The emerging role of phosphate in vascular calcification

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Vascular calcification is recognized as a major contributor to cardiovascular disease (CVD) in end stage renal disease (ESRD) patients. Susceptibility to vascular calcification is genetically determined and actively regulated by diverse inducers and inhibitors. One of these inducers, hyperphosphatemia, promotes vascular calcification and is a nontraditional risk factor for CVD mortality in ESRD patients. Vascular smooth muscle cells (SMCs) respond to elevated phosphate levels by undergoing an osteochondrogenic phenotype change and mineralizing their extracellular matrix through a mechanism requiring sodium-dependent phosphate cotransporters. Disease states and cytokines can increase expression of sodium-dependent phosphate cotransporters in SMCs, thereby increasing susceptibility to calcification even at phosphate concentrations that are in the normal range.

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DEFINITIONS AND CLINICAL SIGNIFICANCE OF VASCULAR CALCIFICATION

Calcification of the cardiovascular system is associated with a number of diseases including end stage renal disease (ESRD) and cardiovascular disease (CVD). Calcium phosphate deposition, mostly in the form of apatite, is the hallmark of vascular calcification and can occur in the blood vessels, myocardium and cardiac valves.[1] Calcium phosphate deposits are found in distinct layers of the blood vessel and are associated with specific pathologies. Intimal calcification is observed in atherosclerotic lesions,[2,3] whereas medial calcification is common to the arteriosclerosis observed with age and diabetes, and is the major form observed in ESRD.[4–6] In ESRD patients, both intimal and medial calcification occurs, but arterial medial calcification is by far the most prevalent.[7,8] Levels of calcium on the order of 20 μg/mg were routinely found in the calcified aortas of uremic patients at autopsy.[7] In valves, calcification is a defining feature of aortic valve stenosis, and occurs in both the leaflets and ring, predominantly at sites of inflammation and mechanical stress.[9]

Some pathological states of vascular calcification are well recognized for their life threatening potential. For example, calcification is recognized as a major mode of failure of native and bioprosthetic valves.[10,11] Furthermore, vascular calcification is responsible for calcific uremic arteriolopathy, a necrotizing skin condition observed in dialysis patients, associated with extremely high mortality rates.[12] Finally, generalized infantile arterial calcification, a genetic disease characterized by deficiencies in ENPP1, the enzyme that generates the potent inhibitor of calcium phosphate deposition, pyrophosphate, causes arterial calcification, fibrosis, and stenosis, which leads to premature death in afflicted neonates.[13]

In contrast, vascular calcifications associated with age, renal and vascular disease were considered benign for the better part of the last century. However, clinical studies in the past two decades have challenged this dogma. Calcification has been positively correlated with coronary atherosclerotic plaque burden[14,15] increased risk of myocardial infarction[16–18] and plaque instability.[19–21] Furthermore, in the Rotterdam Coronary Calcification Study, a large population-based study, graded associations between coronary calcification score and stroke were identified.[22] Likewise, coronary

calcium score was a strong predictor of incident coronary heart disease in four major racial and ethnic groups in the United States.[23] Similarly, medial arterial calcification is strongly correlated with coronary artery disease and future cardiovascular events including lower extremity amputation in type I diabetics[24,25] and is a strong prognostic marker of CVD mortality in ESRD patients.[26] These findings may be explained by evidence that arterial medial calcification in large arteries leads to increased stiffness and therefore decreased compliance of these vessels. These mechanical changes are associated with increased arterial pulse wave velocity and pulse pressure, and lead to impaired arterial distensibility, increased afterload favoring left ventricular hypertrophy, and compromised coronary perfusion.[27] Thus, both intimal and medial calcifications may contribute to the morbidity and mortality associated with cardiovascular disease, and are likely to be major contributors to the 10–100 fold increase in cardiovascular mortality risk observed in ESRD patients.[28] Indeed, both the National Kidney Foundation and American Heart Association have indicated that ESRD patients should be considered at the highest risk for cardiovascular disease (NFK K/DOQI clinical guidelines and Sarnak *et al.*[29]).

