Mechanism of Bone Mineralization

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Mineralized “hard” tissues of the skeleton possess unique biomechanical properties to support the body weight and movement and act as a source of essential minerals required for critical body functions. For a long time, extracellular matrix (ECM) mineralization in the vertebrate skeleton was considered as a passive process. However, the explosion of genetic studies during the past decades has established that this process is essentially controlled by multiple genetic pathways. These pathways regulate the homeostasis of ionic calcium and inorganic phosphate—two mineral components required for bone mineral formation, the synthesis of mineral scaffolding ECM, and the maintenance of the levels of the inhibitory organic and inorganic molecules controlling the process of mineral crystal formation and its growth. More recently, intracellular enzyme regulators of skeletal tissue mineralization have been identified. The current review will discuss the key determinants of ECM mineralization in bone and propose a unified model explaining this process.

The mineralized extracellular matrix (ECM) is a unique feature of the vertebrate skeletal system. The massive load-bearing capacity of the mineralized skeleton permitted the evolutionary emergence of large vertebrates, such as blue whales, which can weigh up to 180 tons. However, the functions of the mineralized tissues are not limited to support the body mass, protect the internal soft organs, or to facilitate locomotion and mastication only; they also serve as a readily accessible reservoir for essential minerals that are indispensable for many physiologic activities. The current review will focus on the mechanism of calcium phosphate biomineralization in the vertebrate skeleton.

The origin of biomineralization has been traced back to the late Precambrian period after tectonic activities caused a marked increase of soluble minerals in the seawater (Wagner and Aspenberg 2011). It is commonly believed that marine organisms first developed primitive exoskeletons made up of calcium carbonate and/or calcium phosphate minerals (Knoll 2003; Wagner and Aspenberg 2011). As part of the process of evolutionary adaptations, the skeletal tissues were internalized, which paved the way for the emergence of organisms with larger body sizes. Whereas it is not clear what prompted some primitive organisms to deposit calcium phosphate instead of calcium carbonate minerals in

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their skeletal tissues, it is conceivable that the deposited calcium phosphate minerals provided certain physiological advantages. Indeed it has been suggested that the presence of calcium phosphate minerals makes the skeleton more stable under acidic conditions (Wagner and Aspenberg 2011). Vertebrate organisms rely on ATP generation via anaerobic glycolysis for sudden rapid movements during “fight-or-flight” situations. The activation of this pathway results in an acidic tissue environment, which would have destabilized a skeleton made up of calcium carbonate more readily.

MINERAL COMPOSITION OF BONE

In 1771, Scheele first reported the presence of calcium phosphate minerals in bone (Scheele 1931). Later, bone mineral was described as a form of hydroxyapatite $[(Ca)_{10} (PO_4)_6(OH)_2]$ comparable to geological apatite (a group of phosphate minerals) by both chemical composition and X-ray diffraction pattern analyses (see Eliasz and Metoki 2017 for a comprehensive review on the historical perspective). However, subsequent studies demonstrated that minerals in bone do not have a uniform composition (Rey et al. 1995; Pasteris et al. 2004). Although there are still some disagreements about the initial phase of the deposited mineral and its time-dependent transition to apatite, it is now accepted that the matured bone mineral is a substituted crystalline phase of calcium phosphate, referred to as carbonated hydroxyapatite (Mahamid et al. 2008). High-resolution transmission electron microscopy (TEM), 3D stereoscopic TEM, and atomic force microscopy on organic matrix-free bone samples provided accurate measurements of bone crystals. These studies have firmly established that bone crystals are nanosized, long, and very thin platelets (Rey et al. 2009).

The source of the dispute regarding the early mineral phase in bone appears to be a simple in vitro experiment performed in the mid-sixties in the laboratory of Dr. Posner (Boskey 1997). The mixing of concentrated solutions of calcium chloride and sodium acid phosphate resulted in the precipitation of calcium phosphate salts, which showed a broad and diffused pattern by

X-ray diffraction analysis. This pattern suggested that the precipitated mineral is amorphous calcium phosphate (ACP), not apatite. Interestingly, when X-ray diffraction analyses were performed on the same samples 2 days later, the precipitates were found to be a poorly formed crystalline apatite (Boskey 1997). This initial finding of mineral phase transition in vitro prompted follow-up experiments using mineralized tissue samples and, subsequently, the presence of ACP in the embryonic chick bones was reported by the same group (Eanes et al. 1965). However, the controversy on whether bone contains ACP continued as later studies concluded that the embryonic bones do not contain ACP (Bonar et al. 1983; Grynpas et al. 1984). Since then, more sophisticated analyses of bone minerals using Raman spectra, Fourier-transform infrared spectroscopy (FTIR), and synchrotron-generated X-ray diffraction techniques have been performed; however, a consensus on the issue is still elusive (Rey et al. 2009).

