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Phosphate: Known and potential roles during development and regeneration of teeth and supporting structures

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Abstract

Inorganic phosphate (P_i) is abundant in cells and tissues as an important component of nucleic acids and phospholipids, a source of high-energy bonds in nucleoside triphosphates, a substrate for kinases and phosphatases, and a regulator of intracellular signaling. The majority of the body's P_i exists in the mineralized matrix of bones and teeth. Systemic P_i metabolism is regulated by a cast of hormones, phosphatonins, and other factors via the bone-kidney-intestine axis. Mineralization in bones and teeth is in turn affected by homeostasis of P_i and inorganic pyrophosphate (PPi), with further regulation of the P_i/PP_i ratio by cellular enzymes and transporters. Much has been learned by analyzing the molecular basis for changes in mineralized tissue development in mutant and knock-out mice with altered P_i metabolism. This review focuses on factors regulating systemic and local P_i homeostasis and their known and putative effects on the hard tissues of the oral cavity. By understanding the role of P_i metabolism in the development and maintenance of the oral mineralized tissues, it will be possible to develop improved regenerative approaches.

Keywords

tooth development; tooth root; cementum; dentin; periodontal ligament; phosphate metabolism; fibroblast growth factor 23; Phex; tissue nonspecific alkaline phosphatase; progressive ankylosis protein

I. Introduction

a. Overview of phosphate metabolism

Phosphorus is abundant in all cells and tissues as an important component of DNA, RNA and phospholipids, a source of high-energy bonds in adenosine triphosphates (ATP), a

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substrate for various kinases and phosphatases, and a regulator of intracellular signaling. Phosphate homeostasis on a cellular level is therefore a significant aspect of normal function for most tissues and organs. Approximately 85% of phosphorus, the second most abundant mineral in the human body, is in bone, primarily compounded with calcium (Ca^{2+}), the most abundant mineral, in hydroxyapatite (HAP) crystals deposited on the collagen matrix (Broadus, 2003). Other mineralized tissues such as teeth also contain calcium phosphate as HAP. The remainder is in soft tissue with only about 1% in extracellular fluids (Drezner, 2002). Therefore, maintenance of "normal" phosphate (inorganic or orthophosphate, P_i) homeostasis is essential for normal development, maintenance, and repair of teeth and skeletal tissues.

Natural foods contain substantial quantities of phosphorus. Deficiency can occur as a result of severe starvation, intake of P_i binders that prevent absorption in the gut, or in diseases associated with renal P_i wasting. Dietary P_i is absorbed in the small intestine where the impact of hormonal regulation, mediated by the active form of vitamin D, 1,25 $(OH)_2$ vitamin D_3 (referred to herein as Vit D), is minor relative to dietary load. From blood, phosphorus is taken into cells, incorporated into mineralized tissue matrices, or excreted from the body in urine. Hormonal regulation is critical to the homeostasis of absorbed P_i, with the primary locus being the kidney, as much of the absorbed P_i is excreted in the urine. Consequently, hormonal regulation of P_i excretion and reabsorption, more so than absorption, maintains circulating plasma concentrations (Drezner, 2002). This "parathyroid-kidney-intestine-bone/tooth" axis of Ca^{2+} and P_i balance is exhibited in Figure 1, with further description of the factors of interest featured in section II.

Renal P_i reabsorption, typically 80-90% of the glomerular filtrate, is mediated principally by the sodium-phosphate co-transporter Type 2a (NPT2a) in the proximal tubules, the expression of which regulates reabsorption (Murer et al., 1999; Murer et al., 1998). The expression and subcellular distribution of co-transporter protein is substantially affected by dietary phosphorus load; hyperphosphatemia decreases expression of NPT2a and hypophosphatemia increases expression. Tubular P_i reabsorption is enhanced by insulin-like growth factor (IGF-1), insulin, thyroid hormone, epidermal growth factor (EGF), P_i depletion, and Vit D, and inhibited by transforming growth factor alpha (TGFα), calcitonin, parathyroid hormone (PTH), parathyroid hormone related peptide (PTHrP), and glucocorticoids. PTH increases endocytosis of NPT2a from the cell membrane (Nashiki et al., 2005). In the proximal tubule, PTH induces mRNA expression of 25-hydroxy-vitamin D 1-α hydroxylase, a key enzyme in the production of Vit D, which will in turn mobilize P_i stored in the bone matrix. However, effects on P_i reabsorption are typically seen as secondary to the primary functions of these hormones. Whereas PTH is considered an important physiological regulator of renal P_i excretion, the balance between dietary intake and renal excretion is maintained in hyper-and hypoparathyroidism, suggesting that the proximal tubule intrinsically regulates excretion in response to dietary load (Levi et al., 1994). The two major classical P_i regulating hormones, PTH and Vit D, are discussed in detail in Section IIa and summarized in Table 1.

Maintenance of normal circulating P_i levels is critical to the normal development, maintenance, healing, and repair (and presumably regeneration) of mineralized tissues,

including teeth. For example, hypophosphatemia during development leads to dental and skeletal deformities (rickets) (Millan, 2006; Whyte, 2002). As stated above, the major determinants of serum P_i levels are dietary load and reabsorption of the renal glomerular filtrate in the proximal tubule. Additionally, other conditions, such as sepsis and insulin therapy, affect circulating P_i levels. Certain diseases, such as tumor-induced osteomalacia (TIO), X-linked hypophosphatemic rickets (XLH), and autosomal dominant hypophosphatemia (ADHR), which involve renal P_i wasting and affect mineralized tissues, have stimulated the search for hormones that specifically regulate P_i homeostasis (the so-called "phosphatonins"). Current candidate phosphatonins include fibroblast growth factor 23 (FGF23), matrix extracellular phosphoglycoprotein (MEPE), and secreted frizzled related peptide-4 (FRP4). The potential roles of phosphatonins during tooth development are discussed under Section IIb and summarized in Table 2.

Another important molecule that is pivotal for regulation of P_i metabolism is pyrophosphate (PP_i). PP_i is composed of two molecules of P_i, which are released upon hydrolysis, in addition to being produced by numerous other physiological reactions. PP_i is ubiquitous in the body, being present in numerous bodily fluids (e.g., blood, urine, saliva, synovial fluid), at the cell and tissue level, and in the mineralized matrices of bones and teeth. The numerous biosynthetic reactions producing PP_i, notably including synthesis reactions coupled to hydrolysis of nucleoside triphosphates (NTPs) such as ATP, have been extensively reviewed (Heinonen, 2001). Measurements of PP_i synthesis and concentrations *in vivo* are difficult due to instability in aqueous solution and rapid turnover by numerous enzymes with PP_i-ase activity. *In vitro* and *in vivo* data strongly support a role for PP_i as a potent inhibitor of HAP crystal growth. The suggested mechanism for inhibition is the binding of PP_i to HAP crystals, inhibiting growth and dissolution of the crystals (Fleisch and Bisaz, 1962; Fleisch et al., 1966; Meyer, 1984; Termine et al., 1970). Interestingly, PP_i is included in tartar control toothpastes to inhibit dental calculus, which results from calcification of dental plaque, a mixture of microorganisms, their metabolic products, and components of saliva, including Ca^{2+} and P_i (Netuveli and Sheiham, 2004; Zacherl et al., 1985).

The metabolic reactions governing P_i and PP_i in the body are inextricably linked—at times concerted, other times antagonistic, but always intimately connected by the close chemical nature of P_i and PP_i, and by enzymes, transporters, and feedback mechanisms that dictate the locations and concentrations of P_i and PP_i in and around the cell. Several proteins have been associated with local/cellular and systemic/humoral regulation of P_i/PP_i homeostasis and these are introduced below and discussed in greater detail in section III.

b. The tooth: A phosphate sensitive organ

The teeth and their supporting tissues are complex epithelial-mesenchymal organs produced by several unique cell types and composed of four mineralized tissues, namely, the highly mineralized *enamel* covering the crown, the resilient *dentin* surrounding the pulp chamber and forming the bulk of the crown and root, the thin layer of *cementum* that covers the root and is essential for attachment of the tooth via the periodontal ligament (PDL), and the metabolically active *alveolar bone* comprising the bony socket and anchoring the tooth via the PDL (Nanci, 2003). The close approximation of four P_i-rich mineralized tissues (the

only such occurrence in the body), as well as the important soft tissue interfaces, makes the tooth an intriguing organ for study of the developmental and homeostatic ramifications of P_i metabolism disorders. This reality is emerging as more studies indicate that the various mineralized tissues of the teeth are differentially regulated by prevailing P_i conditions (Boukpessi et al., 2006; Fong et al., 2008a; Foster et al., 2007; Nociti et al., 2002; Onishi et al., 2007; Toyosawa et al., 2004; van den Bos et al., 2005; Ye et al., 2004; Ye et al., 2008). In other words, systemic and local factors modulating P_i homeostasis seem to differentially control formation of oral mineralized tissues (e.g., pulp/dentin/bone *versus* follicle-PDL/cementum regions) based on a number of developmental disorders in humans, and as a result of gene mutation and knock out (KO) in mouse models, which will be described in detail in the text below and in the included Tables. These models will be described to provide insight into the specific roles of key regulators of P_i homeostasis during development, maintenance, disease, and repair/regeneration of tooth tissues.

c. Potential role for phosphate in regeneration of oral mineralized tissues

Oral health is essential for general health and nutrition as well as being a major determinant for quality of life. While there has been much progress in recent decades in prevention and treatment of oral-dental-craniofacial diseases, pathologies, and disorders, unfortunately they continue to be prevalent global health problems (WHO, 2003). Among approaches to treat congenital or acquired loss of mineralized tissues, repair or therapeutic regeneration may be second only to prevention in terms of importance. The major goal of regenerative therapy is to predictably restore normal tissue structure and function. Regeneration of mineralized tissues such as bones and teeth recapitulates development, at least in part, so that full understanding of the regulation of normal development will provide information key to designing effective therapies for the regeneration of teeth and associated tissues. Recognizing that regeneration of oral tissues is possible has resulted in increased attempts to identify factors and to understand their roles in controlling formation of oral tissues during development and regeneration (Bartold et al., 2000; Bartold et al., 2006a; Bartold et al., 2006b; Fong et al., 2005; Foster et al., 2007; Hu et al., 2006; Papaccio et al., 2006; Popowics et al., 2005; Saygin et al., 2000).

Clues to identifying candidate molecules for use in regeneration of oral tissues have come from studies focused on defining the factors involved in development of these tissues. Expression of specific genes/proteins have been mapped over time during tooth development using rodent models under normal conditions, as well as animal models where genes have been mutated, deliberately knocked out, knocked in, or conditionally expressed. In this regard, much has been learned from detailed analyses of the molecular basis of certain mutant animals where alterations in tooth development have resulted in abnormal adult teeth that significantly affect normal dental/oral function and health. This review is limited to factors involved in controlling P_i metabolism and their known/putative effects on the hard tissues of the oral cavity.

Regulators of P_i metabolism have received considerable attention over the last decade due to the role of P_i as a component of HAP mineralization and because of evidence that P_i may regulate cell behavior and mineralization as a signaling molecule. This review aims to

consolidate and summarize the most exciting research on P_i metabolism and mineralized tissues from recent years, focusing especially on the association with the hard tissues of the oral cavity. The next section, Section II, and Tables 1 and 2 will cover hormones governing serum P_i levels, followed by Section III and Table 3, which introduce local, microenvironmental P_i/PP_i regulatory elements, meaning those elements located in, on, or around the cell that contribute to P_i metabolism. Section IV will discuss accumulating evidence that P_i may act as a cell signaling molecule, modulating gene expression and cell function. Current therapeutics and materials with a basis in P_i metabolism will then be discussed in Section V. Lastly, under Section VI, we will attempt to synthesize this information and provide a vision for applying knowledge of P_i metabolism to regenerative approaches for the oral mineralized tissues.

II. Systemic hormone regulators of phosphate metabolism (Figure 1)

Some P_i metabolism regulating factors, discussed briefly in the introduction, exert their influence by modulating circulating P_i by actions in the kidneys and gastrointestinal tract. These systemic or humoral regulators of P_i include the “classical” regulators of systemic Ca^{2+} and P_i, Vit D, and parathyroid hormone (PTH). Systemic acting factors discovered and characterized more recently include endopeptidases on the X-chromosome (PHEX), and a collection of phosphaturic peptides identified in sera of individuals with disorders/diseases associated with renal P_i wasting. High levels of these peptides have been associated with hypophosphatemia and osteomalacia. These include the phosphatonin-like factors FGF23 (Quarles, 2003; Rowe, 2004; White et al., 2006), FRP4 (Berndt et al., 2003; Berndt et al., 2006; Berndt et al., 2005; Vaes et al., 2005; White et al., 2006), matrix extracellular phosphoglycoprotein (MEPE) (Argiro et al., 2001; Fisher and Fedarko, 2003; MacDougall et al., 2002; Quarles, 2003; Rowe, 2004; Rowe et al., 2000; Rowe et al., 2004; White et al., 2006), and FGF7 (Berndt et al., 2005; Carpenter et al., 2005). Both FGF23 and SFRP4 have been shown to inhibit 25(OH)D_3 1 α-hydroxylase activity (normally increased in conditions causing hypophosphatemia) and are thus considered “phosphatonins,” i.e., a label attributed to factors that increase renal loss of P_i and inhibit Vit D synthesis (Berndt et al., 2005; Econs and Drezner, 1994). Importantly, factors that alter Ca^{2+}/P_i in the sera affect both PTH and Vit D levels, adding an additional complexity to studies targeted at defining the mechanisms by which factors regulating circulating P_i levels modulate mineral homeostasis. Several publications provide excellent reviews of these factors (Berndt et al., 2005; Razzaque et al., 2005; Renkema et al., 2008; Rowe, 2004; Schiavi, 2006; Schiavi and Kumar, 2004; Yu and White, 2005b) and Figure 1 and Tables 1 and 2 provide an overview of the actions of the above-mentioned systemic factors.

a. “Classical” hormonal regulators of phosphate metabolism: 1, 25 $(OH)_2D_3$ and parathyroid hormone (Table 1)

The regulation of serum levels of electrolytes, especially Ca^{2+} and P_i, is of great importance for physiological functions such as nerve impulses, blood clotting, contraction of muscles (Ca^{2+}), and as constituents of cellular membranes, DNA, and signal transduction (P_i). Hypocalcemia may result in seizures and muscle cramping while symptoms of hypercalcemia may include kidney stones, nausea, and fatigue (Gunn and Gaffney, 2004).