VASCULAR CALCIFICATION IS AN ACTIVELY REGULATED, GENETICALLY DETERMINED PROCESS

Growing evidence suggests that vascular calcification, like bone formation, is a highly regulated process, involving both inductive and inhibitory processes (El-Abbadi and Giachelli[30] for review). For example, apatite, bone-related noncollagenous proteins (osteocalcin, osteopontin, alkaline phosphatase, Runx2), and matrix vesicles have all been observed in calcified vascular lesions. Furthermore, outright cartilage and bone formation have also been documented.[2,9,31] Finally, cells derived from the arterial media, including smooth muscle cells (SMCs), adventitial fibroblasts, and pericytes, undergo osteochondrogenic differentiation and matrix mineralization under the appropriate conditions *in vitro*.[32–36] These studies suggest that cell-mediated processes tightly control procalcific and anticalcific mediators in the artery so that ectopic calcification is normally avoided. Under pathological conditions, this balance is upset and leads to ectopic mineralization.

Furthermore, susceptibility to vascular calcification appears to be genetically determined in people and in mice. For example, Table 1 lists human genetic syndromes and mouse mutations that include vascular calcification as part of the disorder, indicating that the affected genes normally regulate this process. Furthermore, a small fraction of ESRD patients enter dialysis without vascular calcification and remain calcification-free suggesting genetic protection against this process.[37] More recently, ESRD patients with ENPP1 121KQ genotype (decreased pyrophosphate-generating capacity) were found to have significantly higher coronary calcium score and pulse pressure than ENPP1 121KK genotype.[38] Likewise, using inbred and B × H recombinant inbred mouse strains, clear differences in the occurrence of coronary arterial medial calcification in response to an atherogenic diet was observed, with the most susceptible strains being DBA/2J and C3H/HeJ, whereas the least susceptible included C57Bl/6 and MRL/lpr.[39] Interestingly, susceptibility to atherosclerotic plaque formation and cartilaginous metaplasia, especially in ApoE-deficient mice, appeared to show the reverse trend, with C57Bl/6 showing much greater lesion formation and ectopic cartilage than C3H mice.[39–41] Furthermore, dystrophic cardiac calcification (DCC), an autosomal recessive complex trait characterized by calcium phosphate deposition in the myocardium following various injurious stimuli, is preferentially induced in the DCC susceptible C3H/HeJ and DBA/2J strains compared with DCC-resistant C57Bl/6 strain.[42,43] Indeed, recent studies have identified a quantitative trait locus (Dyscalc 1) contributing to the DCC phenotype to encode the ATP-binding cassette transporter, ABCC6.[44] Mutations in this gene in people are responsible for pseudoxanthoma elasticum (Table 1), a condition that includes enhanced vascular calcifications.[45] Of note, the substrate for ABCC6, which is expressed in liver, kidney and heart but not blood vessels, has not yet been identified.

Thus, over the past 10 years, our understanding of molecules and processes that regulate ectopic calcification has grown exponentially. As mentioned, much of our understanding comes from the identification of genes through linkage or targeted deletion studies that cause human and/or mouse ectopic calcification disorders, respectively. In addition, a number of *in vitro* and *in vivo* model systems have been developed that mimic important aspects of ectopic calcification. Figure 1 summarizes the current major theories and regulators of vascular calcification that have been put forward based on these studies including failed anticalcific mediators, (2) induction of osteochondrogenesis, (3) apoptosis, (4) abnormal calcium and phosphate homeostasis, (5) circulating nucleational complexes/paracrine factors derived from bone and (6) matrix degradation. However, the key factors and processes important for any specific disease state are still unknown, and it is likely that a different set of processes/molecules may uniquely be involved in different pathologies. In this regard, serum phosphate and cellular phosphate metabolism are emerging as key regulators of vascular calcification in ESRD.

As shown in Figure 1, phosphate may contribute to multiple mechanisms that initiate and/or promote progression of vascular calcification. Although this review focuses on the role of phosphate in promoting osteochondrogenic conversion of vascular cells (mechanism 2), some evidence suggests that elevated phosphate may also contribute to vascular calcification by promoting apoptosis[46] as well as raising the calcium × phosphate product (Ca × P) thereby thermodynamically favoring crystal formation.[47] In addition, Massy *et al.*[48] and others have suggested that elevated phosphate might also contribute