The presence of amorphous mineral phase has been reported in some invertebrates (Becker et al. 1976, 2005). Additionally, ACP has been detected at the sites of ectopic calcification in the vertebrates (Marulanda et al. 2017). In two studies published by Mahamid et al. (2008, 2010), ACP has been identified as a major mineral phase in zebrafish fin bones, suggesting that amorphous to crystalline phase transition occurs in vivo. On the other hand, concerns have been raised about this latter finding on the ground of technical limitations, including possible mineral phase transition during sample collection and processing (Rey et al. 2009).

DETERMINANTS OF SKELETAL TISSUE MINERALIZATION

In human embryos, although primary ossification centers of endochondral bones (e.g., vertebrae and long bones) appear between 8 and 12 weeks of gestation, the bulk mineralization of skeletal tissues does not occur until the third trimester (Kovacs 2003). The type I collagen-rich ECM in the intramembranous bones mineralizes directly, whereas in the endochondral bones, mineralization starts concomitantly by

two sites: within the core region of the cartilage anlagen and along the bone collar surrounding it (Karsenty and Wagner 2002). In the cartilage, the type X collagen matrix synthesized by the hypertrophic chondrocytes serves as the mineral scaffold, whereas in the bone collar (and later in the trabecular bones), the primary mineral scaffolding protein is the osteoblast-derived type I collagen (Karsenty and Wagner 2002). A brief discussion on the key determinants regulating skeletal ECM mineralization is presented below.

Systemic Levels of Calcium and Phosphate Ions

The chemical structure of bone mineral implies that the extracellular levels of ionic calcium (Ca^{2+}) and inorganic phosphate (P_i) will be two critical determinants for bone mineralization. Indeed, data from patients and animal models of human diseases clearly demonstrates that the reduction of systemic P_i levels with or without any alteration of the Ca^{2+} levels lead to osteomalacia with the characteristic increase of unmineralized osteoid volume. The importance of systemic levels of these mineral ions in bone mineralization was demonstrated by impairment of the 1,25 dihydroxy vitamin D_3 signaling pathway. For example, mutations in 25, hydroxy D_3 -1 α -hydroxylase (required for functional vitamin D synthesis) or inactivating mutations in vitamin D receptor, impair the absorption of Ca^{2+} and P_i in the gut (Dardenne et al. 2001; Masuyama et al. 2001; Panda et al. 2001). Additionally, these mutations also reduce the mobilization of these ions from the bone by restricting bone resorption (Suda et al. 1992).

Although Ca^{2+} and P_i are both integral parts of bone mineral, genetic experiments in mice suggest that circulating P_i may have a more prominent role in the regulation of bone mineralization. For example, in *Hyp* mice with a mutation in the *Phex* gene (a model of human X-linked hypophosphatemia [XLH]), the calcium level is normal, but the systemic P_i level is reduced to almost half, causing a severe osteomalacia phenotype (Eicher et al. 1976; Costa et al. 1981). The inactivation of PHEX in the *Hyp* mice

or XLH patients leads to an increase of circulating fibroblast growth factor 23 (FGF23) produced by osteoblasts and osteocytes (Xia et al. 2007). Increased FGF23, in turn, down-regulates the expression of sodium phosphate transporters in the kidney tubules preventing P_i reabsorption and causing hypophosphatemia. As expected, in *ApoE-Fgf23* transgenic mice in which systemic FGF23 level increases in the absence of any PHEX mutations, a reduction of systemic P_i level and osteomalacia comparable to that of *Hyp* mice have been reported (Bai et al. 2004). The similarity of the osteomalacia traits in *ApoE-Fgf23* and *Hyp* mice suggests that the accompanied hypophosphatemia is the main cause of the phenotype in these two models.

