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## NEWS

# Phosphatases and skeletal mineralization: complicated biology yet rapid clinical success

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The Role of Phosphatases in the Initiation of Skeletal Mineralization, a recent *IBMS BoneKEy* webinar presented by Professor José Luis Millán, discussed the path from basic science to a novel treatment.

### Introduction

Of the factors that distinguish a healthy skeleton from a diseased one, the initiation of proper skeletal mineralization—the deposition of hydroxyapatite mineral into the extracellular matrix of growth plate cartilage during endochondral ossification—is one of the most crucial, a basic truth in the bone field to which physicians who treat bone disease can attest. Indeed, patients with hypomineralized skeletons may present with conditions like rickets, osteomalacia or certain arthritic ailments. Meanwhile, those with hypermineralized bones may exhibit osteoarthritis, ankylosis or arterial calcification. The initiation of skeletal mineralization is an intricate process that depends on the proper functioning of a number of enzymes known as phosphatases. When the expression of these molecules is aberrant, mineralization defects causing this variety of diseases can result.

The precise function of the key phosphatases active during the beginnings of skeletal mineralization were the focus of a recent *IBMS BoneKEy* webinar, 'The Role of Phosphatases in the Initiation of Skeletal Mineralization', presented by Professor José Luis Millán, one of the world's leading experts on the basic science of phosphatase function and a professor at Sanford-Burnham Medical Research Institute (La Jolla, CA, USA) (see the webinar at http://www.ibmsonline.org/d/do/131). Moderated by Professor Larry Suva, Director of the Center for Orthopaedic Research at the University of Arkansas for Medical Sciences (Little Rock, AR, USA), the webinar began with a discussion of the molecular activity of the phosphatases, with a particular focus on Professor Millán's work with knockout mice whose phenotypic features have helped propel our current understanding of the enzymes. The webinar then shifted to hypophosphatasia (HPP), a rare disease characterized by low levels of one particular phosphatase, namely tissue-nonspecific alkaline phosphatase (TNAP). The development of an effective enzyme replacement therapy for the treatment of HPP is an all-too-rare success story in medicine, featuring a quick move from the conceptual design of a clinical trial, to the start of that trial, in a span of just 3 years, and Professor Millán underscored the factors that made such rapid advancement possible. Finally, the webinar ended with a discussion of medial vascular calcification, characterized by high levels of TNAP, and efforts to develop alkaline phosphatase inhibitors to treat this symptom that is present in a number of increasingly prevalent conditions. Clinicians can only hope they will be as successful treating vascular calcification as those who treat HPP expect to be, considering the epidemic of vascular calcification that aging, obese and diseased Westernized societies now face.

### TNAP, NPP1 and ANK: Crucial Players in the Mineralization Process, But Not The Whole Story

Skeletal mineralization begins in matrix vesicles, enzyme-filled organelles derived from chondrocytes and osteoblasts that are the sites where calcium and phosphate are first united to form hydroxyapatite crystals. Eventually, rupture of matrix vesicles exposes the hydroxyapatite crystals within them to the extracellular matrix, where the deposition of hydroxyapatite continues along collagen fibrils that serve as scaffolds. This is a process, however, that is opposed by inorganic pyrophosphate. The excess of this molecule results in diseases of hypomineralization, whereas deficiencies result in conditions of hypermineralization. Controlling inorganic pyrophosphate levels are TNAP, which lowers extracellular pyrophosphate concentrations by hydrolyzing pyrophosphate to phosphate; nucleotide pyrophosphatase phosphodiesterase 1 (NPP1), another phosphatase that increases extracellular pyrophosphate levels by producing it from adenosine triphosphate (ATP); and the ankylosis (ANK) protein, a transporter that shuttles pyrophosphate made inside of cells out into the extracellular environment.

The importance of these three players in skeletal mineralization, through their effects on pyrophosphate levels, can be seen from the phenotypes of knockout mice missing them, animals that Professor Millán described in great detail. Animals lacking either NPP1 or ANK exhibit similar phenotypes characterized by soft tissue calcification, including vascular calcification, as the removal of these pyrophosphate-producing and -transporting molecules, respectively, results in lower extracellular pyrophosphate levels and therefore increased mineralization. Both of these knockout models also show evidence of mineral deposition in the intervertebral space as well as in the

perispinal ligaments. Conversely, TNAP knockout mice, now free of that enzyme's pyrophosphate-restricting activity, exhibit the rickets and osteomalacia characteristic of HPP, as pyrophosphate levels are now increased and restrict the deposition of hydroxyapatite.

Based on this knowledge, Professor Millán and colleagues decided to create double knockout animals missing both TNAP and NPP1. 'We bred these mice in the hope that we would now normalize pyrophosphate levels, which would lead to alleviation of the skeletal phenotype, and indeed that is what happened,' he said, noting that the double knockouts no longer exhibited the mineralization defects seen in the skull and phalanges of mice missing only TNAP. *In vitro* work also supported this line of thinking. For instance, Professor Millán noted that osteoblasts from the double knockouts exhibited normal calcification of the extracellular matrix. In addition, just as he had hypothesized, knocking out both enzymes did in fact bring pyrophosphate, measured in the matrix vesicles from the knockout animals, to normal levels.