Table 1 | Genes associated with vascular calcification in mice and men

Gene	Mouse mutant phenotype[a]	Human genetic mutation/phenotype
Matrix Gla protein	Arterial, valve, and cartilage calcification (Luo, 1997)	Keutel syndrome/cartilage and soft tissue calcification (Hur, 2005)
Fetuin	Low serum HA inhibitory activity; enhanced susceptibility to vitamin D overload-induced vascular calcification (Schafer, 2003)	None reported
Osteopontin	Increased calcification of implanted bioprosthetic valve tissue (Steitz, 2002; Ohri, 2005); increased vascular calcification in OPN−/−XMGP−/− mice (Speer, 2002)	None reported
Osteoprotegerin	Vascular calcification and osteoporosis (Bucay, 1998)	None reported
FGF23	Hyperphosphatemia; high serum vitamin D; vascular calcification (Stubbs, 2007)	Familial tumoral calcinosis/vascular calcification, hyperphosphatemia, high-serum vitamin D (Benet Pages, 2005)
Klotho (b-glucuronidase)	Vascular calcification, rapid aging (Kuro-o, 1997)	Tumoral calcinosis, hyperphosphatemia (Ichikawa, 2007)
Nucleotide pyrophosphatase) Enpp1/PC-1/NPP1	Tip-toe walking mouse/vascular and articular cartilage calcification (Okawa, 1998)	Infantile arterial calcification/low pyrophosphate, extensive vascular calcification, neonatal lethal (Rutsch, 2003)
Ank (pyrophosphate transporter)	Progressive ankylosis; articular cartilage calcification; soft tissue calcification (Harmey, 2004)	Calcium pyrophosphate deposition disease/ chondrocalcinosis; high pyrophosphate (Zaka, 2006)
Carbonic anhydrase II	Small artery VC; osteopetrosis; metabolic acidosis (Spicer, 1989)	Osteopetrosis, metabolic acidosis, brain calcifications (Shah, 2004)
Smad 6/Madh6	Endocardial cushion defects; valvular calcification (Galvin, 2000)	None reported
Desmin	Neonatal cardiomyopathy with calcification (Mavroidis, 2002)	None reported
UDP *N*-acetyl-a-D-galactosamine (GalNT3)	None reported	Familial tumoral calcinosis/hyperphosphatemia, vascular calcification, elevated serum vitamin D (Ichikawa, 2005)
Fibrillin 1	Marfan-like syndrome, elastocalcinosis and aneurysm	Marfan syndrome
Fibulin 4	Valve calcification and stenosis, aortic dilatation	Cutis laxa, aortic aneurysm, perinatal lethal (Dasouki, 2007)
ABCC6 transporter (substrate unknown)	Extensive soft tissue and vibrissae calcification (Klement, 2005)	Pseudoxanthoma elasticum/calcification of skin, connective tissue and vasculature (Le Saux, 2000)
BMP and receptor	BMP4 overexpressing transgenic: fibrodysplasia ossificans-like phenotype (Kan, 2004)	ACVR1 BMP receptor, activating mutations: fibrodysplasia ossificans progressiva/muscle/soft tissue calcification (Shore, 2006)
WRN RecQ helicase	Accelerated mortality in p53 null background (Lombard, 2000)	Werner's syndrome/soft tissue calcification (Uhrhammer, 2006)
Lamin A (LMNA)	Cardiac and skeletal myopathy; progressive loss of vascular SMC and calcification (Varga, 2006)	Hutchinson–Gilford progeria/calcification associated with atherosclerosis (Eriksson, 2003)
Glucocerebrosidase (D409H)	Lysosomal storage disorder, valve calcification not found (Xu, 2003)	Gaucher disease/lysosomal storage disease; valvular and aortic arch calcification (McMahon, 2001)
Transcription intermediatery factor 1 (TIF1α)	Ectopic calcification, including arterioles and medium-sized arteries (Ignat, 2008)	None reported
Calcium sensing receptor (CaSR)	Gprc2a*Nuf* mouse, activating mutation: ectopic calcification including arterial calcification and cataracts (Hough, 2004)	Activating mutations: autosomal-dominant hypocalcemia, hypercalciuria, and Bartter-like syndrome (Pollak, 1994)

Abbreviations: ABCC6, ATP-binding cassette transporter 6; BMP, bone morphogenetic protein.
[a]Loss of function mutation is shown unless otherwise stated.

to vascular calcification by inhibiting osteoclastic differentiation,[48–51] though further evidence for this in the context of vessel wall calcification is needed. Whether elevated phosphate contributes to loss of anticalcific molecules, such as decreased fetuin levels in ESRD patients, circulating nucleational complexes or matrix degradation is not yet known.