More recently, osteopontin, a protein belonging to the family of small integrin-binding ligand, N-linked glycoproteins (SIBLINGs), has been shown as a substrate for PHEX (Barros et al. 2013). It has been proposed that in the absence of PHEX, accumulation of osteopontin in the ECM may contribute to the “hard” tissue mineralization defects seen in the XLH patients and in *Hyp* mice. Another SIBLING protein, dentin matrix protein 1 (DMP1), has been shown to be an important regulator of circulating FGF23 levels (Martin et al. 2011). As is the case with the inactivating mutations in PHEX, DMP1 deficiency leads to severe osteomalacia.

A severe osteomalacia phenotype is also seen in the calcium sensing receptor (CaSR) knockout mice. The ablation of the *Casr* gene leads to a marked increase of systemic parathyroid hormone (PTH) levels leading to hypercalcemia accompanied by hypophosphatemia as a result of increased urinary excretion of P_i (Tu et al. 2003). Interestingly, the observed increase of Ca^{2+} in the circulation failed to prevent osteomalacia in these mice. On the other hand, an opposite phenotype has been seen in mice lacking glial cell missing 2 (GCM2), a transcription factor required for the development of the parathyroid gland (Gunther et al. 2000). The ablation of *Gcm2* impairs the development of the parathyroid gland causing a marked reduction of PTH level in the circulation. A basal PTH level is maintained as the thymus acts as a secondary source for the hormone. As expected, the sur-

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viving *Gcm2*^{-/-} mice show a reduction of serum Ca²⁺ levels and an increase of serum P_i levels. However, despite the significant reduction of systemic Ca²⁺ levels, no osteomalacia was observed in these mice (Gunther et al. 2000).

Currently, a mouse model showing isolated hypocalcemia with a normal serum P_i level is unavailable. Considering that Ca²⁺ is an integral component of bone mineral, it is possible that such a model of isolated hypocalcemia, if existed, would have shown impaired bone mineralization. However, phenotypic comparisons of the mouse models with abnormal Ca²⁺ and/or P_i homeostasis discussed above suggest that the osteomalacia phenotype in a model of isolated hypocalcemia may not be as severe as seen in isolated hypophosphatemia. Because of the critical role of Ca²⁺ in many physiologic activities, its serum level is more tightly regulated than that of P_i. Moreover, ubiquitously present inorganic pyrophosphate (PP_i), a potent inhibitor of ECM mineralization, is a P_i derivative (see below) (Terkeltaub 2001). These observations suggest that early organisms depositing calcium phosphate minerals in the skeletal tissues evolved to regulate ECM mineralization by modulating the P_i levels and its incorporation into the growing mineral crystals.

Collagen Scaffold

The organic part of the bone ECM is primarily composed of type I collagen. Two genes, *Col1a1* and *Col1a2*, encode the α1 and α2 chains of type I collagen, respectively. Two α1 and one α2 chains assemble together to form the collagen triple helix in the extracellular spaces of the bone microenvironment. In a hierarchical fashion, these helices are first arranged axially in a staggered manner as collagen fibrils, which are then bundled together to form the collagen fibers (Shoulders and Raines 2009). In a healthy individual, the mineralization of the unmineralized collagen (osteoid) occurs seamlessly in continuation of the existing mineralized matrix on which the newly synthesized osteoid is deposited by the osteoblasts.

The involvement of collagen in bone mineralization came from the initial electron micro-

copy of the mineralizing bones from newborn mice by Sheldon and Robinson (1957). Although earlier studies provided circumstantial evidence that mineral crystals are deposited within the collagen fibrils, this study first demonstrated that there are two sites on the collagen matrix in bone where mineral deposition starts (1) at the intrafibrillar gap spaces, where the carboxy- and amino-terminal ends of two serially arranged collagen triple helices meet, and (2) interfibrillar spaces between the fibrils (Sheldon and Robinson 1957). Since then, numerous studies have confirmed the critical role of collagen matrix as a scaffold for bone mineral deposition. These include the initial studies demonstrating that collagen sponges can be mineralized in vitro, implanted collagen can mineralize in vivo, and demineralized bone collagen can be mineralized under a cell-free condition (Mergenhausen et al. 1960; Bachra and Fischer 1968a,b). More recently, genetic experiments have demonstrated that the reduction of collagen synthesis in bone results in a reduction of mineralized bone mass (Yang et al. 2004).

