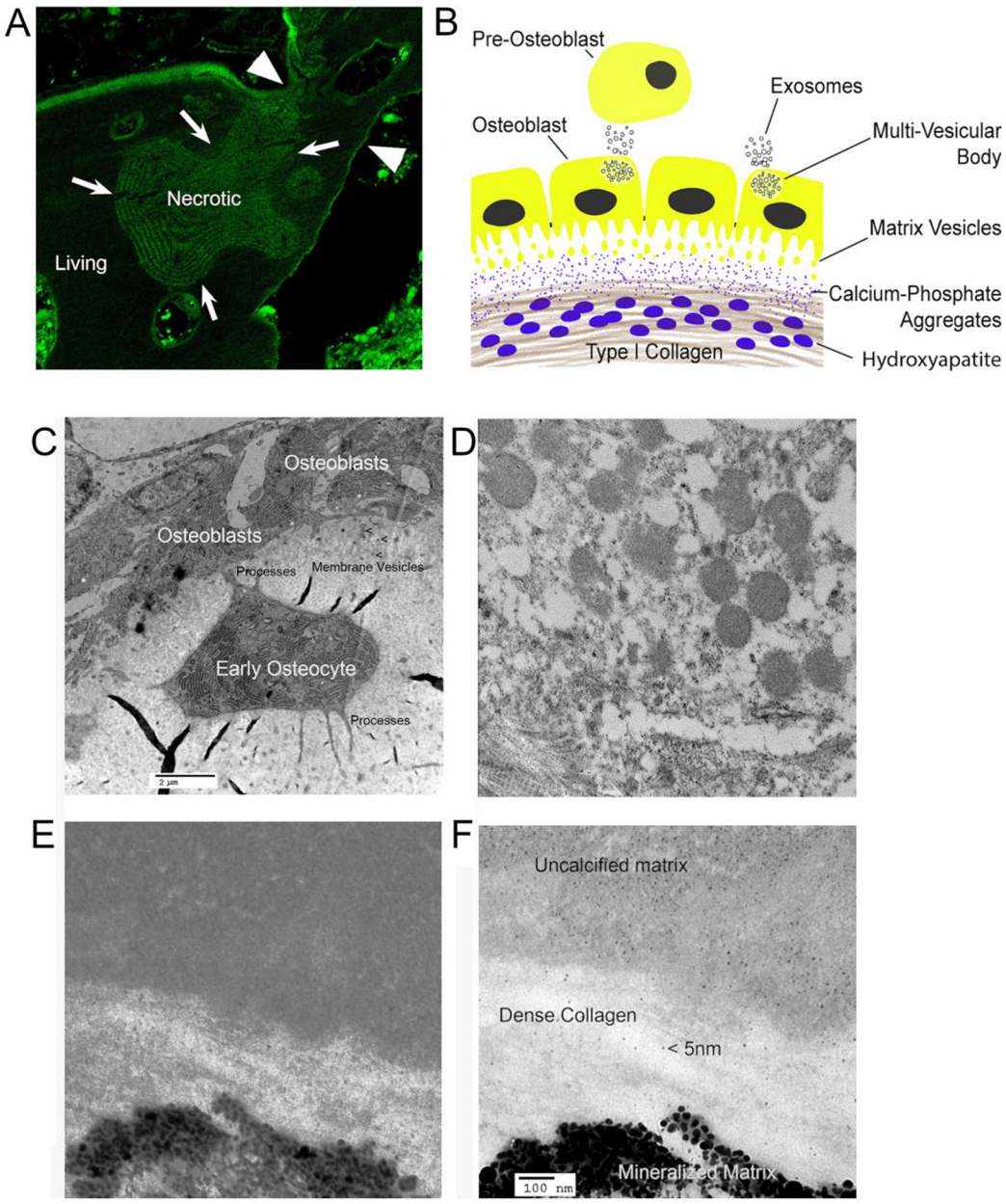

Fig. 5.

Membranes, vesicles, and the apical surface of the bone epithelioid layer. (A) is previously published in [79]; (C–F) are unpublished frames from [40], except (E), from that work, used with permission.

A. Bone is separated from general extracellular fluid by an epithelial-like cell layer. The photograph shows tetracycline labeling of bone formation (top of section) but that tetracycline otherwise is excluded from living (appears dark), but not necrotic (shows labeling) bone; bone is impervious to water and most small ions, but not calcium. For details, see [79].

B. Diagram of bone formation with two types of vesicles. Vesicles, at least in part from multivesicular bodies, are produced on the basolateral surface and serve, at least mainly, to

communicate with other cells. At the apical surface, matrix vesicles [80] as in (C) and (D) increase surface area of the cells at the site of mineralization and express phosphate producing enzymes also found in the osteoblast. Matrix vesicles are excluded from dense collagen. Small calcium-phosphate complexes diffuse into dense matrix and form mineral on collagen strands.