HYPERPHOSPHATEMIA, ELEVATED PHOSPHATE LOAD, AND VASCULAR CALCIFICATION

Evidence for the importance of hyperphosphatemia as a major inducer of vascular calcification comes from studies of genetic syndromes (Table 1) as well as diseases of renal insufficiency. Hyperphosphatemia is observed in two human genetic disorders that cause familial tumoral calcinosis due to mutations in the genes for *FGF23*, a major phosphaturic hormone, and UDP *N*-acetyl-α-D-galactosamine.[52,53] Likewise, in mice, targeted deletion of either FGF23[54,55] or *klotho*, an aging-suppressor gene required for FGF23 function,[56] leads to hyperphosphatemia that is accompanied by vascular calcification.

Hyperphosphatemia is also prevalent in patients with ESRD. Evidence from observational studies reveals that elevated serum phosphate is positively correlated with mortality, and an increase in risk of death is observed in ESRD patients with a serum phosphate greater than

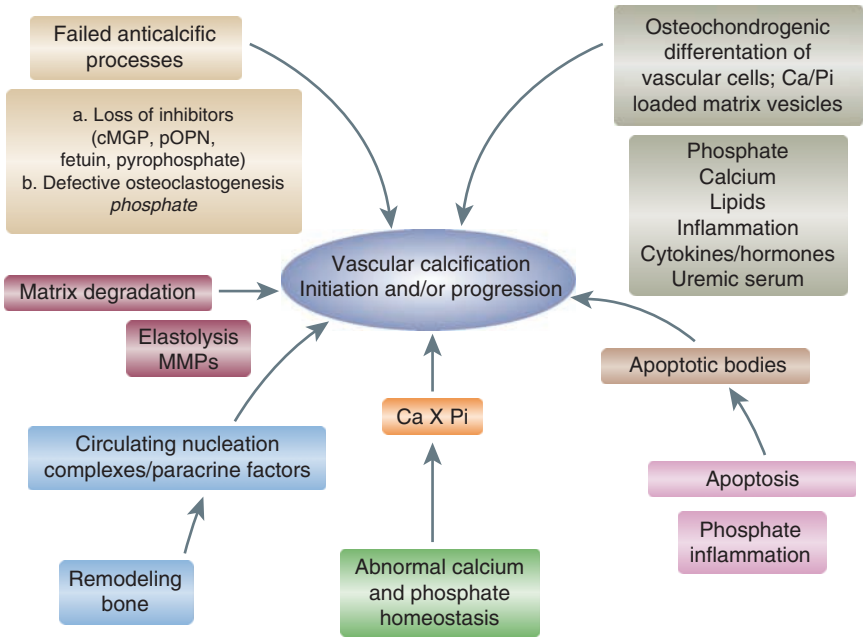

Figure 1 | Major mechanisms of vascular calcification. Six different mechanisms that have been proposed to regulate the initiation or progression of vascular calcification are illustrated, along with key molecular mediators where known. The extent to which each of these mechanisms plays a role in vascular calcification in various disease states, including hyperphosphatemia and ESRD, is currently unknown. cMGP; gamma carboxylated matrix gla protein, pOPN; phosphorylated osteopontin.

6.5 mg/dl.[57] Despite one negative trial,[58] the weight of clinical evidence from three recent randomized clinical trials showed that lowering serum phosphate levels with a non-calcium containing phosphate binder slows progression of vascular calcification in ESRD patients.[59–61] Moreover, even relatively small elevations in serum phosphate in the high normal range (3.5–4.5 mg/dl) have been correlated with increased risk of cardiovascular and all-cause mortality in both chronic kidney disease patients[62] and the general population.[63] Thus phosphate load, even in the absence of outright hyperphosphatemia, may be an important driver of vascular calcification.

Experimental models of renal insufficiency also point to a role of elevated phosphate in vascular calcification. Uremic rats show aortic medial calcification with prolonged (6 months) feeding of a high phosphate diet that could be blocked by treatment with the phosphate binder, sevelamer.[64] Hyperphosphatemia has also been correlated with atherosclerotic calcification in uremic, high fat fed *LDLR* null mice,[65] and sevelamer decreased vascular calcification in this setting. Similarly, Massy *et al.*[66] showed that uremia accelerated atherosclerosis and vascular calcification in *ApoE−/−* mice and sevelamer-prevented uremia enhanced atherosclerotic lesion size as well as calcification in this model. Finally, hyperphosphatemia was correlated with extensive arterial medial calcification similar in type and extent to that observed in ESRD patients in the calcification-prone DBA/2 mouse in response to severe renal insufficiency and high phosphate feeding (CM Giachelli *et al.*, unpublished observations).

