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mouse genome sequence with a dense map of markers already genotyped for the majority of inbred strains require only *in silico* analyses without genotyping efforts and with a resolution that can lead to the single gene.

In conclusion, even if the discovery of genes underlying QTLs has been proven to be extremely difficult, recent advances in the mouse genome and the availability of particular new mouse strains such as CSSs leave promising expectations for the near future. Moreover, the choice of using molecular phenotypes, such as the COLI kidney deposition, that are likely to be closer to pathogenic mechanisms rather than more complex clinical phenotypes such as proteinuria and renal failure, makes the trait chosen by Kato and colleagues[7] a more tractable problem with high potential for shedding light on molecular pathways regulating the most important phenomenon in progression to end-stage renal failure: interstitial fibrosis.

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# Role for alkaline phosphatase as an inducer of vascular calcification in renal failure?

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**Vascular calcification is associated with increased cardiovascular morbidity and mortality. A number of calcification inhibitors have been defined recently, including inorganic pyrophosphate ($PP_i$), an important physicochemical inhibitor of hydroxyapatite crystal growth. Increased hydrolysis of $PP_i$ by tissue-nonspecific alkaline phosphatase (TNAP) may occur in renal failure and act to enhance mineralization of vessels.**

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Heterotopic, extraosseous vascular calcification, a phenomenon known for decades, was once regarded as a passive physicochemical process triggered by excess calcium and phosphate exceeding the solubility threshold, resulting in precipitation and apatite mineral deposition in vessels and soft tissues. Over recent years, this concept has been challenged by a variety of *in vitro* and *in vivo* models of vascular calcification, especially targeted gene deletion studies in animals, which have demonstrated that mineralization is controlled by a balance between procalcific and anticalcific regulatory proteins acting locally in the vessel wall and/or systemically in the circulation. Alterations in this balance induced by injury, disease, or genetic deficiency are postulated to favor heterotopic mineral deposition.[1] Further evidence supporting the hypothesis of calcification as an active, cell-mediated process comes from the observed similarities between vascular calcification and endochondral and intramembranous mineralization in bone. Thus, the molecular events inducing extracellular matrix mineralization may be the same in both physiological and pathological processes. Indeed, within calcifying vascular specimens, cells that are reminiscent of osteoblasts, chondrocytes, or osteoclasts have been detected, derived either from stem cells (circulating or local cells) or from phenotypic transdifferentiation of vessel wall-resident cells such as vascular smooth muscle cells. Furthermore, transcription factors and cytokines with crucial roles in bone homeostasis have been detected in calcified vascular lesions,[1] suggesting that mineralization of vessels may be triggered through similar molecular mechanisms and signaling pathways.

The study by Lomashvili *et al.*[2] (this issue) has investigated the role of tissue-nonspecific alkaline phosphatase (TNAP) in the pathogenesis of vascular calcification. The authors show that TNAP is upregulated under uremic conditions in vessels from rats, which leads to the hydrolysis and therefore inactivation of inorganic pyrophosphate ($PP_i$), a potent inhibitor of hydroxyapatite crystal growth and a potential local and circulating inhibitor of vascular calcification. Although the possibility that the TNAP/$PP_i$ system is involved in vascular calcification is intriguing, what is the evidence for its role or expression in the vascular system?

Firstly, the ectoenzymes PC-1/nucleotide pyrophosphatase phosphodiesterase 1 (NPP1), which hydrolyzes

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adenosine 5′-triphosphate (ATP) to generate $PP_i$, and TNAP are both present on the plasma membrane of calcifying cells[3] and act to tightly regulate tissue levels of $PP_i$. $PP_i$, which is a substrate for alkaline phosphatases, is a small molecule that binds to nascent hydroxyapatite crystals and prevents further incorporation of inorganic phosphate ($P_i$) ions into these crystals. $PP_i$ is present in almost all extracellular matrices, and both *in vitro* and *in vivo* studies have shown the potential for $PP_i$ to prevent pathological mineralization. In animal models, vascular calcification was induced by lowering of the levels of $PP_i$, for example in mice carrying the *ank* mutation (a transmembrane transporter protein that shuttles $PP_i$ between intracellular and extracellular compartments) or in *NPP1*$^{-/-}$ mice, which exhibit cartilage-specific gene expression changes in their vasculature. Moreover, heritable deficiency of NPP1 in humans has been demonstrated to be the cause of idiopathic infantile arterial calcification, a disease affecting children that is associated with severe medial calcification of arteries.[3] Further indirect evidence for a protective role of $PP_i$ comes from other rat models of vascular calcification induced by the vitamin K antagonist warfarin or toxic doses of vitamin D. Bisphosphonates, nonhydrolyzable pyrophosphate analogs, prevented mineralization of vessels, potentially by binding to microcalcifications early in the calcification process.[4] However, bisphosphonates have also been found to stimulate release of a serum mineral complex containing matrix Gla protein (MGP) and fetuin-A, both potent inhibitors of vascular calcification. Additionally, MGP inhibits bone morphogenetic protein-2 (BMP2) induction of alkaline phosphatase activity;[5] therefore, the mechanisms involved in this inhibition by bisphosphonates remain controversial (Figure 1).