The exact mechanism by which the collagen lattice facilitates mineral deposition is still unknown. The amino acid side chains exposed at the intrafibrillar gap space and at the interfibrillar space may regulate this process. However, it is possible that the dense packaging of collagen molecules and their hierarchical organization, rather than the primary structure of the protein is the driving force underlying the nucleation of hydroxyapatite. Indeed, one theory attempts to explain the mineralization of bone ECM by the size-exclusion properties of the collagen scaffold (Toroian et al. 2007; Price et al. 2009). According to this theory, the nanoscale gaps present within a collagen fibril and in between the fibrils arranged in a fiber allow the access of Ca²⁺ and P_i ions, but not the large proteins, which can inhibit the formation and growth of the nascent hydroxyapatite crystals inside the scaffold.

Mineralization Inhibitors

Considering that the concentrations of various ions in all the tissues of the body are at equilibrium to that of blood, it is likely that Ca²⁺ and P_i

levels do not differ significantly between the “soft” and skeletal “hard” tissues. This may raise the question—why do some “soft” tissues, such as blood vessels and some cartilaginous tissues, never mineralize despite their high collagen content? The most straightforward answer to this question can be obtained from several human diseases of pathologic “soft” tissue mineralization and their animal models. For example, generalized arterial calcification in infancy (GACI) and progressive ankylosis in humans are both caused by the reduced level of PP_i in the bone joints (Ho et al. 2000; Albright et al. 2015). PP_i is a mineralization inhibitor that is known for its potent antiminerization properties for over 50 years (Fleisch and Bisaz 1962). A wide distribution of the PP_i synthesis and/or extracellular transport machinery in vertebrate organisms prevents pathologic “soft” tissue mineralization.

PP_i is composed of two inorganic P_i groups joined by an ester linkage (Terkeltaub 2001). This inorganic mineralization inhibitor is produced both intracellularly and extracellularly via enzymatic reactions. Intracellularly, PP_i can be generated as a byproduct of enzymatic activities in numerous metabolic pathways. Intracellular PP_i is transported to the extracellular space through a transmembrane transporter called ANK (Ho et al. 2000). On the other hand, extracellularly, a direct cleavage of the phosphodiester bond in purine and pyrimidine nucleoside triphosphates (e.g., ATP) by nucleotide pyrophosphatase/phosphodiesterase enzymes can also generate PP_i (Hessle et al. 2002).

At a threshold concentration, PP_i prevents the incorporation of Ca^{2+} and P_i into nascent apatitic crystals and inhibits their growth (Terkeltaub 2001). Studies on the genetic models have identified the key proteins involved in PP_i -mediated inhibition of pathologic calcification. In a mouse model of human progressive ankylosis with homozygous mutations in the *Ank* gene (*ank/ank*), massive mineral deposition is seen in the joints and other soft tissues (Ho et al. 2000). A similar phenotype has been seen in *ttw/ttw* mice, which carry homozygous mutations in the ectonucleotide phosphodies-

terase nucleotide pyrophosphatase I (*Enpp1*) gene (Koshizuka et al. 2001).

Interestingly, both *Ank* and *Enpp1* genes are highly expressed in bone, more specifically in the osteoblasts (Murshed et al. 2005). Although the presence of a mineralization inhibitor in bone may appear counterintuitive, this quandary can be explained by the presence of a strong alkaline phosphatase (ALPL) activity in bone. The cardinal role of ALPL during skeletal mineralization in humans was demonstrated by many different mutations identified in the *ALPL* gene in hypophosphatasia patients, causing a wide range of phenotypic severity. Some of these patients show very severe osteomalacia and fetal/perinatal lethality, while others show milder, progressive osteomalacia later in life (Mornet 2000; Tailandier et al. 2000, 2001). The genetic models lacking ALPL activity further confirmed the requirement of this enzyme for bone mineralization (Fedde et al. 1999; Anderson et al. 2004).

ALPL-mediated hydrolysis of PP_i has two implications—first, it reduces the amount of PP_i in the bone microenvironment and, second, it increases the amount of mineralization-promoting P_i ions by liberating them from PP_i . These coupled activities alter the P_i/PP_i ratio in the bone ECM in such a way that the formation and growth of the apatite crystal is promoted (Hessle et al. 2002; Murshed et al. 2005). The critical effect of this ratio in regulating ECM mineralization has been demonstrated in *ank/ank;Hyp* compound-mutant mice, which show a marked reduction of joint mineralization (Murshed et al. 2005). The low extracellular P_i/PP_i ratio in these mice in comparison to the original *ank/ank* mice prevented the ectopic mineral deposition.