Hypophosphatemia results in lowered bone mineralization and rickets, and hyperphosphatemia is characterized by soft tissue mineralization and excess Vit D levels. The appropriate levels of Ca^{2+} and P_i are maintained by the manipulation of a parathyroid-intestinal-kidney-bone axis under the influence of multiple endocrine system effector molecules. These players are featured in Figure 1, with an inclusion of tooth rather than bone as per the focus of this review. The molecules which have been classically believed to regulate Ca^{2+} and P_i levels are PTH, Vit D, and calcitonin, acting via a self-limiting feedback loop mechanism. It had long been perceived that Ca^{2+} levels were paramount in influencing the actions of these molecules, and that P_i levels simply followed the flow of Ca^{2+} in and out of the body, to maintain electrical balance. However, it has now become apparent that the regulation of P_i levels is under as strict control as Ca^{2+} by newly discovered agents such as FGF23 and others.

i. Parathyroid hormone (PTH)

Overview: The primary structure of PTH was first identified in 1970 as an 84 amino acid protein (Niall et al., 1970), of which the biological activity was found to reside in the amino terminal 34 residues (Rosenblatt et al., 1978; Tregear et al., 1973). The PTH receptor was identified in 1991, and was found to be a G-protein coupled receptor using a cAMP second messenger system (Henderson et al., 1992). PTH is secreted from the parathyroid gland based on serum Ca^{2+} levels which are detected by the calcium sensing receptor (CR). Low levels of Ca^{2+} inhibit CR activity, allowing PTH to be released into the circulation to achieve its downstream effects (Riccardi et al., 1995). A major action of PTH is to increase osteoclast activity in order to release Ca^{2+} from bone. This action is indirect in that osteoblasts respond to PTH signals by increasing their production of receptor activator of nuclear factor-kβ ligand (RANKL) (Suda et al., 1999). RANKL binds to RANK on osteoclast precursor cells and directs their differentiation (Kondo et al., 2002). PTH also induces osteoblasts to secrete osteoprotegerin (OPG), a RANK decoy ligand and regulator of osteoclast activation (Fu et al., 2002). In addition, PTH acts in the kidney to stimulate the formation of active Vit D which results in both increased Ca^{2+} absorption from the intestine and reabsorption in the kidneys, while reducing the reabsorption of P_i in the kidney by endocytic removal of NaP_i2a transporters (Nashiki et al., 2005). High levels of Ca^{2+} stimulate the CR which inhibits PTH release, and increases calcitonin release from the thyroid, inhibiting osteoclast activity (Fudge and Kovacs, 2004). The importance of PTH functioning in development was highlighted in the PTH receptor null mouse, which had a phenotype exhibiting enhanced mineralization of the chondrocytes during long-bone formation (Lanske et al., 1996). This in part contributes to the ability of PTH-associated peptides to act in both anabolic and catabolic fashions depending on dose and method of delivery, both *in vitro* and *in vivo* (Jilka, 2007; Pettway et al., 2008; Poole and Reeve, 2005).

Tooth-specific findings: There is some evidence that PTH has direct effects on dental cells. Both odontoblasts and cementoblasts are reported to have cell surface PTH receptors, and odontoblast cells responded to PTH treatment with increased expression of tissue nonspecific alkaline phosphatase (TNAP), *in vitro* (Lundgren et al., 1998; Tenorio and Hughes, 1996). Rats fed a diet which resulted in increased *in vivo* PTH levels had an increase in the width of the predentin, the unmineralized dentin matrix of the tooth,

compared to controls (Engstrom et al., 1978; Engstrom et al., 1977). Several studies have been performed either over-expressing or eliminating the function of the PTH receptor which resulted in dental phenotypes. The PTHR null mouse exhibited a phenotype in which osteoclast activity was greatly reduced with a resultant arrest in the eruption of teeth of normal morphology (Kitahara et al., 2002). Expression of constitutively active PTH receptors invoked abnormal tooth development (Calvi et al., 2004). However, this may not be due to PTH activity *per se*; PTHrP is a peptide with amino terminal homology to PTH and shares the same receptor (Juppner et al., 1991). PTHrP was initially found as the cause of hypocalcemia of malignancy, and has been since reported to be involved in epithelial-mesenchymal signaling in organ development, including teeth (Suva et al., 1987; Wysolmerski et al., 2001). PTHrP has been shown to have direct effects on cementoblast cells *in vitro*, reducing the mRNA expression of both bone sialoprotein (*Bsp*) and osteocalcin (*Ocn*), and inhibiting mineralization (Ouyang et al., 2000).

ii. 1, 25 $(OH)_2D_3$ (Vit D)

Overview: Vitamin D was first discovered in 1920, with its chemical structure elucidated in 1932, and was soon understood to be pivotal for proper bone formation and Ca^{2+} regulation (Brockman, 1936; Mellanby, 1921). The vitamin D precursor cholecalciferol can be produced in response to sunlight (UV light) in the skin, or as part of dietary intake (Goldblatt and Soames, 1923). However, this pre-vitamin D_3 molecule must undergo two sequential hydroxylations in order to produce the active metabolite Vit D (1,25 $(OH)_2D_3$ or calcitriol). Cholecalciferol first moves through the bloodstream to the liver with the vitamin D binding protein (DBP) due to its high degree of hydrophobicity (Cooke and Haddad, 1989). In the liver, cholecalciferol receives a hydroxyl group at carbon 25 from the action of cytochrome P450 25-hydroxylase enzymes such as CYP27A1 (Oftebro et al., 1981). $25(OH)D_3$ then, again with DBP, moves to the proximal kidney tubule to receive another hydroxylation at the 1-α position via cytochrome P450 25-hydroxyvitamin D-1-α-hydroxylase (CYP27B1) (Henry and Norman, 1974). This renal enzyme is stimulated by PTH in response to low Ca^{2+} levels in the blood, or low Vit D levels (Brown et al., 1995). The active metabolite, Vit D, moves with DBP to its target tissues, one of which is the parathyroid gland to inhibit the release of PTH in a negative feedback loop (Russell et al., 1993).

Due to its hydrophobic nature, Vit D readily crosses the cell membrane, and the Vit D receptor (VDR) is located in the cytosol of target cells, similar to receptors for other steroids (Haussler, 1986). In addition to the main Vit D target organs, kidney, intestine, and bone, many other tissues/cell types express the VDR, suggesting Vit D has direct effects in addition to its electrolyte balancing function (Bouillon et al., 1995). Once inside the cell, Vit D binds to VDR which then forms a heterodimer with the retinoid X receptor, which is required for its actions (Sutton and MacDonald, 2003). This complex then moves to the nucleus and binds to the DNA of Vit D response elements present in the promoters of Vit D target genes, acting as either enhancers or repressors (Prufer et al., 2000). Vit D can also act indirectly by reducing the effects of other transcription factors such as NF-kβ (Harant et al., 1997).

Vit D exerts its effects on mineral homeostasis in the intestine to increase the absorption of Ca^{2+} and P_i, and in the kidney to increase their reabsorption in the kidneys from the glomerular filtrate. In the intestine, Vit D stimulates the expression of the Ca^{2+} channel TRPV6, which moves Ca^{2+} into the cell, and also of calbindin-D_{9K} which moves it across the cell and PMCA1b which allows Ca^{2+} into the bloodstream (Hoenderop et al., 2005; Peng et al., 1999). In the kidney, Vit D increases Ca^{2+} reabsorption via up-regulating the expression of TRPV5, calbindin-D_{28k}, and PMCA1b Ca^{2+} transport proteins (Hoenderop et al., 2005). Vit D increases the expression levels of the sodium dependant P_i transporter IIb (NaP_i2b) in the intestine and NaP_i2c in the kidney, which increases reabsorption (Barthel et al., 2007; Capuano et al., 2005). Vit D also stimulates the bone marrow stromal cells to produce RANKL, which induces osteoclastogenesis, thus indirectly increasing the body Ca^{2+} levels (Udagawa et al., 1990).

Apart from its role in mineral homeostasis, Vit D has direct effects on developing bone cells, stimulating the expression of OCN, TNAP, and osteopontin (OPN) in osteoblasts (Chang and Prince, 1993; Mulkins et al., 1983; Yoon et al., 1988). Vit D may also work synergistically with the Wnt and Notch signaling proteins in directing osteoblast development (Fretz et al., 2007; Shen and Christakos, 2005).

With the importance that Vit D plays in both mineral metabolism and osteoblast development, it is not surprising that these systems are affected in Vit D deficiency states. Nutritional deficiency in Vit D is characterized by rickets/osteomalacia, hypocalcemia, and secondary hyperparathyroidism (Nagpal et al., 2005). In mice lacking the VDR, the same findings were present and the mice did not survive past 4-6 months (Nagpal et al., 2005). Mice lacking the 25-hydroxyvitamin D-1-α-hydroxylase gene had a phenotype characterized by rickets at birth, and then osteomalacia upon aging, with secondary hyperparathyroidism, hypocalcemia, and hypophosphatemia (Panda et al., 2001).

Tooth findings: As mineralized structures, the teeth have long been considered as potential target organs for the actions of Vit D, as a consequence of Ca^{2+}/P_i homeostasis abnormalities, as well as via direct effects. The VDR was identified by immunohistochemistry to be present in differentiating ameloblasts and odontoblasts in rat incisors, and was increased following a single dose administration of Vit D (Berdal et al., 1995). Vit D target genes, the calbindins, are expressed in rat incisor ameloblasts and odontoblasts and the expression of these genes also rose following a dose of Vit D. Studies using Vit D deficient animals have shown a reduced expression of amelogenin and enamelin, resulting in a disruption of enamel formation with an increase in interprismatic enamel, which was restored with Vit D treatment (Papagerakis et al., 2002). In Vit D deficient rats, the dentin had mineralization defects which may have resulted from noted decreases in the *OCN* and dentin phosphoprotein (*DPP*) in the rachitic animals (Berdal et al., 1991). *In vitro* studies have shown that dentinogenic cells exposed to Vit D exhibited increased expression of OPN and OCN, and TNAP, while the expression of dentin sialophosphoprotein (DSPP) was not affected (Bronckers et al., 1998; Ritchie et al., 2004; Tsukamoto et al., 1992). Mice lacking VDR expression had a tooth phenotype of thin dentin walls in the incisor, with an increase in pulp chamber space, and a widened predentin layer with an irregular border to the mineralized dentin (Zhang et al., 2007b). Humans with

familial hypophosphatemic rickets, characterized by low circulating Vit D concentrations, have teeth with defects in mineralized dentin resulting from unmerged calcospherites (Chaussain-Miller et al., 2007). Treatment of these patients with Vit D and increased dietary P_i resulted in a normalization of dentin mineralization. Interestingly, as described below (and in Table 2), mice and humans with loss of PHEX or dentin matrix protein 1 (DMP-1) are hypophosphatemic and exhibit a similar tooth phenotype.

b. Phosphatonins: Emerging systemic regulators of phosphate metabolism (Table 2; Figures 1 and 2)

The existence and function of endocrine regulators of Ca^{2+} such as PTH and Vit D have been known and investigated for many decades, with anionic P_i levels being believed to have a somewhat "passive" regulation to maintain electrical balance with cationic Ca^{2+}. There exists, however, a variety of human phosphate wasting conditions characterized by reduced reabsorption of P_i from the urine and mineralization defects of varying degree. These conditions include X-linked hypophosphatemic rickets (XLH), autosomal dominant hypophosphatemic rickets (ADHR), autosomal recessive hypophosphatemic rickets (ARHR), and tumor-induced osteomalacia (TIO) (Bielesz, 2006; Quarles, 2003). The existence of these conditions led to the hypothesis that there is a class of molecules termed "phosphatonins", whose function was to regulate P_i levels in the body (Berndt et al., 2005; Schiavi and Kumar, 2004). Molecular biology techniques have allowed for the elucidation of the genetic aberrations and proteins responsible for the pathological handling of P_i levels in these conditions, and have opened new avenues in research into the endocrine regulation of Ca^{2+}/P_i levels and closed the functional loop began by the PTH/Vit D bone-kidney axis. The primary phosphatonins, to date, include, fibroblast growth factor 23 (FGF23), matrix extracellular phosphoglycoprotein (MEPE), and FRP4, as well as molecules that regulate, albeit indirectly, FGF23 or MEPE function including P_i-regulating gene with homologies to PHEX and (DMP1). As investigations continue, other phosphatonin-like molecules are likely to be identified. This review is limited to phosphatonins and associated factors that have a known tooth phenotype when function is lost, i.e., PHEX, DMP-1, FGF-23, KLOTHO, and MEPE. Actions of these phosphatonins and related factors in conjunction with the classical Ca^{2+} and P_i regulators are summarized in Figure 1.

i. Phosphate regulating gene with homology to endopeptidases on the X-chromosome (PHEX)

General: PHEX is an endopeptidase whose physiological substrates are currently unknown. PHEX expression appears to be limited to osteoblasts, osteocytes, and odontoblasts, suggesting a role in the mineralization process (Ruchon et al., 2000). Positional cloning was used to determine that Phex mutations were responsible for XLH, the most common form of rickets in humans (HYP_Consortium, 1995), which is characterized by rickets and osteomalacia, hypophosphatemia, reduced growth, and altered Vit D levels (Rasmussen and Tenenhouse, 1995). There is a naturally occurring mouse mutation homologous to human XLH, the so-called *Hyp* mouse (Eicher et al., 1976). The *Hyp* mouse has a reduced body weight compared to wild type (WT), with lower serum P_i levels, greatly reduced Vit D levels, and increased *PTH* levels, yet is normo-calcemic (Liu et al., 2006). Morphologically, the *Hyp* mouse has shortened bones with rachitic splaying at the epiphysis, and a widening

of the growth plate due to an expansion of the hypertrophic zone, and an increase in unmineralized osteoid compared to WT. Paradoxically, transgenic expression of *Phex* in the *Hyp* mouse restored the bone phenotype, but did not affect the P_i metabolism deficiencies (Erben et al., 2005). It has been determined that in the *Hyp* mouse there is a dramatic increase in the expression levels of *FGF23* in osteocytes (Liu et al., 2003). The over-expression of *FGF23* has been shown to be responsible for much of the pathology in the *Hyp* mouse (Liu et al., 2006; Sitara et al., 2004). Further discussion of PHEX and its relationship to MEPE and FGF23 will be discussed in detail in sections II.b.ii and II.b.iii below.