'This work was very exciting to us because it provided a potential means of treating alkaline phosphatase deficiency. If we could control the function of NPP1 by downregulating its activity, we might be able to reduce the levels of pyrophosphate present in the alkaline phosphatase knockout mice, and achieve compensation and normalization of the skeletal phenotype,' Professor Millán said. Unfortunately, although experiments supported this hypothesis in the axial skeleton, where the mineralization defects seen in TNAP knockout mice were corrected in the double knockout mice, the double knockouts still exhibited osteomalacia in the appendicular skeleton. 'We abandoned plans to develop small-molecule inhibitors for NPP1 as a mechanism of treating hypophosphatasia,' according to Professor Millán, who noted that differences in the relative concentrations of the enzymes at different skeletal sites may explain the unsatisfactory results.

Along with this disappointment came a mystery: although mice missing TNAP exhibited decreased levels of mineralization outside of matrix vesicles, an examination of the matrix vesicles themselves revealed the presence of hydroxyapatite crystals within them. This conundrum encouraged Professor Millán and his co-workers to think of the beginnings of skeletal mineralization as two distinct processes: an initiation phase of hydroxyapatite deposition within matrix vesicles, followed by a propagation phase of hydroxyapatite deposition in the extracellular matrix. This way of conceptualizing the subject poses an obvious question: what molecule(s) is responsible for the initiation?

### PHOSPHO1, Local Phosphate, and Phosphate Transporters Solve the Puzzle

To answer this question, Professor Millán began to study another phosphatase, called PHOSPHO1, which skeletal tissue contains in abundance, and that has an important role in sphingolipid metabolism. Professor Millán and colleagues first discovered that the use of PHOSPHO1 inhibitors in matrix vesicles isolated from mice lacking TNAP resulted in an additional inhibition of calcification. Because PHOSPHO1 is able to produce phosphate within matrix vesicles from phospholipids, inhibiting this enzyme means that less phosphate would then be available within the matrix vesicles for hydroxyapatite deposition. Studies of knockout mice also support a role for PHOSPHO1 in mineralization, as animals missing this enzyme exhibit features like osteomalacia and scoliosis. However, despite this and other evidence of PHOSPHO1's functioning, the researchers still found hydroxyapatite crystals within the matrix vesicles from mice lacking PHOSPHO1.

'This was one of those eureka moments that one treasures, because it forced us to investigate what we were missing, which was the role of phosphate influx by phosphate transporters into matrix vesicles,' Professor Millán explained. He noted that experiments with double knockout mice missing both PHOSPHO1 and TNAP cinched the case for this hypothesis, as these knockouts exhibited no skeletal mineralization at all, whereas knockout of only PHOSPHO1, or only TNAP, reduced mineralization but calcification was still present. If phosphate was indeed finding its way into matrix vesicles, what was the source? Consideration of this question led him back to previous literature showing that matrix vesicles show evidence of ATPase activity. This recognition of an old insight paved the way for *in vitro* studies showing that TNAP, which sits on the cell surface of matrix vesicles, was not just a pyrophosphatase that hydrolyzes pyrophosphate, but was also an ATPase that can generate phosphate from ATP. Professor Millán concluded that it was this very phosphate generated locally by TNAP from ATP that was the source of phosphate needed for the initiation of mineralization, rather than systemic phosphate, as the double knockouts, as well as both of the single knockouts, show normal systemic levels of this biological molecule.

The result of all of this research, then, is an intricate model of skeletal mineralization. The initiation of this process depends upon PHOSPHO1, as well as on local phosphate, produced by TNAP from ATP, that is transported into matrix vesicles through (still unidentified) phosphate transporters. (To complicate matters, Professor Millán noted that NPP1, in addition to its ability to produce pyrophosphate from ATP, can also produce phosphate from ATP, a mechanism that he said may prevail in the axial skeleton). Then, after the initiation of mineralization, TNAP has a further crucial role in furthering hydroxyapatite deposition in the extracellular matrix.

### A Speedy Success

Perhaps it is not surprising that, with multiple enzymes, with multiple functions, acting at different stages of the mineralization timeline, along with the involvement of additional factors like phosphate transporters, it took years to unravel the complicated process of skeletal mineralization, with many important discoveries still for the future. In contrast, even though previous efforts to treat HPP with enzyme replacement therapy had failed, investigators nonetheless found themselves testing a new bone-targeted TNAP replacement therapy on the first patient in a clinical trial, even though initial discussions of how that clinical trial should be designed took place just 3 years earlier. What were the ingredients for this swift outcome?