The ALPL “knockout” mice were used to validate the therapeutic effectiveness of a genetically engineered ALPL molecule with the ability to bind to the bone matrix in vivo. These mice showed a remarkable improvement of the bone phenotype, as the hypophosphatasia-associated osteomalacia was prevented (Yadav et al. 2011a). This approach of enzyme-replacement therapy has been successfully used to treat the hypophosphatasia-associated skeletal abnormalities in human clinical trials (Kitaoka et al. 2017).

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Matrix Gla protein (MGP) and fetuin, two proteins involved in the prevention of “soft” tissue mineralization are also present in the skeletal hard tissues (Jahnen-Dechent et al. 1997). However, considering that fetuin “knockout” mice do not show any overt bone mineralization defects or skeletal anomalies, the current discussion will focus on MGP only. In humans, the homozygous loss-of-function mutations in *MGP* leads to a rare genetic disorder known as Keutel syndrome (Fryns et al. 1984; Munroe et al. 1999). The patients show abnormal mineralization of their cartilaginous tissues leading to midface hypoplasia, shortening of terminal phalanges, tracheo-bronchial calcification, and vascular abnormalities, including calcification of the arterial media. MGP-deficient mice recapitulated all of these anomalies albeit with a more severe vascular calcification phenotype (Luo et al. 1997).

MGP, a small mineral-binding protein is highly expressed by chondrocytes and vascular smooth muscle cells (Luo et al. 1995, 1997). Although MGP was initially purified from the bovine bones, its expression is markedly lower in the trabecular bones in comparison to the prehypertrophic zone of the developing growth plate (Price and Williamson 1985; Luo et al. 1995). This expression pattern suggests that MGP present in bone might have originated elsewhere (e.g., in the vascular tissues) and transported to bone via blood. Transgenic overexpression of *Mgp* in the osteoblasts resulted in a moderate level of osteomalacia affecting both intramembranous and endochondral bones (Murshed et al. 2004). This observation highlights the importance of the mechanisms to control the expression of mineralization inhibitors in the mineralizing skeletal tissues.

In agreement with the observation that *Mgp* is weakly expressed in bone in comparison to the cartilaginous tissues, the whole-body ablation of *Mgp* in mice does not overtly affect bone mineralization, but shows skeletal anomalies associated with abnormal cartilage mineralization (Marulanda et al. 2017). The most prominent of these anomalies is midface hypoplasia, which is also seen in the patients with Keutel syndrome. Cephalometric analyses of the micro-computed tomography (CT) images of the

head revealed that the phenotype is caused by the impaired growth of the maxillary and palatine bones in comparison to the mandibular bones. Marulanda et al. (2017) reported that the hypoplastic midface was not a result of craniosynostosis as seen in several other mouse models of midface hypoplasia, but a result of ectopic mineralization of the cartilaginous part of the nasal septum, which normally does not mineralize. This abnormal mineralization induced apoptosis of the chondrocytes present in the nasal septum (Marulanda et al. 2017). The loss of the matrix synthesizing active chondrocytes during the early growth phase and the increased rigidity of the ECM upon mineralization might have impaired the growth of the nasal septum and that of the maxillary complex. Interestingly, the deposited minerals in the septal cartilage of the mutant mice show the presence of ACP, suggesting that MGP-mediated inhibition of mineralization occurs during the early phases of mineral precipitation.

THE ROLE OF INTRACELLULAR ENZYMES IN SKELETAL MINERALIZATION

Whereas most of the published work on the mechanism of cartilage and bone mineralization focuses primarily on the extracellular determinants, newer studies have identified several intracellular enzymes as important regulators of this process. The main strength of these studies is the use of in vivo gene ablation models exhibiting obvious “hard” tissue mineralization defects, which are not caused by the alterations of the known determinants discussed above. The intracellular enzymes, sphingomyelin phosphodiesterase 3 (SMPD3) and phosphatase orphan 1 (PHOSPHO1) reported by these studies are both involved in the metabolism of phospholipids and/or associated metabolites (Aubin et al. 2005; Macrae et al. 2010).