Tooth findings: There is a record in the dental literature of dentin/pulp related disorders in XLH patients, including increased predentin, globular dentin, abnormal dentinal tubule distribution, and enlarged pulp chambers (Abe et al., 1988; Baroncelli et al., 2006; Cohen and Becker, 1976; Murayama et al., 2000; Shields et al., 1990). Importantly, *Hyp* mouse molar teeth exhibit a similar defect, as shown in Figure 2 (Abe et al., 1992; Abe et al., 1989; Ogawa et al., 2006; Sofaer and Southam, 1982). In the *Hyp* mouse tooth, an increase in *Ocn* mRNA was noted in the odontoblasts (Onishi et al., 2005). The expression of FGF23 was higher in both developing ameloblasts and odontoblasts in *Hyp* mouse, and the expression of NaP_i2b transporter protein was reduced (Onishi et al., 2007; Onishi et al., 2008). Additionally, in human XLH subjects, there have been some reports of periodontal involvement, including cementum abnormalities, periodontal abscesses, and loss of lamina dura indicating alveolar bone disruption (Cohen and Becker, 1976; Murayama et al., 2000). These reports in humans parallel our recent findings that demonstrate, in addition to defects in dentin as described above, using SEM, a globular cementum morphology was apparent in *Hyp versus* WT, as well as a similar pattern in incisor cementum from a 5 yr old girl diagnosed with hypophosphatemic rickets (Chu et al., 2007; Fong et al., 2008a).

ii. Matrix extracellular phosphoglycoprotein (MEPE)—MEPE was identified in a tumor-induced osteomalacia (TIO) patient, and is a member of the SIBLING (Small Integrin-Binding Ligand N-linked Glycoprotein) family, a group also including DMP1, dentin sialophosphoprotein (DSPP), bone sialoprotein (BSP), and osteopontin (OPN), which are implicated in mineralization (Fisher and Fedarko, 2003; Rowe et al., 2000). Physiologic expression of MEPE is seen in osteocytes, odontoblasts, hypertrophic chondrocytes, and in the callus of bone fractures, which suggests it has a function in bone and dentin mineralization (Argiro et al., 2001; Fisher and Fedarko, 2003; Lu et al., 2004; MacDougall et al., 2002; Nampei et al., 2004; Rowe et al., 2000). MEPE has been shown to be suppressed by an injection of Vit D in mice, and it is up-regulated in the VDR null mouse (Rowe et al., 2004). Increased expression of MEPE protein has been noted in XLH patients and the homologous *Hyp* mice (Argiro et al., 2001; Guo et al., 2002; Liu et al., 2005; Rowe, 2004; Rowe et al., 2004). The MEPE null mouse had an increase in both cortical and trabecular bone, indicating that MEPE functions as an inhibitor of mineralization under conditions of normal P_i and Vit D (Gowen et al., 2003). A tooth-specific phenotype has not been identified in *Mepe* null mice. MEPE may play a role in the hypophosphatemia in TIO patients as it has been shown to reduce NaP_i expression in opossum kidney cells *in vitro*, and lowers serum P_i in mice when injected (Gowen et al., 2003; Rowe et al., 2000). The

functional mineralizing portion of MEPE is the acidic serine-aspartate rich MEPE associated Motif (ASARM) (Martin et al., 2008). It was formerly thought that MEPE was a PHEX substrate that could release the ASARM moiety upon cleavage, however, further studies showed that MEPE is not a substrate of PHEX, and in fact PHEX seems to protect MEPE (and possibly DMP1) from cleavage by cathepsin B, which frees ASARM (Campos et al., 2003; Rowe et al., 2004). In fact, newer investigations suggest that functional PHEX is capable of cleaving ASARM peptides, thus regulating the inhibitory effect of ASARM on HAP crystal growth (Addison et al., 2008). The decrease in mineralization noted in the *Hyp* mouse may therefore be a combination of increased *MEPE* expression, coupled with a lack of *PHEX*-mediated protection from degradation of MEPE and inability to control ASARM degradation, producing an excess of ASARM that may in turn play a major role in the decreased mineralization characterizing the *Hyp* mouse (Rowe, 2004). Mice null for *Phex* (*Hyp*) and for *Mepe* were crossed, and the resultant offspring did not have a correction of the mineralization inhibition, or hypophosphatemia and low Vit D levels seen in the *Hyp* mouse (Liu et al., 2005). This indicated that *MEPE* was not the prime phosphatonin defining the mineralization phenotype in *Hyp* mice. Dental findings from mice lacking *MEPE* have not been reported to date.

iii. Fibroblast growth factor 23 (FGF23) and Klotho

General: FGF23 is a member of the FGF family, a group of 22 proteins having various effects on cellular development, differentiation, and function. The Fgf23 gene localized to human chromosome 12p13 encodes the 251 amino acid FGF23 protein (Yamashita et al., 2000). Autosomal dominant hypophosphatemic rickets (ADHR) is a disorder characterized by low serum P_i, low Vit D levels, rickets and osteomalacia, and normal PTH levels. FGF23 was identified as the target of the genetic defect responsible for ADHR, and also found to be highly expressed in tumors responsible for TIO, another condition characterized by P_i wasting (ADHR_Consortium, 2000; Shimada et al., 2001; White et al., 2001). *FGF23* was over-expressed in Chinese hamster ovary (CHO) cells that were implanted into SCID mice. The resulting tumors generated FGF23 and these mice developed the ADHR/TIO phenotype of osteomalacia, low serum P_i, low Vit D (with low levels of 25-hydroxyvitamin D-1-α-hydroxylase in the kidney) (Shimada et al., 2001). These data suggested that FGF23 functions as a phosphatonin, affecting P_i levels directly or by regulating the actions of Vit D. FGF23 has since been documented to negatively regulate renal expression of NaP_i2a and NaP_i2c, thereby decreasing P_i reabsorption in the kidney, and decreasing expression of 25-hydroxyvitamin D-1-α-hydroxylase in the kidney, thus reducing the levels of active Vit D as well as stimulating expression of 24-hydroxylase, which is catabolic for Vit D (Roy et al., 1994; Segawa et al., 2003; Shimada et al., 2004).

FGF23 expression has been detected by RT-PCR in multiple organs including heart, skeletal muscle, intestine, and liver, but it has been determined that the primary source is bone forming cells, especially the osteocytes, and in the bone fracture repair callus (ADHR_Consortium, 2000; Riminucci et al., 2003). Immunohistochemical results suggest that FGF23 expression is mainly in osteoblasts and osteocytes in bone, odontoblasts and cementoblasts in teeth, and in growth plate chondrocytes (Yoshiko et al., 2007). This study also indicated that the expression of FGF23 was greater in adult than fetal tissues. Evidence

for responsiveness of FGF23 levels to serum P_i levels has been inconsistent, with reports showing little if any response by FGF23 to increases in dietary P_i in human subjects, to several reports in which *FGF23* levels increased with dietary P_i load in mice (Berndt and Kumar, 2007; Ferrari et al., 2005; Larsson et al., 2003; Perwad et al., 2005). The expression of FGF23 has been shown to be increased by Vit D, although this action may be indirect, as the increase could be blocked by the administration of cycloheximide (Kolek et al., 2005). An FGF-inducing factor may also be produced by chondrocytes in response to Vit D signaling (Masuyama et al., 2006).

The pathologic over-expression of FGF23 is responsible for the development of multiple disease states characterized by P_i wasting, low levels of Vit D, and bone mineralization defects including rickets/osteomalacia, such as ADHR, TIO, XLH, and ARHR in humans, and the *Hyp* phenotype in mice with a *Phex* mutation. In ADHR, FGF23 is expressed in a mutated form resistant to proteolytic degradation (White et al., 2001). When full length FGF23 or either of its two breakdown fragments were injected into rats, only the full length form reduced serum P_i levels, suggesting that an accumulation of active FGF23 in ADHR patients results in the observed pathology (Shimada et al., 2002). The tumors present in TIO have been determined to frequently express high levels of FGF23, and conditioned media from the tumors injected into mice caused increased renal P_i clearance, and the P_i wasting in patients was resolved following resection of the tumors (Shimada et al., 2001). In XLH patients and *Hyp* mice, there is a mutation in Phex which appears to cause an increase in FGF23 levels. It was hypothesized that FGF23 was a PHEX substrate, and the lack of PHEX degradation of FGF23 led to its increase. However, FGF23 does not appear to be a PHEX substrate, and there is a frank over-expression of FGF23 in osteocytes examined from *Hyp* mice (Benet-Pages et al., 2004; Quarles, 2003). It is hypothesized that a natural substrate of PHEX regulates FGF23 expression and the buildup of this factor in the absence of PHEX results in the higher expression levels of FGF23 seen in the *Hyp* mouse. It is unclear if FGF23 has direct actions on bone forming cells, but FGF23 expression has been noted in the repairing callus of fractured bones, and *Hyp* osteoblasts transplanted into WT mice demonstrated mineralization defects (Ecarot et al., 1992; Liu et al., 2003; Riminucci et al., 2003). Autosomal recessive hypophosphatemic rickets (ARHR) is a newly classified P_i wasting disorder, in which mutations have been found in the Dmp1 gene, following the recognition that the *Dmp1* null mouse has a phenotype similar to the *Hyp* mouse and to that of individuals afflicted with ADHR and XLH (Farrow et al., 2007). Osteocyte production of *FGF23* is increased in the *Dmp1* null mouse, which could account for some of the observed phenotype (Feng et al., 2006). DMP1 and its role in mineralization and effects of its deletion in mice will be discussed under section II.b.iv below.

Human tumoral calcinosis (TC) is the result of the loss of function of FGF23. Patients having TC are reported to have high serum P_i levels, increased Vit D levels, calcification in vascular and soft tissues, and mineralized tumor masses (Mitnick et al., 1980; Prince et al., 1982). There are two different mutations which result in the observed reduced FGF23 function. One is a mutation in the Galnt3 gene, which encodes an enzyme responsible for protein glycosylation, suggesting that inappropriate glycosylation of FGF23 inhibits its function (Topaz et al., 2004). A mutation in Fgf23 at serine residues, different from that in

ADHR, also results in the development of TC (Araya et al., 2005). Serine residues are potential sites for glycosylation, which strengthens the notion that proper glycosylation is a requirement for FGF function.

Molecular biology techniques have been employed to further understand the function of FGF23 and the pathology associated with gain or loss of function. Several groups have generated transgenic mice that over-expressed *Fgf23*. These mice recapitulated the TIO phenotype of rickets and osteomalacia, abnormally low levels of Vit D, low serum P_i with renal P_i wasting, and growth reduction, confirming the involvement of FGF23 in the pathology seen in TIO, XLH, ADHR, and ARHR patients, and in *Hyp* mice (Larsson et al., 2004; Shimada et al., 2004). Other groups have produced mice in which the *Fgf23* gene has been removed (*Fgf23* KO). These null mice had some characteristics of TC with soft tissue calcifications, hyperphosphatemia, high levels of Vit D, as well as multiple atrophic organs such as thymus, testis, uterus, and skin, shortened lifespan, reduced growth, and a reduction in the hypertrophic zone of the growth plate (which remained at normal width) of endochondral bones (Liu et al., 2006; Shimada et al., 2004; Sitara et al., 2004). These null models were further investigated to determine the participation of *FGF23* in the *Hyp* mouse by producing a *Hyp/Fgf23* KO cross (Liu et al., 2006; Sitara et al., 2004). As described under section II.b.i., the *Hyp* mouse has a phenotype of rickets and osteomalacia, shortened long bones with rachitic splaying, increased *FGF23* and *PTH*, low Vit D, low serum P_i, and normal Ca^{2+}, and expanded growth plate due to an expansion of the hypertrophic zone (Liu et al., 2006). The compound mice had no detectable expression of *FGF23*, high levels of Vit D, low *PTH*, hyperphosphatemia, normal Ca^{2+}, shortened bones, and a resolution of rickets, but not osteomalacia, with growth plates similar to the *Fgf23* KO (Liu et al., 2006; Sitara et al., 2004). It is clear that much of the phenotype in the *Hyp* mouse is due to the *Fgf23* over-expression, and its removal superimposes the *Fgf23* KO phenotype onto the *Hyp* mouse. It was then determined that much of the phenotype of the *Fgf23* KO is the result of the over-expression of Vit D when the *Fgf23* KO was crossed with a mouse null for 25-hydroxyvitamin D-1-α-hydroxylase (Sitara et al., 2006). The resultant mouse showed a change from hyperphosphatemia in the *Fgf23* KO mouse to hypophosphatemia in the double KO, with a reduction in renal expression of NaP_i2a, and loss of the ectopic calcifications noted in the *Fgf23* KO, and a slight restoration in size of the animal. The phenotype of the 25-hydroxyvitamin D-1-α-hydroxylase null mouse of hypophosphatemia and rickets seemed to be superimposed on the *Fgf23* KO phenotype, suggesting that the excess Vit D in the *Fgf23* KO is a major contributor to the noted pathology (Dardenne et al., 2001). An additional study crossed the *Fgf23* KO with a *VDR* null mouse. In addition to the resultant phenotype just noted, this group also reported a recovery in the organ atrophy in the *Fgf23* KO (Hesse et al., 2007). It is interesting to note that in the various endocrine conditions characterized by mishandling of P_i metabolism, existing evidence suggests that the observed phenotypes may not result from direct effects of the mutated genes in question. The similar phenotypes resulting from loss of PHEX in humans (XLH) and mice (*Hyp* mutants) and loss of DMP1 in humans (ARHR) may have a stronger association with noted high levels of FGF23 (as opposed to loss of PHEX or DMP1). Additionally, the phenotype expressed in the absence of *FGF23* in the *Fgf23* KO mouse may be in large part a consequence of the increase in active Vit D.

The signal transduction mechanisms by which FGF23 exerts its effects have begun to be dissected. Signaling by FGFs is mediated by FGF receptors (FGFR), which are type I transmembrane proteins that dimerize upon binding their cognate FGF ligand extracellularly and then autophosphorylate intracellularly and propagate a signal cascade which stimulates the FGF effector genes in the nucleus. There are four FGFRs with alternative splice types a-c, with the "c" type believed to be limited to mesenchymal cells (Ornitz et al., 1996; Werner et al., 1992). FGF23 has shown affinity to FGFR 1c, 3c, and 4c, and activates the MAP kinase (ERK1/2) system (Urakawa et al., 2006; Yamashita et al., 2002; Yu et al., 2005). The binding of FGF23 to FGFRs is enhanced by klotho, and may be required for its actions (Kurosu et al., 2006; Urakawa et al., 2006). Klotho is a transmembrane protein which may be related to glucuronidsases and is expressed in the kidney, parathyroid, pituitary gland, and choroid plexus, but may also exist in a circulating form (Liu and Quarles, 2007; Xiao et al., 2004). Mice null for *klotho* have a phenotype of advanced aging with defects in hearing, osteoporosis, ectopic calcifications, and skin atrophy (Kuro-o et al., 1997). The phenotype of the *klotho* null and *Fgf23* KO, including hyperphosphatemia and increased Vit D, and ectopic tissue calcification, closely resemble each other; lending credence to the idea that klotho plays an integral role in FGF23 signal transduction (Memon et al., 2008).