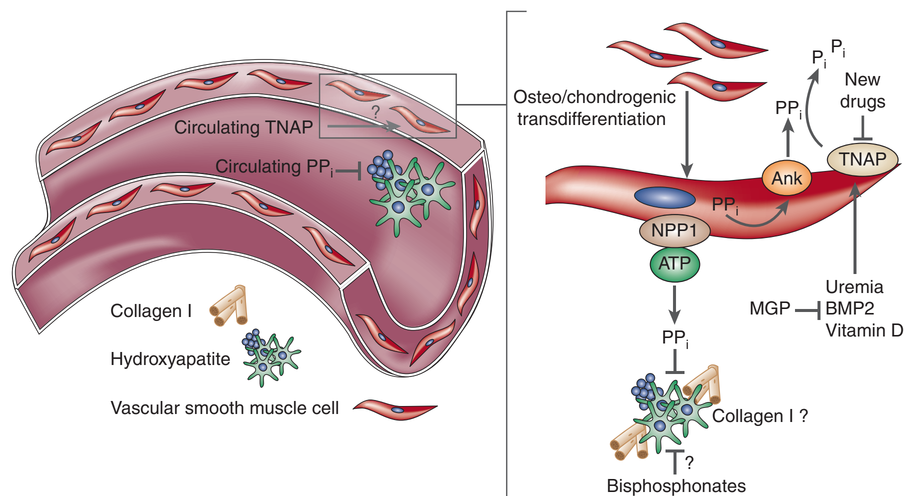


**Figure 1 | Possible mechanism for TNAP-induced vascular calcification.** Alkaline phosphatases are membrane-anchored ectoenzymes that catalyze the hydrolysis of inorganic pyrophosphate ($PP_i$), small molecules made of two phosphate ions linked by an ester bond that bind to nascent hydroxyapatite crystals and prevent further incorporation of inorganic phosphate ($P_i$) ions into these crystals. Tissue-nonspecific alkaline phosphatase (TNAP) is induced by bone morphogenetic protein-2 (BMP2) and vitamin D agents that induce osteo/chondrogenic transdifferentiation of vascular smooth muscle cells. Uremia may be another condition to induce TNAP. $PP_i$ is released by vascular smooth muscle cells via Ank, a protein that shuttles intracellular $PP_i$ to the extracellular milieu. The enzyme PC-1/nucleotide pyrophosphatase phosphodiesterase 1 (NPP1) can generate $PP_i$ via hydrolysis of adenosine 5′-triphosphate (ATP). Mineral deposition may also be inhibited by bisphosphonates, $PP_i$ analogs. Collagen I, a cofactor for tissue mineralization in orthotopic bone formation, may play a role in vascular calcification as well. Matrix Gla protein (MGP), which can be released by bisphosphonates in the animal model, can interfere with BMP2 signaling.

Alkaline phosphatases themselves comprise a heterogeneous group of dimeric metalloenzymes that have a wide tissue distribution but are particularly concentrated in the liver, kidney, or bone. They are encoded by at least four different gene loci—namely, tissue-nonspecific, intestinal, placental, and germ-cell alkaline phosphatase—with post-translational modifications of the tissue-nonspecific isoenzyme producing liver, kidney, and bone forms of the enzyme. Alkaline phosphatases have dual functions, acting to catalyze the hydrolysis of phosphomonoesters with the release of $P_i$ as well as acting as pyrophosphatases to hydrolyze $PP_i$; however, their functions outside bone are poorly understood. They appear to be able to act on bone both locally and systemically. Increased serum levels of a soluble form of TNAP are associated with altered osteoblast activity and bone disease. Conceivably, the circulating form of TNAP could also affect vascular mineralization, although increasing the serum concentration of circulating TNAP in normophosphatemic mice did not induce heterotopic mineralization of any tissue.[6] In patients with hyperphosphatasia, a group of disorders that also feature elevated serum TNAP activity, such as Paget's disease of bone, there are preliminary studies, but, to date, no clear evidence for an association with vascular calcification. This may indicate that only membrane-bound TNAP plays a role in vascular calcification, or that other vascular-specific cofactors are necessary to induce vascular calcification in the context of elevated TNAP. Since physiological TNAP function takes place in bone, whereas TNAP expression is not bone specific, this suggests that the spatial restriction of extracellular matrix mineralization to bone could be explained by additional requirements. One such component was postulated to be collagen I expression by osteoblasts.[6] The only tissues in which both collagen I and TNAP are physiologically coexpressed are bone and teeth. Interestingly, *in vitro*, the extracellular matrix produced by rapidly mineralizing vascular cells was also found to contain higher amounts of collagen I than non-mineralizing cells.[7]