SMPD3 (also known as neutral sphingomyelinase 2) is a cell membrane-bound lipid-metabolizing enzyme expressed in high amounts in the brain, cartilage, and bone. Among various acid, basic, and neutral sphingomyelinases, only SMPD3 deficiency leads to impaired mineralization of the skeletal tissues (Stoffel et al. 2005,

2007; Macrae et al. 2010; Khavandgar and Murshed 2015). SMPD3 cleaves sphingomyelin present in the cell membrane to generate ceramide, a bioactive lipid molecule, and phosphocholine, an essential nutrient. Emerging data from the analysis of genetically altered mouse models suggest that SMPD3 activity during embryonic development plays a critical role in normal skeletogenesis. This insight originally came from two different mouse models lacking SMPD3. One of these models, *frolfro*, carries a chemically induced deletion encompassing part of intron 8 and most of exon 9 of the *Smpd3* gene, whereas the other mouse model (*Smpd3*^{-/-}) was generated by the conventional gene-targeting approach (Aubin et al. 2005; Stoffel et al. 2005, 2007).

The *fro* mutation completely abolishes the enzymatic activity of SMPD3, but does not affect its membrane localization (Khavandgar et al. 2011). A severe mineralization defect affecting both intramembranous and endochondral bones, and an abnormal delay of apoptosis of the hypertrophic chondrocytes during the early stages of skeletal development are the hallmarks of the skeletal phenotypes in *frolfro* mice. Recently, Li et al. have demonstrated that SMPD3 activity in both chondrocytes and osteoblasts are required for a normal bone development (Li et al. 2016). The poor mineralization of the skeletal tissues in *frolfro* mice is seen without any alterations of the homeostasis of Ca²⁺, P_i, and PP_i (Khavandgar et al. 2011). This observation suggests that the loss of SMPD3 function affects ECM mineralization through a novel, yet unknown, mechanism.

As mentioned above, SMPD3 cleaves sphingomyelin to generate many different species of ceramides that regulate a myriad of cell functions. SMPD3 activity also liberates an essential nutrient, phosphocholine. Whereas the role of ceramide is well documented in the regulation of apoptosis (Obeid et al. 1993), it is not yet clear whether both ceramide and phosphocholine or only one of these metabolites is involved in skeletal tissue mineralization. Interestingly, gene knockout experiments reducing ceramide biosynthesis via an alternative pathway without involving SMPD3 activity did not report any ECM

mineralization defects (Holland et al. 2007). These findings lead to two conclusions: it is possible that the ceramides generated from these two pathways contribute to different pools with distinct cellular functions; alternatively, it is possible that ceramide does not play a role in the process of bone mineralization. While at present, no experimental data directly suggests the involvement of ceramide in ECM mineralization, the importance of phosphocholine in this process has been convincingly shown by animal experiments (Macrae et al. 2010; Yadav et al. 2011b). Phosphocholine generated from sphingomyelin by SMPD3, or from dietary choline by two isoforms of choline kinases, can be cleaved by PHOSPHO1, an intracellular enzyme with phosphatase activity. The deficiency of PHOSPHO1 in mice has been shown to cause similar bone mineralization defects as seen in *frolfro* mice (Yadav et al. 2011b).

INTRACELLULAR REGULATORS OF ECM MINERALIZATION AND THE “MATRIX VESICLE THEORY”

Whereas the regulatory roles of two intracellular enzymes, SMPD3 and PHOSPHO1, in ECM mineralization are now well established, the question remains how these intracellular enzymes may regulate a process that occurs outside the cells. A possible mechanism may involve matrix vesicles (MVs), which are nanosized (20–200 nm) vesicles released by the cells in a mineralizing tissue (Anderson et al. 2004, 2005; Golub 2009; Wu et al. 2002). Since their initial discoveries as chondrocyte-derived vesicular bodies promoting the initiation of mineral nucleation, there have been considerable efforts to explain various types of ECM mineralization, including pathologic mineralization of “soft tissues” using the “matrix vesicle theory” (Anderson 1984). Although there is no general consensus, it has been suggested that MVs provide an isolated microenvironment to facilitate the initial nucleation of apatite mineral. Mineral crystals formed inside the MVs grow progressively in size by the addition of Ca²⁺ and P_i ions and eventually rupture the MV membrane to be deposited on the collagen scaffold.