Tooth findings: The lower incisor of the *Klotho* null shows a disturbance in the dentin development on the labial side, with an apparent defect in odontoblast function (Suzuki et al., 2008). The continually erupting mouse incisor tooth features a crown analogue on its labial aspect and a root analogue on its lingual aspect (Ohshima et al., 2005; Tummers and Thesleff, 2008). The labial incisor dentin had a reduction in the predentin width, with the development of an irregular bone-like mass of matrix having cells entrapped within. Many of these entrapped cells were shown immunohistochemically to contain high levels of *DMP1* protein, and an increase in apoptosis was noted in these cells. A similar phenotype has been noted in incisors and molars obtained from *Fgf23* KO mice (Figure 2), including narrow pulp chambers with ectopic matrix deposition, as well as accumulation of osteoid, increased incidence of apoptosis in cells, and disorganization and decreased width of the PDL (Blethen et al., 2008; Chu et al., 2007). Detailed descriptions of these findings will be featured in a forthcoming publication from our lab.

iv. Dentin matrix protein 1 (DMP1)

General: DMP1, another member of the SIBLING protein family (Fisher and Fedarko, 2003), is a highly phosphorylated acidic protein, initially identified as a product of odontoblasts. However, DMP1 has been identified as highly expressed in mature osteoblasts and in the bone-encased osteocytes (George et al., 1994; Toyosawa et al., 2001). DMP1 expression has also been noted in soft tissues, where it has been co-localized with MMP-9 for functions currently unknown (Ogbureke and Fisher, 2007). The acidic domains of DMP1 were seen *in vitro* to form β-like sheets capable of binding Ca^{2+} ions and initiating crystal nucleation, and *in vivo* could play a major role in the mineralization of predentin to dentin (He et al., 2003). In addition to its role in mineralization, DMP1 has also been implicated as a signaling molecule. Overexpression of Dmp1 in mesenchymal cells induced Dspp mRNA expression and an odontoblast phenotype, and DMP1 protein was observed to be localized to the nucleus (Narayanan et al., 2003; Narayanan et al., 2001). Intact DMP1 protein has not

been recovered, only an amino and carboxyl terminal fragment, suggesting that DMP1 requires proteolytic cleavage to become active or is rapidly cleaved; however, the protease responsible for this is still unknown (Qin et al., 2003). The exact nature of DMP1 regulation of expression remains unknown, but many transcription factor response elements have been identified in the Dmp1 promoter region, and it is reported that in a cementoblast cell line treated with P_i, *Dmp1* expression was up-regulated by 30-fold (Foster et al., 2006b; Narayanan et al., 2002). When mouse ulna were placed under strain, the osteocytes expressed high levels of *DMP1*, suggesting DMP1 plays a role in osteocyte function in the response to mechanical forces in bone (Yang et al., 2004).

The *Dmp1* null mouse has both dental and skeletal phenotypes. Postnatal *Dmp1* null mice develop skeletal defects including rickets and osteomalacia/mineralization defects, a widened hypertrophic zone resulting in an enlarged growth plate (Feng et al., 2006; Ling et al., 2005). Many of the defects observed in the *Dmp1* null mouse may be attributed to the surprising finding that the *Dmp1* null mouse is hypophosphatemic, and was a mouse model for ARHR (Feng et al., 2006; Lorenz-Depiereux et al., 2006; Ye et al., 2005). The hypophosphatemia is the result of an increase in the expression of *FGF23* by osteocytes, by an unknown mechanism, similar to the *Hyp* mouse (Feng et al., 2006). This suggests that FGF 23 is under the control, directly or indirectly, of DMP1. The connection, if any, between DMP1 and PHEX is unknown. However, it is worth noting that the many factors discovered so far to play a role in P_i regulation, such as FGF23, MEPE, PHEX, and DMP1, are all expressed in osteocytes in normal or pathological conditions. Thus, it may be hypothesized that the osteocytes have an as yet undiscovered role in regulating P_i levels in the body.

Tooth findings: In the tooth of the *Dmp1* KO mouse, there is a reduction in mineralization of dentin, with a widened predentin layer, thin dentin walls with an enlargement of the pulp space, dentinal tubule abnormalities, a reduced expression of *Dspp*, and periodontal defects, including changes in the thickness of alveolar bone, periodontal ligament, and cementum (Ye et al., 2004; Ye et al., 2008). Tooth abnormalities in ADHR patients have been reported for one individual and were described as observed defects of dentin and multiple caries in a 5-year-old subject (Lorenz-Depiereux et al., 2006).

v. Other factors—FRP4 was found to be a highly expressed gene in many tumors of TIO patients (Berndt et al., 2003; Kumar, 2002). FRP4 is structurally similar to the extracellular portion of the Wnt receptor, frizzled, and functions as an antagonist to Wnt binding to frizzled (Hsieh et al., 1999). Mounting data support a role for *Wnt* signaling in bone formation and remodeling (Gaur et al., 2005; Glass et al., 2005; Li et al., 2005; Vaes et al., 2005), as well as tooth development (Nadiri et al., 2004; Pispa and Thesleff, 2003). Gain of function in Wnt signaling mutations has been noted in patients with an increase in bone mass (Boyden et al., 2002). The injection of recombinant FRP4 into rats or mice resulted in an increase in P_i excretion and hypophosphatemia as early as 2 and 4 hrs, respectively, in rats, and in mice at 60 minutes (Berndt et al., 2003; Berndt et al., 2005). Opossum kidney cells treated with FRP4 *in vitro* exhibited reduced P_i uptake (Berndt et al., 2003). In addition

to induction of phosphaturia and hypophosphatemia, FRP4 induced 25-hydroxy-vitamin D1-α hydroxylase activity (Berndt et al., 2003; Berndt et al., 2006).

McCune-Albright syndrome (MAS) is a skeletal disease caused by *Gnas1* mutation and characterized by polyostotic fibrous bone dysplasia, endocrine hyperfunction, and café-au-lait skin pigmentation. Oral manifestations of MAS reported include enamel hypoplasia and hypomineralization, delayed tooth eruption, taurodontism, and craniofacial bone (mandible and maxilla) dysplasia (Akintoye et al., 2003; Akintoye et al., 2004; Gomes et al., 2002). MAS is sometimes complicated by hypophosphatemia and abnormally low Vit D levels, resembling the presentation of XLH and OOM. Increased plasma levels of FGF23 suggest a mechanism underlying the hypophosphatemia and abnormal Vit D metabolism (Yamamoto, 2006; Yamamoto et al., 2005).

c. Closing the Ca^{2+}/P_i endocrine loop

The data derived from FGF23 studies have allowed for the endocrine regulatory loop of Ca^{2+}/P_i homeostasis to be closed (as outlined in Figure 1). Low Ca^{2+} or low P_i stimulates PTH expression. PTH drives osteoclastogenesis, mobilizing Ca^{2+} (and P_i) from bone and the production of active Vit D, which allows for increased absorption of Ca^{2+} and P_i from the intestine and reabsorption in the kidney. PTH reduces the reabsorption of P_i in the kidney, but PTH is soon down-regulated as Ca^{2+} levels approach normal, so has a short term role in regulating P_i. The increase in Vit D directs an increase in the level of FGF23, which feeds back to reduce both Vit D levels and levels of NaP_i Type 2a and 2c P_i transporters in the kidney to act in a more long term fashion, to balance the actions of Vit D, to ultimately prevent the development of a hyperphosphatemic state.

III. Local cellular regulators/ion transporters regulating P_i/PP_i homeostasis (Table 3 and Figure 2)

While P_i metabolism functions on a systemic level via modulation of circulating concentrations, there are also several cell-localized regulators of both P_i and PP_i that strongly influence local, microenvironmental concentrations. These factors transport P_i and PP_i across cell membranes and govern the P_i/PP_i ratio by generation and hydrolysis of PP_i. The primary regulators of P_i metabolism at the microenvironmental level include mouse progressive ankylosis protein (*ANK*, as well as human homolog, ANKH), a putative transporter of PP_i from the intracellular compartment to the extracellular space (Gurley et al., 2006a; Harmey et al., 2004; Ho et al., 2000; Johnson et al., 2003; Nociti et al., 2002; Nurnberg et al., 2001; Pendleton et al., 2002; Reichenberger et al., 2001; Sweet and Green, 1981; Terkeltaub, 2001), the PP_i-generating nucleoside triphosphate pyrophosphohydrolase plasma cell membrane glycoprotein-1 (*NPP1* or *PC-1*) (Fong et al., 2005; Goding et al., 1998; Harmey et al., 2004; Johnson et al., 2003; Murshed et al., 2005; Nociti et al., 2002; Okawa et al., 1998; Rutsch et al., 2000; Rutsch et al., 2001; Terkeltaub, 2001; van den Bos et al., 2005), and tissue nonspecific alkaline phosphatase (*TNAP*), an enzyme proposed to cleave PP_i substrate to its P_i constituents (Beertsen et al., 1999; Chapple, 1993; Fedde et al., 1999; Groeneveld et al., 1996; Hessle et al., 2002; Murshed et al., 2005; Narisawa et al., 1997; van den Bos et al., 2005; Whyte, 2002; Whyte et al., 1995). Details regarding the

functions of these factors are provided in Table 3, including implications for their roles based on mouse models where genes have been mutated or deleted. Existing data indicate that local control of P_i/PP_i is critical for normal cementum/periodontal tissue development, an idea that will be explored in more detail in the sections below.

The importance of maintaining appropriate concentrations of P_i in the extracellular environment for regulation of mineralization was highlighted by Murshed and colleagues (Murshed et al., 2005). Examining a variety of KO mice, the modulation of extracellular P_i concentrations was found to be critical in both regulating physiological mineralization and preventing pathological calcification. However, these studies lacked details related to teeth and surrounding tissues, essential for defining the precise role of P_i and its regulators in controlling mineralization. Results from studies to date suggest that local control of PP_i/P_i is critical for normal root/periodontal tissue development, and further, that cementum may be a uniquely sensitive tissue to PP_i and P_i in the local area.

a. Tissue nonspecific alkaline phosphatase (TNAP)

General—TNAP is one of four alkaline phosphatase isozymes expressed in mammals, and is found in liver, kidney, and bone, as well as tooth (for an excellent and extensive review see Millan, 2006). TNAP activity produces P_i from hydrolysis of PP_i, and other natural substrates (e.g., pyridoxal 5'-phosphate (PLP) and possibly phospoethanolamine (PEA)). TNAP function is important in skeletal mineralization by removing a mineralization inhibitor, PP_i, and increasing a required HAP mineral building block, P_i (Murshed et al., 2005). In this sense, the tendency towards biological mineralization may be regulated by the ratio of P_i:PP_i, with regulators like TNAP, ANK, and PC-1 (the latter two are discussed below) central in determining microenvironmental P_i and PP_i levels. TNAP is present on cell membranes of osteoblasts and matrix vesicles (MVs), and circulating levels are also measurable.

The condition hypophosphatasia (HPP) results from dysfunctional or low levels of the TNAP causing poor mineralization and the conditions rickets and osteomalacia (Whyte, 1994; 2002). In *Tnap* (*Akp2*) KO mice, there was decreased mineral density in the bones and dentition (Beertsen et al., 1999; Millan, 2006).

Mice deficient in *ANK* or *PC-1* exhibit decreased extracellular PP_i, resulting in pathological calcification in the soft tissues (see Gurley et al., 2006a; Ho et al., 2000; Okawa et al., 1998, and below). Combinations of deficiencies in *ANK* or *PC-1* with lack of *TNAP* resulted in partial improvements to the hypophosphatasia and mineralization defects (Anderson et al., 2005; Harmey et al., 2004; Hessle et al., 2002). Tooth phenotypes have not been described in these compound mutants. These data underscore the concerted nature of the interactions between PC-1, ANK, and TNAP in regulating P_i and PP_i in the cellular milieu. The authors also described an apparent signaling effect of PP_i in regulating gene expression of *Ank, Enpp1,* and the osteopontin (*Opn*) gene. This is discussed in more detail in section IV below.

A recombinant *TNAP* enzyme replacement therapy approach targeting bone was able to rescue skeletal (and tooth) developmental defects in *Tnap* null mice (Millan et al., 2007). This success, when contrasted to failures of previous IV infusions of TNAP into HPP

patients (Weninger et al., 1989; Whyte et al., 1984; Whyte et al., 1982), suggests that local, pericellular function of TNAP around bones and teeth is more physiologically critical than circulating serum levels during development of mineralized tissues. This conclusion, however, is at odds with findings from another study in which increased circulating *TNAP* produced by the liver rescued the bone mineralization phenotype in *Tnap* KO mice (Murshed et al., 2005).

Tooth—TNAP is highly expressed in the periodontia, including cementoblasts, osteoblasts, and PDL fibroblasts (Groeneveld et al., 1995). HPP afforded the first linkage of a P_i metabolism disorder with a developmental cementum phenotype, and has been consistently linked to premature loss of deciduous teeth (Bruckner et al., 1962; Chapple, 1993; van den Bos et al., 2005). TNAP deficiency causes cementum aplasia or severe hypoplasia, especially in the more coronal acellular cementum region, compromising periodontal attachment due to defects in attachment of Sharpey's fibers, ultimately resulting in premature exfoliation of teeth (Bruckner et al., 1962). Reports of effects of HPP on cellular cementum have varied, with some indication for hypoplasia (van den Bos et al., 2005).

In addition to decreased mineral density in the teeth, the *Tnap* (*Akp2*) KO mouse was reported to feature delayed incisor eruption (Beertsen et al., 1999; Millan, 2006). Molars developed relatively normally, though a mild enamel hypoplasia and slightly delayed root dentin mineralization was reported. The most striking defect noted was aplasia or severe hypoplasia of acellular cementum, including both matrix and mineral phase, accompanied by a suggestion of defective PDL attachment (Figure 2). No overt differences were described in cellular cementum structure. To understand the mild effect or lack of effect of TNAP deficiency on dentin, van den Bos et al. (2005) assessed PP_i levels and PP_i-associated enzyme regulators in dentin/pulp *versus* PDL of normal teeth. The authors reported decreased expression of Enpp1 and also decreased NPP1 (PC-1) activity in pulp *versus* PDL, and also lower levels of PP_i in pulp *versus* PDL, reinforcing findings of others regarding differences in the role of regulators of P_i metabolism among a variety of tissues, and highlighting the need to understand genes dictating differences between hard tissues.

An increase in TNAP levels and activity has been linked to other human mineralized tissue diseases via case reports in the literature, some including dental phenotypes. Generalized hypercementosis has long been associated with Paget's disease (osteitis deformans). Paget's disease is a typically late onset disorder of focally increased bone turnover resulting in disorganized, sclerotic, and weak bone (Helfrich and Hocking, 2008). Paget's disease has been linked to a number of gene mutations, all involved in osteoclast function. Serum calcium and P_i have been reported to lie within normal limits, but TNAP levels are highly elevated, usually attributed to rapid turnover of bone. Pendred Syndrome is an autosomal recessive inherited disorder of the thyroid gland. In addition to goiter, hearing impairment, and an apparent neutrophil defect, a case report has linked Pendred Syndrome with an oral manifestation of cementum hyperplasia (based on assessment of radiographs) and a thickened lamina dura (Sharma and Pradeep, 2007). These findings are intriguing in light of the observed elevated serum TNAP, P_i, and Ca^{2+}, though a single case report must be considered cautiously. While the underlying mechanism for cementum phenotypes

associated with these conditions is unclear, increased TNAP activity in the periodontia may be related to an altered P_i/PP_i ratio and consequent tissue changes.

b. Progressive ankylosis protein (ANK, ANKH)

General—The mouse progressive ankylosis gene (*Ank*), analogous to Ankh in humans, transports intracellular PP_i to the extracellular space (Ho et al., 2000). Loss of *ANK* function results in low levels of PP_i in the local extracellular environment, with high intracellular PP_i, in contrast to *TNAP* deficiency, which causes increased PP_i outside cells (Ho et al., 2000; Terkeltaub, 2001). The *ank/ank* mutant mice have been described as a model for a progressive arthritis-like condition characterized by ectopic calcifications in cartilage and joint tissues, with mice exhibiting degeneration in joints, tendons, and ligaments, and impaired mobility manifested as an arthritis-like condition (Ho et al., 2000; Sweet and Green, 1981; Terkeltaub, 2001). While the phenotype had been characterized since 1981 (Sweet and Green, 1981), the gene and mutation were not identified until 2000, when a publication by the Kingsley laboratory described the mechanism of the observed pathology (Ho et al., 2000).