C. Plastic embedded thin section of mouse bone in EM (~20 μm across) [40] showing membrane vesicles at the osteoblast apical membrane, the bone secreting side. To retain calcified material, 2 mm thick calvarial bone was fixed 5 min in 4% paraformaldehyde in phosphate buffer at 4 °C and moved directly to dehydration for plastic embedding. An early osteocyte, recently separated from the osteoblast layer, is seen with processes connecting the cell to osteoblasts, and below the osteocyte connecting to deeper osteocytes (not seen here). Irregular black areas are tears in the section.

D. The appearance of this section of the apical surface at higher power. Many vesicles from the membrane are seen. Average vesicle size is ~300 nm. Collagen fibers with crosslinking are seen below the membrane vesicles. Vesicles are excluded from dense collagen fibers, where mineral accumulates (see text).

E. Early mineralized bone on EM of a similar section with dense collagen. Hydroxyapatite crystals ~30 nm associated with collagen are seen. No bilayer vesicles occur in dense crosslinked collagen.

F. The section (E) at higher power. Amorphous calcium phosphates (based on Posner complexes) of small size, ~5 nm, occur in the non-mineralized and dense collagen layer, hypothetical diffusible calcium phosphate aggregates [36,37].

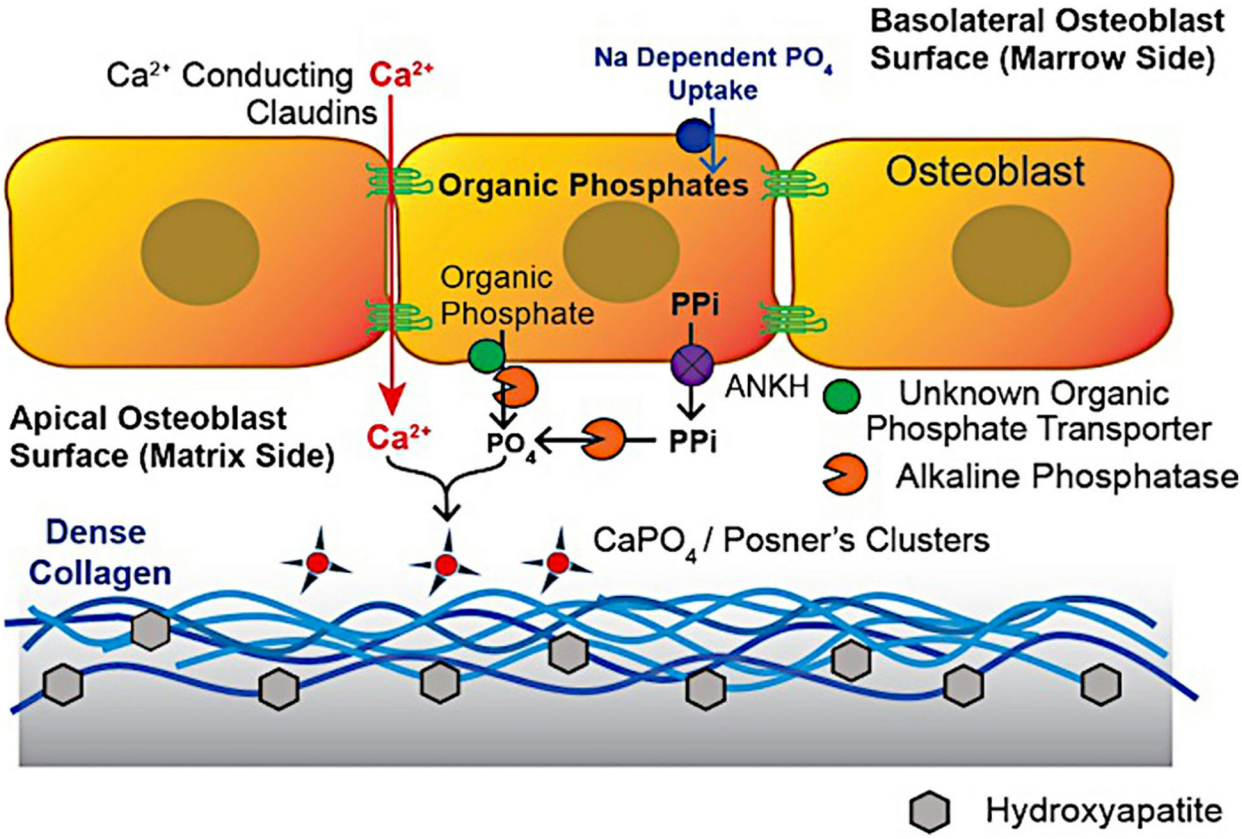

Fig. 6.

Major mineral transport mechanisms in bone. For Ca^{2+}, transport is largely trans-cellular [82]. Phosphate from sodium-dependent uptake at the basolateral surface [76] is converted to organic phosphate [119] and exported from the apical surface by unknown transport mechanisms. This is followed by release of phosphate by an ecto-enzyme (extracellular membrane attached) alkaline phosphatase [110]. The osteoblast also massively expresses the pyrophosphate transport regulator ANKH [121], possibly capturing PPi produced during collagen synthesis: one pyrophosphate is produced per amino acid added. In the absence of alkaline phosphatase, accumulation of organic phosphates including pyridoxal phosphate [110] and of pyrophosphate [112] occurs, both related to attenuation of mineralization.