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Wu et al. (2002) showed extensive phospholipid degradation in the mineralizing MVs with a concomitant increase of free fatty acids. This observation suggested the presence of phospholipase activity inside the MVs and demonstrated a link between phospholipid metabolism and the initiation of ECM mineralization. Phospholipase activity generates phosphocholine and phosphoethanolamine, which can be cleaved by PHOSPHO1 releasing free P_i inside the MVs. The increase of P_i inside the MVs may alter the P_i/PP_i ratio to favor the seeding of early mineral crystals.

Being a membrane-bound enzyme with an intracellular catalytic domain, SMPD3 is expected to be present inside the MVs. The cleavage of sphingomyelin present in the MV membrane by

SMPD3 may generate additional phosphocholine to be cleaved by PHOSPHO1. Although further studies will be needed to establish that SMPD3 and PHOSPHO1 works in a relay in the MVs, such a possibility is supported by the observation that both PHOSPHO1 and SMPD3 are present in the MV preparations (Mebarek et al. 2013). Apart from its role described above, SMPD3 might be involved in the biogenesis of the MVs.

SUMMARY

Based on the discussion above, a unified model for bone mineralization can be proposed. When present at physiologic concentrations, two mineral ions— Ca^{2+} and P_i —will promote hydroxy-

Figure 1. Model of bone mineralization. Serum calcium (Ca^{2+}), inorganic phosphate (P_i) levels, and a mineral scaffolding collagen-rich extracellular matrix (ECM) are important determinants of bone mineralization. Alkaline phosphatase (ALPL), an ectoenzyme tethered to the osteoblast cell membrane, cleaves inorganic pyrophosphate (PP_i), a small, but potent mineralization inhibitor. This facilitates bone ECM mineralization in two ways: first, it reduces the level of a mineralization inhibitor, and second, in the process generates P_i , an activator of ECM mineralization. This coupled ALPL activity alters the P_i/PP_i ratio in the bone microenvironment to favor bone mineralization. Compact hierarchical assembly of collagen molecules in the fibrils and fibers results in both intra- and interfibrillar nanoscale gaps. These gaps are accessible by Ca^{2+} and P_i ions, but not by the large protein inhibitors of ECM mineralization. This may explain why there are mineral deposits both inside and in the gaps in between the collagen fibrils. Matrix vesicle (MV)-mediated mineralization may serve as an auxiliary mechanism for bone mineralization. These nanoscale vesicles carrying the intracellular mineralization-promoting enzymes bud off from the mineralizing cells. Enzymes like SMPD3 and phospholipases present inside the MVs may cleave the phospholipids (e.g., sphingomyelin [SM]) to generate phosphocholine, which in turn can be cleaved by another cytosolic enzyme PHOSPHO1 liberating free P_i . An increase of intravesicular P_i leads to its precipitation with Ca^{2+} to form the nascent hydroxyapatite (HA) crystals.

apatite crystal growth within and between the newly synthesized collagen fibrils in the skeletal ECM. PP_i , a chemical derivative of P_i , can inhibit the mineralization process. The presence of a scaffolding matrix and a defined extracellular ratio of P_i to PP_i are two critical determinants of ECM mineralization. ALPL, an ectoenzyme bound to the osteoblast cell membrane, cleaves PP_i to generate P_i and alters the local P_i to PP_i ratio to favor mineral precipitation (Fig. 1).

The above model suggests that the specificity of skeletal mineralization can be explained, in part, by the unique coexpression of tissue-non-specific genes encoding type I collagen and ALPL. Indeed as in vivo proof for this hypothesis, it was demonstrated that when the PP_i cleaving enzyme, ALPL, was mis-expressed in the dermis and arterial media, tissues rich in fibrillar collagen, it caused rapid ECM mineralization (Murshed et al. 2005). However, it should be noted that this finding does not rule out the existence of auxiliary mechanisms working together to mineralize the skeletal ECM. The possibility of additional mechanism(s) is supported by the fact that both SMPD3- and PHOSPHO1-deficient mice show bone mineralization defects. These enzymes may work in concert to increase the P_i levels to facilitate the nucleation of minerals inside the MVs (Fig. 1).

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