Mutation in the human ortholog of the mouse gene, Ankh, has been associated with a variety of skeletal defects and human disease. While PP_i acts as a potent inhibitor for growth of HAP crystals, excess levels (as in conditions such as adult hypophosphatasia) result in pathological calcium pyrophosphate dihydrate (CPPD) elaboration (Netter et al., 2004; Ryan, 2001). The condition chondrocalcinosis (CC) is caused by deposition of CPPD within articular cartilage and causes joint pain and arthritis. Mutations associated with CCAL2 cluster to the N terminus of ANKH and result in alterations of 1-4 amino acids (Pendleton et al., 2002). Like *ank/ank* and *Ank* KO mice, CCAL2 patients experience crystal deposition in the articular cartilage and synovial fluid, however the crystals are CPPD rather than HAP. Evidence suggests gain of function of ANKH as a result of the CCAL2 mutations, causing excess extracellular PP_i and pathological CPPD deposition (Gurley et al., 2006b; Pendleton et al., 2002; Ryan, 2001).

Additional dominant mutations in Ankh have been linked to the condition craniometaphyseal dysplasia (CMD), a congenital disorder resulting in cranial and long bone mineralization defects (Nurnberg et al., 2001; Reichenberger et al., 2001). CMD is characterized by overgrowth and sclerosis of the craniofacial bones and abnormal modeling of long bone metaphyses, and in contrast to CCAL2 patients and *Ank* mutation/KO in mice, there is no apparent joint phenotype. Ankh mutations associated with CMD affect single amino acids in a region distinct from the mutations that cause CCAL2, and have been linked to significantly less PP_i transport (Gurley et al., 2006b).

Tooth findings—Our group became interested in the periodontal status of the *ank/ank* mice, hypothesizing that the PDL space perhaps exhibited some ectopic ossicles or even ankylosis. However, an unexpected and intriguing tooth phenotype was observed in the *ank/ank* mice, and more recently in *Ank* KO mice (Figure 2). Rather than observing ossicles or ankylosis as expected, a marked increase in cementum formation was instead exhibited, while PDL, dentin, and alveolar bone appeared unaffected (Nociti et al., 2002). An increased

rate of cementogenesis and increased cellularity of the cementum (in the normally acellular cervical region) were observed over the entire course of root formation, from initiation of cementogenesis (26 dpc) through adulthood. The same hypercementosis phenotype was identified in adult mice with a *Pc-1/Enpp1* mutation (see more on this factor below), suggesting that the increased cementum was a direct result of the decreased extracellular PP_i common to both mutations. Studies to characterize the mechanical properties for the *ank/ank* mutant *versus* WT tissues have not identified any significant difference in the hardness, elastic modulus, or structure, as observed by SEM and TEM (Fong et al., 2008b).

The first detailed dental clinical report regarding CMD was recently published and described the dental features of a 3½ yr old patient (Zhang et al., 2007a). In this case, primary teeth were marked by dysmorphic and discolored surfaces, especially mandibular central incisors and maxillary 1st molars. The only indication for excess mineralization was an observation of enamel pearl-like material on the buccal surfaces of maxillary 2nd molars. The enamel pearls were evaluated at a gross level but their appearance was not inconsistent with the mineral accumulation observed in mice lacking *ANK* function; this is an area for further investigation to determine if there is a cementum phenotype and if there are any clinical dental ramifications. A previous CMD case report of a 10 yr old patient reported delayed tooth eruption, but did not report malformation or discoloration (Hayashibara et al., 2000). No tooth phenotype has been reported for CPPD, though cementum hypoplasia or aplasia might be expected in light of observations from HPP patients.

c. Plasma cell membrane glycoprotein 1 (PC-1)

General—Like *ANK*, the protein *PC-1* (ectonucleotide pyrophosphatase/phosphodiesterase 1, *ENPP1*) regulates extracellular PP_i. Mutations in the gene for *PC-1*, a membrane-bound enzyme that generates PP_i from triphosphates, result in low extracellular PP_i, and as a result, a joint phenotype in mice resembling those with *ANK* deficiency (Johnson et al., 2000; Terkeltaub, 2001). The hypermineralization observed in *PC-1* deficient mice (tiptoe walking, or *ttw* mice), while similar to that described in *ank/ank* mice, has been reported to be more severe, and one explanation for this may be localization of *PC-1* but not *ANK* in matrix vesicles (MVs), cell buds proposed to serve as focal points for mineralization (Hessle et al., 2002; Johnson et al., 2001; Vaingankar et al., 2004). Humans with mutations in these genes also present pathologies resulting from deficient PP_i, including craniometaphyseal dysplasia (CMD) and idiopathic infantile arterial calcification (IIAC) (Nurnberg et al., 2001; Reichenberger et al., 2001; Rutsch and Terkeltaub, 2005; Rutsch et al., 2001).

While *ANK* and *PC-1* functions increase PP_i in cell environment, *TNAP* activity decreases PP_i to release P_i. In this sense, *PC-1* and *ANK* can be thought of as natural antagonists to *TNAP* in terms of regulating P_i/PP_i levels and mineralization (Harmey et al., 2004). Crossing *Ank* mutant mice with *Akp2* null mice resulted in partial correction of both PP_i levels and mineralization defects.

Tooth findings—The tooth phenotype of *ttw* mice was described alongside *ank/ank* mice and presented a strikingly similar hypercementosis phenotype at 77 dpc (Nociti et al., 2002).

This is presumably the result of relatively lower levels of extracellular PP_i and/or the altered ratio of P_i/PP_i. The tooth phenotype of *ttw* mice has not been more fully characterized.

d. Phosphate transporters: P_iT1 and 2

Sodium dependent P_i transporters types I and II have been identified in several organs; type II NaP_i2a and NaP_i2b are the predominant transporters responsible for P_i reabsorption in the kidneys (as described above). Type III sodium-dependent P_i transporters, *PiT-1* and *PiT-2*, are widely expressed and likely perform housekeeping functions in regulating cellular P_i entry (Kavanaugh and Kabat, 1996; Li et al., 2006; Ravera et al., 2007). However, *Pit-1* expression in osteoblasts and other cells is regulated by P_i availability and other factors (Foster et al., 2006b; Suzuki et al., 2006; Suzuki et al., 2001; Zoidis et al., 2004), suggesting that *PiT-1*, but not *PiT-2*, may be of more physiological importance due to responsiveness to physiological cues (Zoidis et al., 2004). This hypothesis on *PiT-2* was supported by a survey of the temporospatial expression during mouse tooth development, which mapped *Pit-2* to secretory ameloblasts, dental papilla, and stratum intermedium, but not odontoblasts or cementoblasts (Zhao et al., 2006). Using tooth bud organ culture, it was found that *Pit-2* expression does not respond to P_i starvation or treatment with PTH or Vit D. No tooth defect has been identified with loss of P_iT function.

e. Other phosphatases

Other regulators of P_i/PP_i levels include those more recently identified and of unknown importance in mineralized tissue formation. There is evidence that TNAP is not the sole enzyme with phosphatase activity in the osteoblast; in *Tnap* null mice, initial skeletal mineralization proceeds normally, and only later are hypomineralization defects manifested (Hessle et al., 2002; Narisawa et al., 1997). *TNAP* has been reported to be restricted to the basolateral domain of the osteoblast, and a phosphatase localized to the osteoidal aspect was characterized as a plasma membrane Ca^{2+} transport ATPase (*PMCA*, or *PMCA1*) (Francis et al., 2002; Kumar et al., 1993; Meszaros and Karin, 1993; Nakano et al., 2004; Nakano et al., 2003; Prasad et al., 2004; Stains et al., 2002), a protein identified previously in osteoblasts (Meszaros and Karin, 1993; Stains et al., 2002).

PHOSPHO1 is a cytoplasmic phosphatase linked to bone and cartilage mineralization (Houston et al., 2002; Houston et al., 2004; Stewart et al., 2006; Stewart et al., 2003) and hypothesized to contribute to P_i requirements for mineralization by cleaving phospholipid metabolites (Roberts et al., 2004; Roberts et al., 2005). PHOSPHO1 is associated with matrix vesicles of murine mineralizing cells, including osteoblasts and chondrocytes (Roberts et al., 2007; Stewart et al., 2006). No tooth phenotypes have been described to date in animal models, making it unclear if these phosphatases play a significant role in tooth mineralized tissue development.

IV. Phosphate as a signaling molecule

Disruptions in P_i metabolism leading to hyperphosphatemia, hypophosphatemia, or local disruptions in P_i/PP_i ratio clearly have a considerable effect on bone and tooth development and maintenance, as outlined in the previous sections. Studies of mouse models are yielding

a great deal of information about how local and systemic factors interact and have an impact on developing tissues. In parallel, *in vitro* experiments focused on effects of P_i may provide us with information of a more unambiguous and mechanistic nature. This section reviews the evidence for signaling properties of P_i in studies predominately done using *in vitro* culture systems. The combination of knowledge from such *in vitro* studies and *in vivo* observations in animal models should provide powerful and incisive insights into the role of P_i metabolism in health and disease, as well as potential for use in regeneration.

a. Phosphate regulates mineralized tissue cells

Phosphate present in the extracellular milieu of mineralizing cells may serve as a signal for a shift in cell function/differentiation, in addition to acting as one of the building blocks for hydroxyapatite mineral. The ability of P_i to regulate gene expression may represent an extracellular signaling mechanism, which subsequently informs the cell about its environment and provides cues for directing cell functions. Studies by several groups have begun to explore the mechanisms of P_i regulation of cell gene expression. The enzyme TNAP is up-regulated early in osteoblast differentiation, and a requirement of *TNAP* activity for *Opn* expression in MC3T3-E1 pre-osteoblast cells was observed by Beck et al. (1998). In a subsequent study exploring the *TNAP-Opn* link, P_i was shown to be a direct inducer of *Opn* expression in MC3T3-E1 cells (Beck et al., 2000), and was hypothesized to act as a signal to cue these precursor cells to undergo the coordinated program of cellular changes necessary for differentiation and matrix mineralization (Beck, 2003). The ability of 300 μM foscarnet to block *Opn* induction suggested that P_i internalization was necessary for signaling activity. Regulation of P_i-mediated *Opn* expression was further indicated to be ERK 1/2 and PKC mediated (Beck and Knecht, 2003). Subsequent *in vitro* studies employing microarray analyses identified several genes up- and down-regulated by 10 mM P_i, including those for extracellular matrix proteins and transcription factors, like *Nrf2* (Beck et al., 2003). The increase in *Nrf2* expression occurred by 2 hrs and did not require protein synthesis, suggesting it is an early response gene in MC3T3-E1 cells.

A standard procedure for promoting mineral nodule formation by osteoblast-like cells *in vitro* is by adding P_i or β-glycerophosphate. P_i is actively transported into osteoblasts via transporters, including *P_iT-1/P_iT-2* (Guicheux et al., 2000; Nielsen et al., 2001; Palmer et al., 1999; Suzuki et al., 2000). In addition to the critical role of P_i for normal mineralization, it has become apparent that P_i also plays a major role in several other biological processes, including enzyme regulation, nucleotide metabolism and signal transduction (Beck et al., 2003; Berndt et al., 2005; Foster et al., 2006b; Fujita et al., 2001; Rutherford et al., 2006). New and established data provide evidence that calcitropic hormones, growth factors, and cytokines have the ability to modulate P_i/PP_i levels, with significant impact on the extent of mineral formation (Bielesz, 2006; Rowe, 2004; Yu and White, 2005a). A recent example demonstrating the role of growth factors comes from the studies of Suzuki et al. (2006) where it was reported bone morphogenetic protein (*BMP)-2* enhanced *Pit-1* mRNA and functions in MC3T3-E1 cells, a murine osteogenic cell line.

Shapiro et al. suggested elevated P_i may signal stage-specific apoptosis of terminally differentiated chondrocytes (Mansfield et al., 2003; Mansfield et al., 1999; Mansfield et al.,

2001; Teixeira et al., 2001), and additional studies showed 5 and 7 mM Pi significantly promoted apoptosis in human alveolar bone osteoblasts as well as MC3T3-E1 cells in culture (Adams et al., 2001; Meleti et al., 2000). These studies also indicated the requirement for P_i transport across the cell membrane by using foscarnet. The authors hypothesized that under normal physiologic conditions osteoblasts may encounter high P_i in areas of local bone resorption and be directed toward apoptosis as a result. Of course, the ramifications of such programming should be considered under pathological conditions as well. In the *Hyp* mice, which are hypophosphatemic as a result of *Phex* mutation (described in detail in section II.b.i), a widened and irregular hypertrophic zone was described in the growth plate cartilage (Miao et al., 2004). In contrast, *Klotho* KO mice (described in detail in section II.b.iii), which are hyperphosphatemic due to lack of *Fgf-23* signaling, feature positive staining for apoptosis in odontoblasts and pulp cells in the incisor (Suzuki et al., 2008). Our studies of the similarly hyperphosphatemic *Fgf-23* KO mice indicate not only apoptosis in odontoblasts embedded in matrix in the labial aspect of the incisor, but also widespread apoptosis in the alveolar bone osteoblasts (personal observations to be published in forthcoming manuscript).

Studies in the Shapiro laboratory showing that 10 mM P_i is sufficient to promote apoptosis in MC3T3-E1 cells must be reconciled with studies by Beck et al. treating the same cells with 10 and even 20 mM P_i for up to 96 hrs without reports of apoptosis or cell death. One possible explanation lies with the amount of fetal bovine serum (FBS) used in cell culture; FBS is known to contain proteins that can bind and prevent precipitation if Ca^{2+} and P_i even under supersaturated conditions. It is plausible that interactions between these binding proteins and P_i effectively limit the amount available to cells and reduce the tendency of P_i to induce apoptosis. Other factors to consider in this matter may include pH of P_i used, concentration of Ca^{2+} in the media, and stage of cell differentiation (Beck, 2003; Mansfield et al., 2003). The role for Ca^{2+} ions in conjunction with P_i for signaling events has been discussed by George (Narayanan et al., 2003) and Shapiro (Adams et al., 2001; Mansfield et al., 2003), and is a facet that we did not examine here.

b. Effect of phosphate signaling on tooth cells

In light of the dramatic tooth root phenotypes resulting from disruption of local Pi metabolism regulators, we have undertaken studies to understand the role of P_i in tooth root development and its potential use in therapeutic regenerative applications. *In vitro* studies employed an immortalized mouse cementoblast cell line, OCCM-30 (D'Errico et al., 2000), to identify downstream events resulting from exposure of cementoblasts to elevated P_i. Exposure of cementoblasts to 5 mM P_i regulated several genes known to be involved in formation of cementum (as well as other mineralized tissues). Some of the most significantly regulated genes included members of the SIBLING family. *Opn* and *Dmp1* were both strongly up-regulated by P_i, while *Bsp* was down-regulated (Foster et al., 2005; Foster et al., 2006b). The SIBLING family includes multiple genes having in common chromosomal location (4q21 in mice), gene structure, and post-translational modifications, and all were initially identified by their association with mineralized tissues (Fisher and Fedarko, 2003; Qin et al., 2004). Originally described as extracellular matrix proteins and regulators of mineralization, SIBLING family proteins have since been linked with several

additional functions. In particular, BSP, OPN, and DMP1 partner with and activate *in vitro* matrix metalloproteinases (MMP)-2, -3, and -9, respectively, suggesting an additional tissue turnover function for these SIBLINGs (Fedarko et al., 2004). All three MMPs are regulated by P_i in these cementoblasts *in vitro* (Foster et al., 2006a). Interestingly, *Opn* and *Dmp1*, up-regulated by P_i *in vitro*, have been reported to be up-regulated in the hyperphosphatemic *Fgf-23* KO mice. In OCCM-30 cementoblasts, P_i also regulated gene expression of *Ank*, *Pc-1* (*Enpp1*), *Pit-1*, and *Tnap/Akp2*, suggesting that the cementoblasts were sensitive and responsive to P_i levels.

