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Review

# Animal models with pathological mineralization phenotypes

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ABSTRACT

Extracellular matrix mineralization is important for mechanical stability of the skeleton and for calcium and phosphate storage. Professional mineral-disposing cell types are hypertrophic chondrocytes, odontoblasts, ameloblasts and osteoblasts. Since ectopic mineralization causes tissue dysfunction mineralization inhibitors and promoting factors have to be kept in close balance. The most prominent inhibitors are fetuin-A, matrix-Gla-protein (MGP), SIGBLING proteins and pyrophosphate. In spite of their ubiquitous presence, their loss entails a specific rather than a stereotypic pattern of ectopic mineralization. Typical sites of pathological mineral accumulation are connective tissues, articular cartilage, and vessels. Associated common human pathologies are degenerative joint disorders and arteriosclerosis. This article gives a summary on what we have learned from different mouse models with pathologic mineralization phenotypes about the role of these inhibitors and the regulation of mineralization promoting factors.



## 1. Introduction

Life is unthinkable without calcium ions that bind to and stabilize phospholipid bilayers and influence the function of a plethora of proteins and cellular processes. Its high affinity for inorganic phosphate, with whom it tends to form insoluble precipitates (the solubility constant is $1 \times 10^{-26}$), however, puts the cells at risk of becoming petrified. Therefore cells had to develop strategies to, on the one hand, inhibit calcium phosphate crystallization in supersaturated physiological solutions and, on the other hand, separate calcium and phosphate by compartmentalization.

## 2. Calcium and phosphate concentrations in different compartments

In mammals typical extracellular inorganic phosphate (Pi) concentrations are between 1 and 3 mM and intracellular phosphate levels are in the same range. In rat kidney cells, for example, intracellular Pi was measured to be between 1 and 2.7 mM [1]. Values for different subcellular compartments are difficult to obtain. However, in all these compartments protein function needs to be regulated by addition and removal of phosphate moieties. Furthermore, a sufficient supply of Pi is needed in mitochondria to enable oxidative phosphorylation. This constitutive intracellular phosphate turnover probably prevents Pi concentrations from being dramatically lower than 1 mM in any subcellular compartment. In contrast to phosphate, calcium concentrations are known to be 10,000-fold lower (100 nM) in the cytoplasm than in the extracellular environment (1 mM). Several intracellular calcium stores exist. The best known is the endoplasmic reticulum and its derivative in muscle cells, the sarcoplasmic reticulum. Here calcium levels can reach around 1 mM. Another significant calcium store is the mitochondrium. Low calcium increases lead to a stimulation of mitochondrial respiration while calcium overload entails apoptosis [2]. The so-called acidic calcium stores mainly comprise endosomes, lysosomes, secretory granules and the Golgi apparatus.

## 3. Inhibitors of calcium phosphate formation

Given the omnipresence of Pi in all these calcium-storing compartments inhibitors are necessary to prevent calcium phosphate precipitation. These can be inorganic ions, organic ions and proteins. Polyphosphates are crucial inhibitors of calcium phosphate precipitation and especially abundant in secretory granules [3]. Pyrophosphate (PPi) also is known to potently inhibit the formation of calcium phosphate crystals. An important difference between polyphosphate and PPi is that the latter forms insoluble crystals with calcium at high concentrations while polyphosphates even at tremendously high concentrations as found in acidocalcisomes only form amorphous precipitates. The PPi concentrations in human plasma and cell culture supernatants are usually 2–3 μM, at least 300-fold lower than Pi concentrations [4]. However, higher concentrations in microenvironments cannot be excluded. The most important organic inhibitors are oxalate and citrate, which

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chelate calcium ions. In addition, many proteins exist that, often via γ-carboxyl groups, prevent calcium phosphate deposition mainly in the extracellular space. Fetuin-A (alpha 2 Heremans-Schmidt glycoprotein), matrix-Gla-protein, osteopontin, and osteocalcin are the most prominent examples. Some of these inhibitors, e.g. albumin, bind calcium ions, which has the effect that in plasma only 50% of calcium is freely diffusible. However, other inhibitors like fetuin-A seem to bind to small basic calcium phosphate particles and inhibit their growth [5].

## 4. Physiological mineralization processes

Mineralized cartilage, bone, and teeth are the only tissues in which deposition of calcium phosphate in the form of hydroxyapatite (HA) is intended. The biomineralization process in all three examples is very similar, but–as discussed below–might also bear some differences. Three conditions must be fulfilled for mineralization to occur: (1) presence of a suitable extracellular matrix, (2) low concentrations of mineralization inhibitors, and (3) high local concentrations of calcium and phosphate ions.

In vertebrates, all mineralizing tissues are characterized by high collagen content, which is thought to provide a lattice for the growing HA crystals (Figs. 1–3) [6]. However, closely adjacent collagen-rich tissues such as ligaments inserting into bone have to be protected from mineralization. The spatial specificity of tissue mineralization is achieved by restricting the degradation of mineralization inhibitors. Tissue nonspecific alkaline phosphatase (TNAP) plays a key role in this process [7] (Fig. 1). It is highly expressed by hypertrophic chondrocytes, ameloblasts, odontoblasts and osteoblasts and cleaves off Pi not only from PPi and ATP, but also from polyphosphates, thereby converting the inhibitor into a promoter of mineralization [8]. Through a GPI-anchor TNAP is inserted into vesicular membranes and transported to the plasma membrane. Specialized matrix vesicles released by mineralizing chondrocytes and, probably to a lesser degree, also by osteoblasts also contain TNAP (Fig. 1A). Phospho1 is a phosphatase that is not anchored to membranes. According to the current model by the Millán group, this protein resides in the lumen of the matrix vesicle where it produces Pi by hydrolyzing phosphoethanolamine and phosphocholine to nucleate apatite crystal formation [9]. Targeted ablation of murine Phospho1 aggravates the mineralization defect elicited by the loss of TNAP [10]. The calcium ATPase PMCA not only provides Pi close to the plasma membrane of osteoblasts through ATP cleavage, but also transports calcium into the extracellular space and could therefore contribute to mineralization [11].