One of the most important factors was the laboratory's success in creating an excellent animal model of HPP, a rare disease that has a range of manifestations: it can be lethal perinatally yet only produce mild abnormalities in other cases, a variety that results from the large number of different possible mutations—more than 260—at different sites in the TNAP enzyme. The animal model that Professor Millán and his colleagues had

worked to generate very closely mimicked the infantile form of HPP, with knockout mice missing TNAP showing evidence, for instance, of a hypomineralized skull, rickets and a lack of secondary ossification centers in the phalanges.

The existence of an animal model that was a good representation of the human disease gave investigators confidence that the treatment being developed for HPP would in fact be effective in people. Researchers had speculated that previous failures of enzyme replacement therapy—past attempts in the 1980s and 1990s had used infusions of purified liver TNAP, placental TNAP, or TNAP-rich plasma from patients suffering from Paget's disease—may have resulted in part from the inability of the enzyme used in those investigations to reach the site where mineralization is initiated. Thus, the new treatment—a TNAP dimer fused to a human antibody to allow better purification of the enzyme—was also engineered to contain a peptide that allowed for targeting to bone specifically, and indeed, TNAP-knockout mice that received subcutaneous injections of the engineered enzyme at 1 day of age exhibited striking improvements.

First, they lived longer: compared with TNAP knockout mice, all of which died around 3 weeks after birth if they did not receive treatment, mice that received the new treatment survived for nearly 2 months (at which point the experiment had ended). Second, the skeletons of the treated knockout mice looked normal, compared with the untreated mice. Third, treated mice did not exhibit the teeth abnormalities that have been documented in HPP. Indeed, although untreated mice showed evidence of unmineralized dentin and alevolar bone matrix in the incisor and molar, as well as abnormal cementum (the tissue that covers the root of the tooth), treated mice showed no evidence of these defects. With regard to dental abnormalities, in research that has been submitted for publication, Professor Millán and colleagues have found that ameloblasts, the cells that make dental enamel, express TNAP and that knockout mice missing TNAP exhibit abnormalities in incisor and molar enamel, defects that are corrected with TNAP replacement treatment. Finally, the knockout mouse model of HPP also showed evidence of a dose response, with higher doses of the TNAP replacement enzyme resulting in better survival and a better skeletal phenotype, which helped investigators to determine the dose of enzyme that would be appropriate in clinical trials.

Clearly the TNAP knockout mouse model showed that enzyme replacement therapy was feasible, but Professor Millán stressed that basic science alone did not speed this treatment so quickly to clinical trials. The clinical expertise of Michael Whyte, the world's leading clinical expert on HPP, was crucial, as were non-scientific factors including the focus and drive of Enobia Pharma, the Canadian company that developed the engineered enzyme, the work of advocacy groups that educated patients about the enzyme replacement therapy, as well as HPP's status as an orphan disease that sped up the regulatory process that new drugs face. All of these factors resulted in an unusual coup for a bench scientist. 'As a molecular biologist and as a basic scientist, it is very rare that one can contribute to a treatment that actually works and that in this case saves the lives of children. I am extremely proud to be part of this highly collaborative effort,' Professor Millán said.

### The Future

Although various chapters of the HPP story have not yet unfolded—for instance, studies are underway to determine whether viral vectors can be employed to deliver the TNAP enzyme replacement therapy to bone—Professor Millán has his sights set on vascular calcification, and in particular, the medial vascular calcification that affects arterial smooth muscle cells. Medial vascular calcification is a growing problem in numerous diseases, including increasingly common conditions like diabetes, obesity and end-stage renal disease, so there will be a great need for treatments that can alleviate the bone-like calcification observed in the arteries of these patients.

Models of medial vascular calcification include mice missing NPP1 or ANK, as well as uremic rats, all of whom exhibit upregulated levels of TNAP. Medial vascular calcification is also seen in generalized arterial calcification of infancy, a rare disease in infants, and knock in of TNAP in a mouse model of this disease results in very high levels of TNAP and a high degree of vascular calcification. All of these models suggest that TNAP inhibitors could have an important role in preventing the vascular calcification associated with increased expression of TNAP.

Professor Millán and colleagues have identified a number of compounds that inhibit TNAP activity, and they have also made progress in understanding the 3-D structure of the TNAP enzyme, in particular its active site, and how potential inhibitors would interact with the enzyme there. Some of the inhibitors that have been tested thus far show the ability to inhibit calcium deposition by vascular smooth muscle cells in culture, and to decrease calcification in *ex vivo* aorta cultures. The hope is that TNAP inhibitors can one day be used not only for generalized arterial calcification of infancy but for other diseases where medial vascular calcification is a problem, though one issue that will have to be watched carefully is whether TNAP inhibitors used to treat vascular calcification may also have adverse effects on bone. Finally, TNAP inhibitors could also have positive effects, potentially, on the bone pain resulting from the osteoblastic metastases of prostate cancer, and could have a useful role in Paget's disease and in cases of heterotopic ossification as well.