Further studies of the effect of P_i signaling on cementoblasts have focused on cellular response mechanisms. Microarray time course studies identified early response elements to elevated P_i in OCCM-30 cells, including transcription factors (TF) and *Wnt* pathway factors (Rutherford et al., 2006) that are the subjects of current research. Intriguingly, cementoblasts exposed to P_i up-regulated the zinc TF *Egr1 (Egr1* was the TF found to be up-regulated by *FGF23* in the presence of *Klotho)*, known to be expressed in teeth, by 1 hr, and Yasutake et al. (2006) and Yu et al. (2006) have shown FGF23 up-regulates *Egr1* mRNA in kidney cells and modulates expression of *Klotho*. Further, the dramatic response of *Opn* to elevated P_i has been studied by employing murine *Opn* promoter constructs, revealing a necessity for the glucocorticoid response element for the *Opn* response (Fatherazi et al., 2008; Fatherazi et al., 2006).

Studies of direct effects of P_i on other tooth mineralized tissue cells are relatively uncommon. Lundquist et al. characterized Ca^{2+} and P_i uptake in rat pulp MRPC-1 cells as they were stimulated to differentiate to an odontoblast-like phenotype (Lundquist, 2002; Lundquist et al., 2002). While Ca^{2+} uptake was at a maximum during proliferation, P_i uptake increased four-fold following 4 mM P_i addition and just prior to mineral nodule formation. Although it was hypothesized that Na-P_i transport was increased in order to translocate P_i ions for matrix mineralization, it is possible that P_i may have modulated Na-P_i uptake and gene expression in relation to differentiation and mineralization in these cells.

c. Effect of phosphate on osteoclasts

Alveolar bone has been described as featuring the highest degree of turnover of bone turnover in the human body. At sites of mineralized tissue resorption, active osteoclasts are likely exposed to high concentrations of P_i (and Ca^{2+}) as HAP is dissolved in the acidic environment under the osteoclast ruffled border. The mechanism of P_i transport in osteoclasts has also been a topic of interest due to the presumably large concentrations of P_i generated under the ruffled border during active resorption. Gupta et al. (1997) reported the presence of a Na-dependent P_i transporter in rabbit osteoclasts. The transporter was localized in intracellular vesicles in undifferentiated osteoclasts, but upon polarization was found in the basolateral membrane (not in contact with bone) opposite the H-ATPase. This suggests that this Na-P_i transporter is not the primary element responsible for removing P_i after bone mineral dissolution. An acid-dependent high capacity P_i transport system was later identified in activated mouse RAW 264.7 osteoclasts (Ito et al., 2007; Ito et al., 2005). The unique P_i uptake was found to be regulated by intracellular Ca^{2+} and efflux was stimulated by P_i or its analogs.

Effects of alterations in extracellular P_i have also been studied for possible effects on osteoclasts and osteoclastogenesis. Inhibition of osteoclast formation and activity by elevated P_i has been observed *in vitro* using bone marrow derived precursors and cell lines from several species (Kanatani et al., 2003; Takeyama et al., 2001; Yates et al., 1991). One study suggested that differentiating osteoblasts may specifically down-regulate osteoclastogenesis via increased TNAP and subsequent increase in local P_i, however no mechanistic data were presented to support this and other factors could have been responsible for the *in vitro* observations (Takeyama et al., 2001). Doi et al. (1999), in studies of osteoclastic ability to resorb various types of calcium phosphate substrates, hypothesized that increases of Ca^{2+} and P_i in the culture media may have down-regulated osteoclast activity, though observed increases were relatively small (0.5 to a maximum of about 2.5 mM P_i) and no mechanism for P_i regulation of the rabbit osteoclasts was discussed. In further *in vitro* studies on human and mouse osteoclast precursors, it was demonstrated that increased P_i (1.5-4.5 mM) inhibits osteoclastic differentiation in the presence of RANKL and M-CSF, possibly by disrupting DNA binding of critical transcription factors AP-1 and NF-κB (Mozar et al., 2008). In addition to findings that high P_i inhibits osteoclastogenesis and function, studies on mice under hypophosphatemic conditions have suggested that low P_i may also inhibit osteoclasts. By employing both *Hyp* mice and mice maintained on a low P_i diet (low, 0.02%; control, 0.6%), Hayashibara et al. (2007) demonstrated fewer osteoclasts by histomorphometric measurements. By putting *Hyp* mice on a high P_i diet (3.0 %), the authors demonstrated partial correction in numbers of osteoclasts as well as bone mineralization. The authors tested whether osteoclast formation *in vitro* paralleled observations in hypophosphatemic mice. Osteoclastogenesis from WT marrow cells was decreased under both low (0.5, 0.75 mM) and high (1.5-2.5 mM) P_i conditions, while osteoclastogenesis was not impaired in *Hyp* mouse marrow cells under normal P_i conditions (1 mM). In contrast to studies already discussed (Mozar et al., 2008), P_i levels (low and high) did not significantly affect osteoclastogenesis when directly induced by RANKL and M-CSF addition. Therefore, to date there has been evidence of P_i effects on osteoclasts and their precursors, including in hypo- and hyperphosphatemic mouse models, however the extent to which this impacts mineralized tissues, especially those of the tooth, remains to be seen.

d. Pyrophosphate (PP_i) and polyphosphates (polyP) as signaling molecules

More recently, a potential signaling role for PP_i distinct from that of P_i has been proposed. While data compiled to date are intriguing, it is difficult to conclusively show PP_i specifically is eliciting cellular effects when the possibility for hydrolysis to P_i is so likely (in the presence or absence of TNAP and other enzymes). Moreover, the hypothesis for PP_i signaling also currently lacks an identified channel or cell surface sensor to convey PP_i concentration information to the cell interior to effect expression changes. Nonetheless, further study is warranted to clarify initial data in this area of study.

PP_i is a profound inhibitor of HAP mineral formation, but *in vivo* and *in vitro* studies have been cited for evidence that PP_i additionally regulates cell function. *Opn* levels were altered in primary calvarial osteoblasts from both *Ank* mutant and *Akp2* null mice (Harmey et al., 2004). Loss of *ANK* function decreased *Opn*, while loss of *TNAP* increased *Opn*. Crossing

these two mutants results in partial normalization of *Opn* expression, in addition to mineralization. Addition of μM amounts of exogenous PP_i was reported to increase Opn mRNA in calvarial osteoblasts. The authors hypothesized the effect must be from PP_i rather than a P_i hydrolysis product because the effective dose was in the μM range. Regulation of *Ank* and *Enpp1* by PP_i was interpreted as evidence for a concerted feedback loop in which *ANK* and *ENPP1/PC-1* regulate *OPN* expression by modulating PP_i levels, and thus control mineralization. While this model is compelling, it is difficult to separate out P_i and PP_i effects, and unclear why *Opn* should be lower in *Ank* mutant mice where intracellular PP_i is elevated.

MC3T3-E1 cells were shown to respond to added μM-mM levels of PP_i by increasing *Opn* expression in an ERK1/2 dependent response (Addison et al., 2007). This regulation of gene expression was not sensitive to foscarnet or levamisol (a *TNAP* inhibitor), again suggesting a role for PP_i distinct from P_i. PP_i also successfully blocked the ability of *TNAP* to release P_i from β-glycerophosphate (βGP) and *OPN* protein, *in vitro*. The authors thus suggest PP_i may serve as a negative regulator of mineralization by multiple mechanisms, including direct binding to mineral, increase of *OPN* expression, and inhibition of *TNAP* activity.

Polyphosphates (polyP) are long polymers of Pi that have been found in cells and plasma that are suggested to have myriad functions including energy storage, nucleotide regulator, and P_i donor for kinases. Like PP_i, polyP may also serve as a regulator of calcification. PolyP and exopolyphosphatase activity was detected in primary human osteoblasts in relatively high concentrations compared to non-mineralizing cell types (Leyhausen et al., 1998). In studies with MC3T3 E1 pre-osteoblasts, polyP has been cited as contributing to an osteoblast-like phenotype (Hacchou et al., 2007; Kawazoe et al., 2004), and polyP effects are being further explored for therapeutic potential (Kawazoe et al., 2008; Yamaoka et al., 2008). It remains unclear how much of the polyP effect may be attributed to liberation of P_i and PP_i from the longer polymer chains.

V. Phosphate-based drugs and materials

The rationale for using P_i directly or regulating interstitial P_i for the therapeutic regeneration of mineralized tissues is partially informed by the extant phosphate-based therapeutics. These include the bisphosphonates, calcium phosphate bone cements, and bioactive glass. Of these, the bone cements and bioactive glasses have utility in the promotion of tissue repair, particularly bone. These compounds appear to function as scaffolds supporting tissue formation, i.e., osteoconductive rather than osteoinductive, in the case of bone. Broader utility would be obtained if a phosphate-based therapeutic device could provide a signal which initiated a regeneration cascade. Today this vision is an as yet unrealized goal.

a. Bisphosphonates

Current treatments for osteoporosis, metastatic disease in bone, Paget's disease of bone, and hypercalcemia of malignancy frequently include bisphosphonates, originally conceived of as synthetic analogs of PP_i. The amino bisphosphonates, e.g., zolendronate, pamidronate, and alendronate, are currently preferred due to faster onset, greater potency, and longer duration

of action. The amino bisphosphonates are also reported to have anti-neoplastic activity (Santini et al., 2003).

Bisphosphonates (BPs) are synthetic analogues of PP_i in which the pyrophosphate (P-O-P) bond has been replaced by a phosphoether (P-C-P) bond, essentially making a non-hydrolyzable surrogate for PP_i. BPs were observed to strongly bind hydroxyapatite of bone, and had the effect of reducing bone resorption by osteoclasts. Early generation BPs were simple in structure, resembling PP_i with simple side-chains at the R_2 position which tuned their pharmacological properties. Later generation BPs incorporated bulkier side chains often containing nitrogen (known as amino-BPs), exhibiting much greater potency and persistence in inhibiting osteoclastic resorption (Rogers, 2003).

While BPs potently block bone resorption, the molecular mechanism of action was only elucidated recently. Convincing X-ray diffraction data have now demonstrated amino-BPs form an inhibitory complex with the enzyme farnesyl pyrophosphate synthase (FPPS) (Kavanagh et al., 2006; Rondeau et al., 2006). FPPS catalyzes a key step in the mevalonate synthesis pathway, and is also part of the larger HMG CoA reductase pathway for steroid and cholesterol synthesis. The mevalonate synthesis pathway provides isoprenoids for protein prenylation, essential for proper function of small GTPases such as Ras and Raf. These GTPases play important roles in cellular functions such as intracellular signaling, and intra/extracellular trafficking. By homing to mineralized surfaces, BPs become concentrated in osteoclasts during active resorption and are thought to severely impair osteoclast function.

One of the earliest clinical applications of BPs was to use etidronate as an inhibitor of calcification in fibrodysplasia ossificans progressiva and in patients who had total hip replacement to prevent heterotopic ossification (Russell, 2007). Due to their high affinity to bone mineral, especially at locations of increased bone turnover rate, bisphosphonates are also used as agents for “bone scanning” to detect bone metastasis and other bone lesions (Francis and Fogelman, 1987). However, the most extensive clinical applications of BPs relate to their antiresorption property.

Paget’s disease has been treated with BPs for many years (Devogelaer, 2000). A dose-dependent inhibition of bone resorption by using BPs was observed in human (Delmas and Meunier, 1997). Pamidronate infusion was used initially and later replaced by a new and more potent zoledronate with even more profound suppression of disease activity (Siris et al., 2006).

The use of BPs is popular in oncology since many human cancers are associated with hypercalcemia and/or bone destruction. The goals of such treatments are to prevent skeletal complications, palliate pain, and maintain quality of life (Russell, 2007). BP treatment (e.g., intravenous administration of ZA) has been demonstrated to be effective in multiple myeloma (Sirohi and Powles, 2004) and in patients with bone metastases from breast cancer (Coleman, 2005; Pavlakis et al., 2005) and prostate cancer (Parker, 2005).

The other successful clinical application for BPs is treatment of osteoporosis, a major public health problem (Rodan and Reszka, 2003). BPs are one of the major classes of drugs in treating postmenopausal and other forms of osteoporosis at present. Etidronate was the first

BP used in osteoporosis treatment (Storm et al., 1990; Watts et al., 1990). Other BPs, including alendronate and risedronate, were shown to be effective in reducing fracture rates in osteoporosis patients by Meta-analysis (Boonen et al., 2005). To increase patient compliance to the therapy, recent research has been focused on improving formulations to reduce administration frequency, from a once-daily (alendronate, risedronate), to a once-weekly (alendronate, risedronate), to a once-monthly (ibandronate) tablet (Chesnut, 2006; Reginster et al., 2006). Now with new and more potent BPs and new routes of administration (IV), the treatment could be done even once-quarterly (ibandronate) (Croom and Scott, 2006) or once-yearly (ZA) (Reid et al., 2002).

Recently, bisphosphonate treatment of bone neoplasia has been associated with osteonecrosis of the jaws (ONJ) (Marx et al., 2005; Migliorati et al., 2005; Ruggiero et al., 2004; Woo et al., 2005). ONJ is relatively rare with an apparently increasing incidence rate which presents as a serious clinical complication to routine oral/dental procedures such as tooth extraction. Jaw bones are affected primarily, and various reasons for jaw selectivity have been proposed. Hypotheses include impaired healing of soft and hard tissues after trauma or periodontal (alveolar) procedures [as reviewed in (Bertoldo et al., 2007; Woo et al., 2006)], accumulated microcracks in jaw bones (Li et al., 2001; Mashiba et al., 2000; Mashiba et al., 2001), changes in vascularity in jaw bones (Wood et al., 2002), and association with infection (Marx et al., 2005). The exact reason for this selective and severe effect of BPs on jaw bones remains unknown and highlights the phosphate sensitivity of the dental-oral-craniofacial complex.

b. Bioceramics

Calcium phosphate bioceramics are salts of orthophosphoric acid and constitute a wide group of compounds, including dicalcium phosphate dihydrate (DCPD), octacalcium phosphate (OCP), tricalcium phosphate (TCP), and HAP (Barrere et al., 2006). Each calcium phosphate bioceramic has a different chemical composition and crystal structure, resulting in specific physicochemical properties. These calcium phosphate bioceramics could be either of natural origin (freeze-dried bone or derived coral HA), or synthetic origin (obtained after aqueous precipitation or after sintering) (Barrere et al., 2006). Due to their superior osteoconductivity, calcium phosphate bioceramics have been successfully used in cranio-maxillofacial, dental, and orthopedic surgery as bone fillers (cement or granules), coating materials on metallic orthopedic and dental implants (de Jonge et al., 2008; Le Guehennec et al., 2007), or scaffold materials for bone tissue regeneration (Barrere et al., 2006; El-Ghannam, 2005; Matsumoto et al., 2007; Warren et al., 2004).