Investigations on tissue mineralization in molluscs indicated that the mineral is first deposited as an amorphous precursor before it matures into crystals [12]. Accordingly, also in vertebrates, evidence was found for the existence of amorphous calcium phosphate (ACP) [12]. Using cryo-EM Mahamid et al. showed that globular ACP is formed and released by intracellular vesicles [13] (Fig. 2). It can be speculated that these vesicles contain large amounts of polyphosphates to keep the calcium phosphate precipitate in an amorphous state and that their degradation by TNAP or Phospho1 in the extracellular space induces maturation [8]. This model is in contrast to the classical matrix vesicle hypothesis according to which crystal nucleation occurs inside these small vesicles after they budded off from microvilli [14]. It should be kept in mind that matrix vesicles were originally described in growth plate cartilage [15]. Most of the studies on osteoblast-derived matrix vesicles were performed in vitro. The extracellular matrix of cartilage contains type 2 (and type 10) collagen, but also high amounts of proteoglycans which give it sponge-like properties and are the basis for its elastic resistance to compression forces. As a consequence, the cartilage collagen network might be too loose to restrict access of large inhibitory

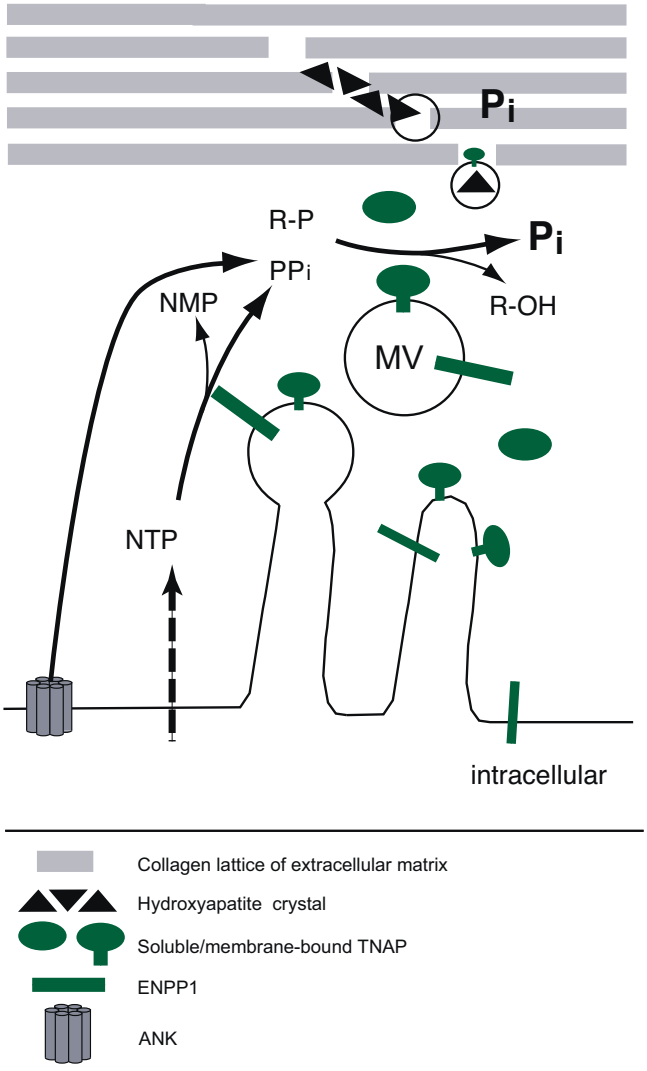


**Fig. 1.** Initiation of mineralization by matrix vesicles and regulation of the inhibitor pyrophosphate (PPi). Matrix vesicles (MV) bud off from microvilli on the surface of chondrocytes and osteoblasts. They thus carry ENPP1 and GPI-anchored tissue nonspecific alkaline phosphatase (TNAP) on their surface. ENPP1 generates the inhibitory pyrophosphate (PPi) by cleavage of nucleotides (NTP) which are released by channels, e.g. connexins, or by vesicular exocytosis. The membrane protein ANK is thought to transport PPi from the cytosol to the extracellular space. TNAP cleaves PPi and phosphoproteins (collectively represented as R-P) thus producing inorganic phosphate (Pi). Matrix vesicles might diffuse into the collagen matrix (grey bars), carrying calcium phosphate mineralization foci which subsequently increase in size and disrupt the vesicle membrane.

proteins (see below). Therefore, in contrast to bone with its tight collagen type 1 network, cartilage might require matrix vesicles for mineralization. In secretory vesicles travelling to the plasma membrane TNAP necessarily resides in the lumen. In contrast, for the function of in matrix vesicles it is thought that TNAP has to be localized outside where it produces Pi to enhance growth of the apatite crystals [15]. However, loss of TNAP's GPI anchor as seen in hereditary hyperphosphatasia with mental retardation caused by *PIGV* mutations, which makes the enzyme float away from the mineralizing cell as soon as it is secreted, does not at all entail skeletal mineralization problems [16].

Mineralization of newly synthesized osteoid is a very slow process which takes several weeks. By this time, the bone-forming osteoblasts have been replaced by inactive bone lining cells. Therefore, the osteoblasts apparently initiate the mineralization process which later on continues largely independent of cellular activity.