TNAP expression favors bone mineralization by removal of $PP_i$ from the extracellular milieu. The defective mineralization of bone in TNAP deficiency can be corrected solely by reduction of $PP_i$ levels. However, we have less

information about TNAP and $PP_i$ in the vasculature. Studies have shown that TNAP is upregulated *in vitro* during phenotypic transdifferentiation of vascular smooth muscle cells into osteogenic cells induced by β-glycerophosphate, which in turn is associated with downregulation of smooth muscle lineage markers.[8] Furthermore, TNAP was found to be expressed in calcified vessels of patients and colocalized with osteo/chondrogenic transcription factors that regulate expression of the gene.[1]

The study by Lomashvili *et al.*[2] supports and extends these studies, strengthening the contention of a role of TNAP/$PP_i$ in uremic vascular calcification. However, there still remain a number of unanswered questions. Although the study investigated the role of TNAP in vascular calcification, the animal model used did not induce vascular calcification. In fact, the authors mention that aortic calcification was not seen with their diet. Therefore, the link between vascular calcification and upregulation of TNAP is hard to prove. Previous studies have shown that injury is required to induce TNAP in vessels and to inhibit $PP_i$; however, in this model, uremia was sufficient only to induce TNAP. This strongly suggests that reduction of $PP_i$ in the context of vascular injury may be required for calcification, and studies to investigate this idea would be important. Also, it may be that TNAP has distinct functions not only related to hydrolysis of $PP_i$, especially if its upregulation occurs prior to osteo/chondrogenic differentiation. The authors did not show that interfering with the supposed pathomechanism could rescue animals from vascular calcification, which would support their concept. For example, it would have been interesting to see if chondrogenic conversion of vascular smooth muscle cells occurred in this model, as was seen in *NPP1*$^{-/-}$ animals. As TNAP plays a major role in bone homeostasis, one may wonder whether, in the animals, TNAP was increased in serum or indeed other tissues by uremia, and whether this had any effects on bone formation. Furthermore, what were the circulating levels of $PP_i$ in the animals? Circulating levels of $PP_i$ are reduced in renal-failure patients, as shown by the same group;[9] however, a direct relationship between this reduction and vascular calcification has not yet been shown. Although decreased $PP_i$ levels may contribute to vascular calcification in this patient group, the source of circulating $PP_i$ may not be the vascular system, and it is unclear whether lower circulating levels of $PP_i$ reflect increased TNAP activity in the vessel wall. These important questions could potentially be answered in a slightly modified animal model and would be highly informative.

Strategies aimed at selective inhibition of TNAP may have clinical benefits in vascular calcification, although other organ systems such as bone may be adversely affected, and it seems not entirely clear whether the role of TNAP is conserved between mice and humans. Alternatively, $PP_i$ analogs such as bisphosphonates developed for osteoporosis might be optimized for activities that inhibit vascular calcification. To date, the efficacy of bisphosphonates has not been proved in large prospective trials enrolling patient cohorts with vascular calcification, and smaller studies have yielded ambiguous results. Of note, new compounds that block the hydrolytic pyrophosphatase capacity of TNAP have been developed that were effective in an *in vitro* system and also in an *ex vivo* animal model to inhibit calcification.[10] Greater understanding of both the mechanisms and the clinical consequences of vascular calcification may aid the development of future therapies more effectively designed and applied to inhibit or even reverse the deleterious clinical consequences of the condition. The TNAP/$PP_i$ system may be a potential candidate to link the combined phenotype of osteoporosis and vascular calcification, entities that may share a common pathomechanism. The idea that treatment might be feasible is perhaps premature. Systemic vascular treatment may prove difficult, and other enzymes, not targeted by specific inhibitors of TNAP, may also be involved in regulation of $PP_i$ levels.

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