To date, HAP has been the most widely used bioceramic material for bone substitution (Hak, 2007; Kokubo et al., 2003; Matsumoto et al., 2007; Warren et al., 2004). HAP can be fabricated to low porosity blocks and granules by sintering, or to high porosity structure by either a low heat alkaline extraction process producing xenograft (Caffesse et al., 2002), or by hydrothermal conversion of coral (Roy and Linnehan, 1974). The high porosity HAP structures have been cited for their advantages of favorable bone ingrowth and increased resorption in comparison to low porosity counterparts (Garg, 1999). However, since strength is inversely related to porosity, highly porous HAP structures are only suitable for non-load

bearing conditions (Garg, 1999). An analogue of HAP bioceramic being used as a bone graft material is biphasic calcium phosphate (BCP), which is a mixture of HAP and TCP. Studies have established BCP as a viable graft material for use in treating periodontal osseous defects (Garg, 1999; Nery et al., 1992).

Porous HAP and BCP have been studied as a scaffold material for use in guided tissue regeneration (to exclude epithelial down-growth). These materials are desirable for scaffolds for their effectiveness in promoting cell/bone ingrowth and integration, and their bioresorbable properties. The use of collagen and other resorbable barrier membranes in conjunction with xenografts or porous BCP has demonstrated success in periodontal tissue repair and regeneration (Caffesse et al., 2002; Reynolds et al., 2003; Wang and Cooke, 2005; Zafiropoulos et al., 2007).

Calcium phosphate bioceramics are too brittle to be used as direct load bearing prostheses. Therefore, they are frequently used as coating materials on metal prosthesis surfaces to provide biological compatibility (de Jonge et al., 2008). Calcium phosphate coatings for metal implants were introduced about 2 decades ago due to their osteoconductive property (de Groot et al., 1987; Geesink et al., 1987). These coatings are demonstrated to increase bone-to-implant contact (Barrere et al., 2003a; Barrere et al., 2003b; Leeuwenburgh et al., 2006), to improve the implant fixation (Soballe et al., 2003), and to facilitate the bridging of small gaps between implant and surrounding bone (Soballe et al., 1991).

Another class of bioceramics is bioactive glass consisting of calcium oxide, sodium oxide, and phosphate mixed in a silicon oxide matrix. Bioactive glass is not porous and therefore is not susceptible to any tissue ingrowth. However, its highly bioactive surface is favorable for cell and tissue attachment. When introduced to the wound site, the glass surface is readily colonized by osteogenic cells, then by a collagen network attaching the particulate to the surrounding tissue, enabling further bone growth and attachment. Bioactive glass has been reported to be of value in treating intrabony periodontal defects when applied alone, or in combination with resorbable membranes or enamel matrix proteins (EMD) (Garg, 1999; Kuru et al., 2006; Mengel et al., 2006; Mengel et al., 2003; Miliauskaite et al., 2007; Park et al., 2001).

VI. Discussion and future directions (Figure 3)

Currently, most regenerative therapeutic regimens for mineralized tissues employ some variation of the principles of tissue engineering as articulated by Langer and Vacanti (Langer and Vacanti, 1993; Lanza et al., 2007), e.g., local implantation of combinations of biological factors (cells, proteins) with engineered polymer-based scaffolds (e.g., poly-esters). Variations on this basic approach can range from employing scaffolds without added biological factors to cells, and/or proteins added to engineered scaffolds. Whereas stem cell therapy may restore a partial loss of organ function, the initial goal of tissue engineering is complete conversion of the engineered therapeutic combination to new tissue(s) or organs, e.g., bone (Herford and Boyne, 2008). The long-term goal is for regenerated tissues or organs to redress acquired or congenital deficiencies and become fully responsive to all the normal relevant physiological homeostatic mechanisms, i.e., become fully functional within

the context of complete organism. Predictably, the development, structural and functional maturation, and maintenance of such tissue/organs would be adversely affected by malfunctions in key homeostatic regulatory systems. A case in point is the abnormalities in tooth development associated with alterations in local or serum P_i levels as described above. Hence, understanding of such systems is key to diagnosing, and if necessary, clinically managing any such malfunctioning systems to attempt to insure optimal outcomes to regeneration therapy.

Dental tissues and teeth form through a complex series of temporally and spatially regulated reciprocal interactions between oral epithelium and neural crest ectomesenchyme (Thesleff, 2003; Thesleff and Sharpe, 1997). Such interactions require like-cells to aggregate and join to interact with the other cell types for tissue formation to proceed normally. Gene expression studies of developing teeth, principally in mice, using normal and genetically altered animals has produced a large database (http://bite-it.helsinki.fi/) of genes implicated in the regulation of these processes. Members of major developmental signaling pathways, including FGF, sonic hedgehog (Shh), BMP, and Wnt signaling networks are involved at various and multiple stages (Cobourne and Sharpe, 2003; Dassule et al., 2000; Jarvinen et al., 2006; Jernvall and Thesleff, 2000; Laurikkala et al., 2003). The molecular basis of tooth agenesis has recently been reviewed (Matalova et al., 2008), identifying several genes, the loss of which can lead to agenesis of one or more teeth.

Strategies for whole tooth regeneration commonly start by separating tooth buds into epithelial and mesenchymal tissue components at the correct stage of development. Dental epithelium may be recombined with a competent dental mesenchyme (or mesenchymal substitute) prior to culture or implantation (Ohazama et al., 2004), the epithelial and mesenchymal tissues disaggregated into cell suspensions, mixed, added to engineered scaffolds and these constructs implanted *in vivo* (Young et al., 2002). Alternately, the separate epithelial and mesenchymal layers may be dissociated into high-density cell suspensions and placed in close approximation, permitting like-cells to reaggregate and the two cell types to interact and organize themselves (Nakao et al., 2007). Such recombined tooth buds have the potential to develop into structures resembling whole teeth. These data reveal that whole tooth regeneration will likely require cells, competent by nature or by manipulation, to interact in order to initiate the development cascade. It seems highly unlikely that manipulation of a single factor will provide sufficient signal to initiate whole tooth regeneration. The next challenge will be to produce a fully functioning tooth using readily available substitutes for competent dental epithelia and ectomesenchyme. Ultimately, to have real world applications, the bioengineered tooth will need to be “tunable” for characteristics of tooth-type (molar, incisor, etc.), size, shape, color, and must be integrated in its functional location.

To date, among the dental tissues, the periodontium comprising the cementum, PDL, and bone, has received the most effort toward developing regenerative therapy. Currently, partially characterized mixtures of enamel proteins, absent a clear developmentally based mechanism, are commercially available for regeneration of the periodontium (Esposito et al., 2005; Gestrelius et al., 2000; Giannobile and Somerman, 2003). Platelet-derived growth factor (PDGF), a mesenchymal cell mitogen and angiogenesis factor, combined with a

calcium phosphate based carrier is also commercially available (Hollinger et al., 2008). Such first-generation products provide the “proof-of-principle” of dental tissue regeneration and inform refined subsequent efforts.

Cementum, based on the data reviewed above, appears to be particularly sensitive to local P_i concentration. TNAP deficiency results in increased local concentrations of PP_i and cementum aplasia or severe hypoplasia. Mice with loss of function in *ANK* develop low local extracellular PP_i, resulting in hypercementosis while other tissues apparently remain unaffected (Figure 2). *In vitro* data reveal that extracellular P_i can signal changes in gene expression in osteoblasts (Adams et al., 2001; Beck, 2003) and cementoblasts (Foster et al., 2006b; Rutherford et al., 2006), modulating cell phenotype. Taken together, these findings suggest that therapeutic regulation of extracellular P_i may have utility in the regeneration of the periodontium (Figure 3).

This idea immediately raises a number of practical questions. What is the optimum extracellular *in vivo* P_i concentration? What is the best method to achieve this concentration of extracellular P_i? How long must that concentration be sustained? Initially, *in vitro* washout experiments to determine the minimum time necessary for elevated P_i need to be undertaken. How could appropriate levels of P_i be maintained in the periodontal lesion for sufficient time to affect cells? Biodegradable thermoset or chemoset hydrogels containing the appropriate P_i concentration or active TNAP might be useful. Do sufficient populations of cementoblasts or their precursors exist in diseased periodontal tissues to enable cementogenesis? Would cementoblasts need to be included in the P_i/hydrogel implant? Would this signal be sufficient to induce regeneration of the PDL and bone to complete the periodontium? Would therapeutic stimulation of angiogenesis and neovascularization be necessary?

A concerted research program dedicated to these and other questions may bring a new facet to the fascinating and diverse biology of P_i. These types of studies may also further understanding of cementum and dentin formation and inform potential regenerative therapies for treatment of a broader group of diseases, including not only dental diseases, but also hypophosphatemic conditions, ectopic calcification, and osteoporosis.

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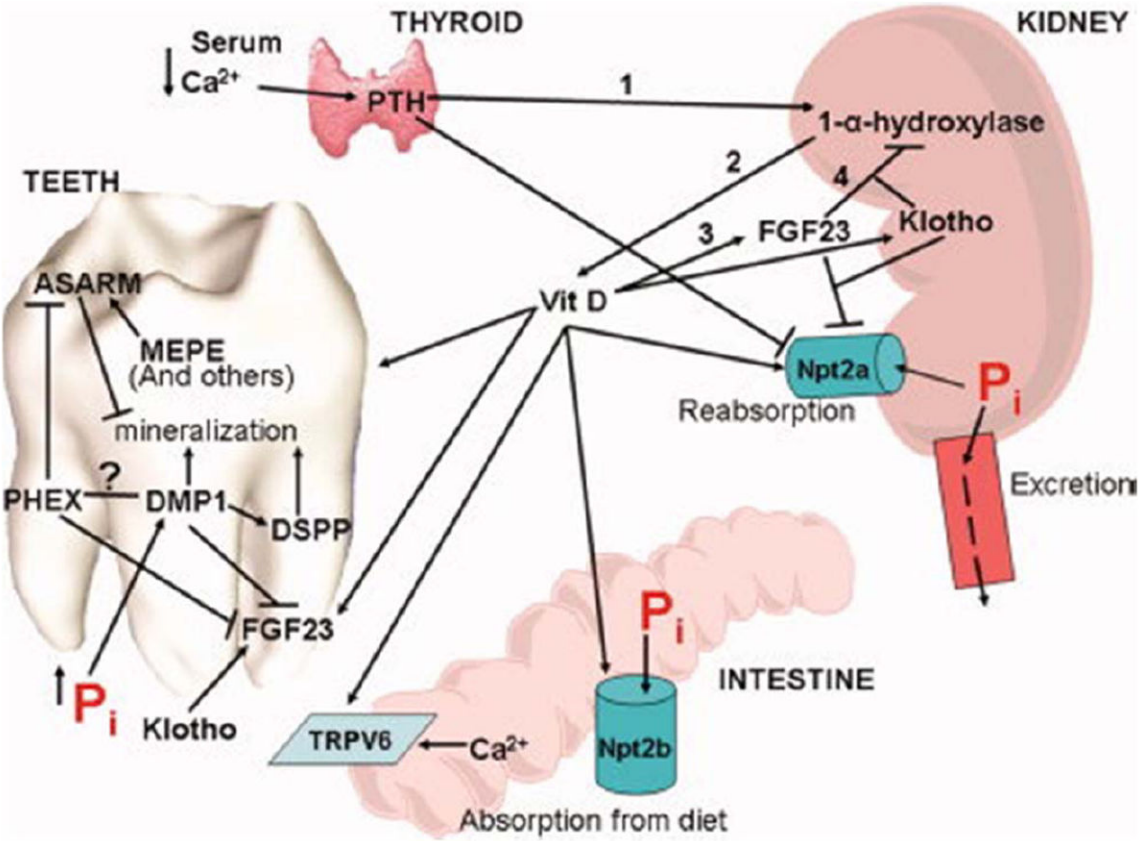

Figure 1. Serum calcium (Ca^{2+}) and phosphate (P_i) levels regulate gene expression in the parathyroid-kidney-intestine- tooth axis

Decreases in serum Ca^{2+} induce the calcium sensing receptor in the parathyroid glands, embedded in the thyroid gland, to secrete PTH into the bloodstream. PTH stimulates the activity of 1-α-hydroxylase in the kidney which catalyzes the formation of the active 1,25 dihydroxy form of Vit D (line 1). PTH potently stimulates osteoclast activity to release Ca^{2+} from bone. Active Vit D increases intestinal absorption of Ca^{2+} via the TRPV6 Ca^{2+} channel and of P_i through the Npt2b ion channel. Renal reabsorption of P_i is increased by Vit D through increased Npt2c activity (line 2). PTH acts to reduce P_i reabsorbtion by down-regulation of Npt2a, while the induced increase in serum Ca^{2+} reduces secretion of PTH. Vit D has effects on tooth mineralization as evidenced by dentin defects under Vit D deficient states. Vit D acts to increase the expression of FGF23 in bone and the FGF23 receptor binding partner Klotho in the kidney (line 3). FGF23 reduces the activity of 1-α-hydroxylase, decreasing the formation of active Vit D (line 4), closing the loop began by PTH demonstrated by the lines numbered 1-4. SIBLING protein expression in bones and teeth are affected by P_i levels. Mutations in Phex induce expression of FGF23 in osteocytes and ameloblasts and odontoblasts, with concurrent increases in MEPE expression and ASARM formation. Loss of function mutations in Dmp1 induce an increase in FGF23 and decrease in DSPP expression levels. The gene expression changes in these mutations result in decreased mineralization in bones and teeth. The hyperphosphatemia in the FGF23 loss of function mutant may induce the observed increased expression of DMP1 in bone and tooth in a compensatory attempt to increase mineralization.

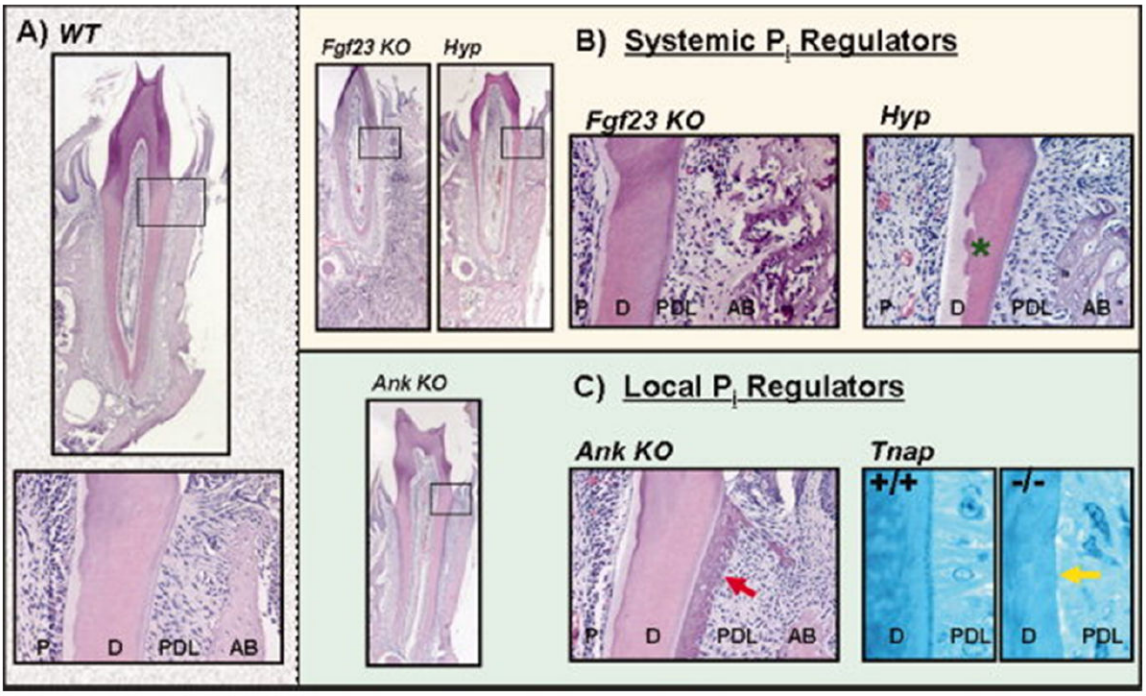

Figure 2. Phosphate disregulation and tooth root tissues

Mouse first mandibular molars here serve as models for effects of P_i disregulation on tooth roots and supporting tissues. All buccal-lingual sections shown are from 45 dpc mice, except *Tnap* images, which are from mice age 61 dpc. (A) Wild-type (WT) mouse shown for comparison. Top: Low magnification, Bottom: High magnification of the cementum-enamel junction (CEJ). (B) Systemic P_i regulation: Ablation of *Fgf23* resulted in narrowed PDL space and a marked increase in alveolar bone. Alveolar bone exhibited high cell density and appeared to be primarily composed of osteoid. *Phex* mutations (*Hyp* mouse) resulted in enlarged pulp chambers, widened predentin, reduction in mineralized dentin, interglobular dentin (green star), and an increase in alveolar bone with pockets of osteoid. (C) Local P_i regulation: Loss of *Ank* resulted in dramatically thicker cementum (red arrow) while dentin, PDL, and alveolar bone appeared normal. Alternately, *Tnap* mutations (-/-) resulted in a failure to form cementum (yellow arrow). *Tnap* images courtesy of Dr. Wouter Beertsen. P=pulp, D=dentin, PDL=periodontal ligament, AB=alveolar bone.

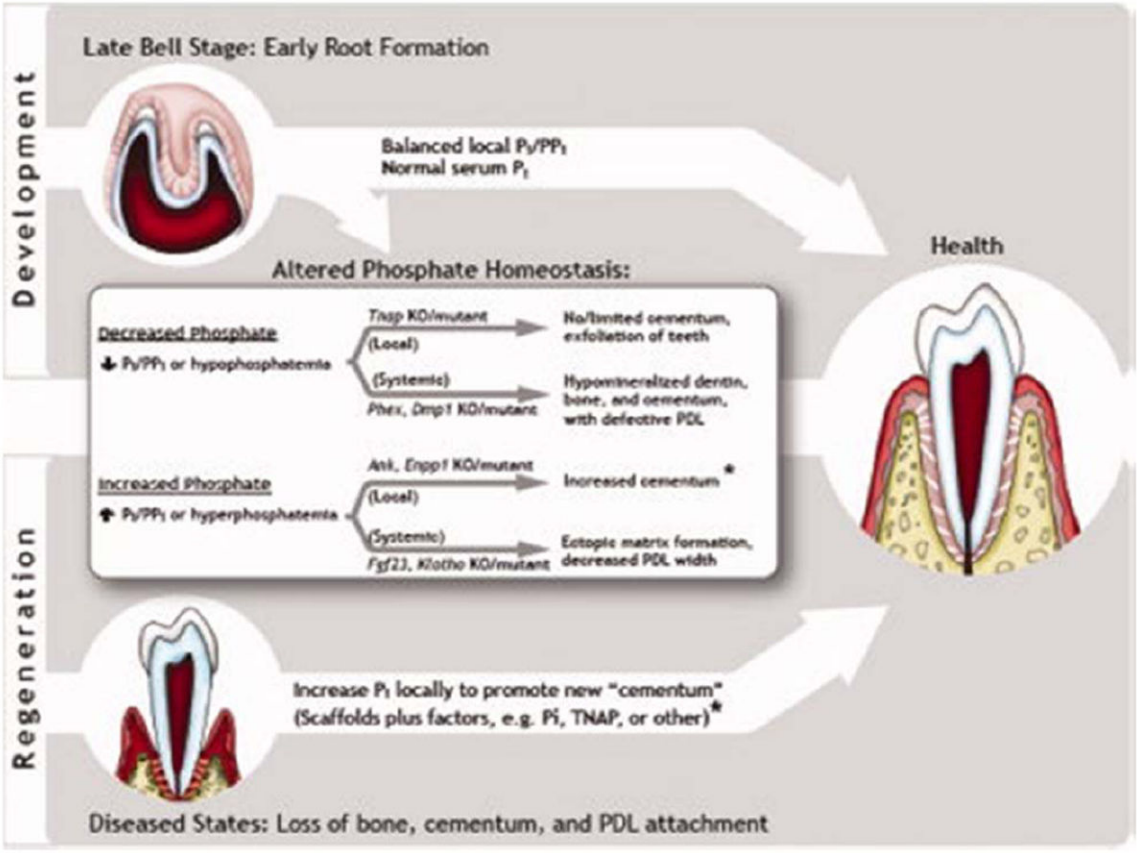

Figure 3. Phosphate: Applying knowledge gained from developmental studies toward designing therapies to regenerate periodontal tissues

TOP PANEL (Development): As discussed throughout this chapter, an appropriate balance of P_i/PP_i locally, coupled with physiological serum levels of P_i, are required for development of teeth and supporting tissues.

MIDDLE BOX: Alterations in P_i metabolism, summarized in this box, result in defective tooth development

BOTTOM PANEL (Regeneration): Potential approaches for regenerating oral tissues include increasing levels of P_i at local sites.

* Note: Ank/PC-1 KO/mutant teeth with increased P_i/PP_i suggest that increasing levels of P_i locally during early phases of wound healing may promote new cementum formation and subsequently promote new ligament attachment and new bone formation.

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Table 1

"Classical" regulators of calcium (Ca^{2+}) and phosphate (P_i) homeostasis.

Factor	Expression Pattern Cells/Tissues	Known/Putative Function	Murine KO/Mutation Models	Phenotype: General	Phenotype: Teeth
PTH: Parathyroid hormone	Secretion from parathyroid gland based on serum Ca^{2+} levels.	Secreted into circulation in response to low serum Ca^{2+} in order to increase osteoclast activity, releasing Ca^{2+} from bone stores. Acts indirectly by increasing RANKL expression in osteoblasts to stimulate osteoclasts in turn.	*PTH* receptor null	Mineralized tissues: Enhanced mineralization of chondrocytes during long bone formation	Alveolar bone: Osteoclast activity reduced, delayed eruption of teeth
	Cells with PTH receptors include: Osteoblasts, odontoblasts, cementoblasts	Acts in kidney to stimulate formation of active Vit D (see below)			
		Clinically, PTH has anabolic activity when delivered intermittently at low dose			
Vit D: 1,25-dihyrdoxy vitamin D_3	Kidney	Stimulates, indirectly, increased Ca^{2+} and P_i absorption through intestine, and increased Ca^{2+} and P_i reabsorption in kidneys. Stimulates bone marrow stromal cell RANKL expression to free Ca^{2+} from bone matrix via osteoclast activity.	Nutritional deficiency	Mineralized tissues: Rickets/osteomalacia, hypocalcemia, hyperparathyroidism	Reduced expression of amelogenin and enamelin, disrupted enamel formation. Dentin mineralization defects including decreases in *OCN* and *DPP*
		Stimulates osteoblast development by regulating expression of non-collagenous marker proteins	VDR null	Mineralized tissues: Rickets/osteomalacia, hypocalcemia, hyperparathyroidism	Thin dentin, widened and irregular predentin, increased pulp chamber
			25-hydroxyvitamin D-1-α-hydroxylase null	Mineralized tissues: Same as above, also secondary hyperparathyroidism, hypophosphatemia	Not reported

Table 2

Systemic/humoral regulators of phosphate (P_i) homeostasis: Phosphatonins, phosphatonin-like factors, and co-factors

Factor	Expression Pattern Cells/Tissues	Known/Putative Function	Murine KO/Mutation Models	Phenotype:	
				General	Teeth
FGF23: Fibroblast growth factor 23	Highest expression in bone and highly expressed during active bone remodeling. Noted in osteocytes, osteoblasts, cementoblasts, odontoblasts, and low levels in chondrocytes, cementocytes, and osteoclasts	Regulates P_i homeostasis by controlling renal reabsorption via modulation of expression of P_i co-transporters (i.e., NPT2a, NPT2c). Inhibits expression of α-hydroxylase required for formation of Vit D. DMP1 may modulate *Fgf23* expression. Vit D regulates Fgf23 expression.	*Fgf23* KO	General: Premature aging-like features, e.g., shortened lifespan, hypogonadism, emphysema, organ atrophy	Alveolar bone: Accumulation of unmineralized osteoid associated with ankylosis, apoptotic cells
	Lower levels in fetal vs. postnatal and adult tissues	Reports indicate that FGF23 signals through FGF type I receptors and requires Klotho binding for activation. Low FGF23 levels in fetal tissues, with high levels in young adult and adult tissues, suggests a role in mineral homeostasis vs. skeletal development.		Mineralized Tissues: Disorganized growth plate, accumulation of unmineralized osteoid, suppressed bone turnover, ectopic calcification, decreased mass & volume in long bones	Enamel: Defective in continually erupting rodent incisors, cyst-like appearance and lack of polarity to ameloblasts
		Altered circulating levels used to diagnose P_i-wasting diseases.			Pulp: Narrow chamber, ectopic matrix formation Cementum: Normal appearance PDL: Disorganized, and decreased width Dentin: Normal appearance
Klotho	Expressed predominantly in kidney, also in choroid plexus, parathyroid gland, and other tissues.	Single-pass transmembrane domain and short cytoplasmic domain having b-glucuronidase activity	*Klotho* KO	General: Similar to *Fgf23* KO mouse, e.g., rapid aging syndrome including shortened lifespan, skin and muscle atrophy, osteoporosis, and emphysema.	Incisors: Ectopic calcification, altered odontoblast morphology (with defects in predentin and dentin)
	Soluble form in circulation	Extracellular domain: Shed and secreted in blood, considered a cofactor of FGF-23 signaling and suppressor of insulin/IGF-1 signaling.		Mineralized tissues: Accelerated osteoblast aging, ectopic calcification	Molars: Ankylosis with alveolar bone

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Factor	Expression Pattern Cells/Tissues	Known/Putative Function	Murine KO/Mutation Models	Phenotype:	
				General	Teeth
PHEX: Phosphate regulating gene with homology to endopeptidases on the X chromosome	Highest expression in osteoblasts, osteocytes, and odontoblasts	Interacts with (cleaves) small circulating factors outside of the kidney to control renal P_i homeostasis and mineralization (e.g., MEPE, DMP-1, and other SIBLINGs), releasing acidic serine and aspartic acid-rich MEPE-associated motif (ASARM) peptides ("minhibins"), potent inhibitors of mineralization Regulates expression *of Fgf-23* (may be indirect)	*Hyp* mutant mouse (3' deletion in *Phex* gene)	Growth retardation, osteomalacia, reduced mineralization in growth plate, reduced bone volume and osteoclasts in long bone	Alveolar bone: Hypomineralization, increased osteoid Dentin: Hypomineralization, interglobular dentin, widened predentin, irregular dentinal tubules Pulp chamber: Enlarged Enamel: Appears normal Cementum: Disrupted globular appearance at SEM level (appears normal by H&E)
DMP1: Dentin matrix protein 1	High levels in osteocytes, also found in odontoblasts, cementoblasts/cytes	Considered to be required for mineralization; may regulate odontoblast and osteoblast/cyte specific genes; in osteocytes, considered to have a role in protection during mechanical loading; processed to NH_2 and COOH fragments that are distributed differently in dentin and may have unique roles; suggested role as a transcription factor (regulates *Dspp*); may regulate *Fgf-23* expression	*Dmp1* KO	Increased osteoid, hypomineralized bone, defective osteocyte maturation, osteomalacia rickets, decreased bone mineral, increased crystal size	Dentin: Similar to *Hyp* mouse, e.g., hypomineralized with widened predentin PDL: Disorganized, with detachment from cementum

* No tooth phenotype reported for *Mepe* KO mouse; No animal models for loss of *FRP4, FGF7*.

Table 3

Local/cellular regulators of phosphate (P_i) and pyrophosphate (PP_i) metabolism.

Factor	Expression Pattern Cells/Tissues	Known/Putative Function	Murine KO/Mutations Models	Phenotype:	
				General	Teeth
TNAP: Tissue non-specific alkaline phosphatase (ALP, TNSALP, ALKP) Gene: *Akp2*	Expressed by diverse cells including osteoblasts, PDL cells, odontoblasts, follicle cells, cementoblasts, and chondrocytes, among others. Also present in matrix vesicles (MVs).	Marker of osteoblast differentiation, catalytic function in mineralization, cleaves the mineralization inhibitor, PP_i	*Tnap* mutation/KO	Hypophosphatasia: Increased local levels of PP_i, osteopenia	Cementum: Hypoplasia or aplasia with compromised PDL attachment, exfoliation of teeth
ANK: Progressive ankylosis gene/ protein. (Human homologue, ANKH)	Expressed by diverse cells including osteoblasts, PDL cells, odontoblasts, follicle cells, cementoblasts, and chondrocytes, among others.	Transporter/co-transporter of PP_i from intracellular to extracellular space; inhibits HAP deposition via increased extracellular PP_i	*Ank* mutation/KO	Decreased extracellular PP_i, ectopic calcification	Cementum: Hypercementosis (increase ~10 fold), unusually cellular cementum may result from rapid matrix accumulation
PC-1: Plasma cell membrane glycoprotein 1 (NPP1-nucleotide pyrophosphatase phosphodiesterase–1) Gene: *Enpp1*	Expressed by diverse cells including osteoblasts, PDL cells, odontoblasts, follicle cells, cementoblasts, and chondrocytes, among others. Also present in matrix vesicles (MVs).	Increases intra/extracellular and MV PP_i, inhibits HAP deposition via increased extracellular PP_i	*Enpp1* mutation (tiptoe walking, *ttw/ttw*)	Decreased extracellular PP_i, ectopic calcification	Cementum: Similar to *Ank* mutant and KO (e.g., hypercementosis)