POTENTIAL ROLE OF THE SODIUM-DEPENDENT PHOSPHATE COTRANSPORTER, PIT-1, IN VASCULAR CALCIFICATION

Consistent with clinical and animal studies, phosphate levels comparable to those seen in hyperphosphatemic individuals[57] induce SMC calcification and osteochondrogenic phenotype change *in vitro*.[67–69] No calcification occurs in human SMCs cultured in a medium containing 1.4 mM phosphate, but calcification is induced in a dose- and time-dependent manner when phosphate levels are increased from 1.6 to 3.0 mM. The finding that elevated phosphate induces SMC calcification was confirmed by other investigators.[68,70] In addition to SMC cultures, rat aortas cultured in elevated phosphate medium undergo medial calcification.[71] Together, these studies demonstrate that elevated phosphate is a strong inducer of vascular calcification.

Phosphate transport into cells is primarily mediated by sodium-dependent phosphate cotransporters, and three types of cotransporters have been identified based on structure, tissue expression and biochemical characteristics.[72,73] The type I and type II sodium-dependent phosphate cotransporters are primarily expressed in kidney and intestinal epithelium, and their functions are important for the maintenance of phosphate homeostasis in body.[72,73] The type III sodium-dependent phosphate cotransporters, Pit-1 and Pit-2, were originally identified as cell-surface receptors for the gibbon ape leukemia virus and the amphotropic murine retrovirus, respectively. Type III members are widely expressed in tissues such as kidney, liver, lung, heart, brain, osteoblast, chondrocyte, and SMCs.[67,74–79]

A functional sodium-dependent phosphate transport system has been characterized in vascular SMCs.[67,79,80]

RT-PCR revealed expression of type III sodium-dependent phosphate cotransporters, Pit-1 and Pit-2, in vascular SMCs, whereas no transcripts for type I and type II sodium-dependent phosphate cotransporters were detected. Real-time PCR indicated that Pit-1 mRNA levels were higher than Pit-2 levels in SMC.[79] Treatment of vascular SMCs with a competitive inhibitor of sodium-dependent phosphate co-transporters, phosphonoformic acid, caused a dose-dependent inhibition of phosphate uptake, calcification, and osteochondrogenic phenotype in SMCs.[67,68,70] These results suggested that phosphate transporter activity was necessary for mineralization as well as osteochondrogenic transition in human SMCs.

As phosphonoformic acid has lower affinity for type III than type II receptors,[80] the requirement of phosphate uptake for SMC calcification was further examined using SMCs that were stably transduced with a Pit-1-specific small hairpin RNA.[79] Pit-1 small hairpin RNA-expressing cells had reduced Pit-1 mRNA and protein levels as determined by Northern and Western blots, respectively. Sodium-dependent phosphate uptake in the cells was reduced compared with that in control cells. After incubation with elevated phosphate for 7, 10 or 14 days, there was substantially reduced calcification in Pit-1 knockdown cells compared to control cells. Of interest, restoration of phosphate uptake in Pit-1 knockdown cells by overexpression of mouse Pit-1 rescued elevated phosphate-induced mineralization. Similar to phosphonoformic acid, inhibition of phosphate uptake by Pit-1 small hairpin RNA blocked the expression of phosphate-induced osteogenic differentiation markers, Runx2 and osteopontin. Furthermore, we determined that neither phosphate loading of matrix vesicles nor cell death was mediated by Pit-1 in SMC. These studies indicated that sodium-dependent phosphate cotransporters, in particular Pit-1, might be a major mechanism for controlling vascular calcification and SMC phenotypic state. Taken together, these results demonstrate that phosphate transport through Pit-1 is required for calcification of human SMC *in vitro*. A model for the possible functions of elevated phosphate and Pit-1 in SMC calcification is shown in Figure 2.

Recent studies have supported a role for increased phosphate uptake through Pit-1 in vascular calcification *in vivo*. Mizobuchi *et al.*[81] showed that mRNA levels of Pit-1 and Runx2 were increased in calcified aorta of uremic rats with severe hyperparathyroidism, whereas no increase was observed in non-calcified aorta of control animals. Likewise, *LDLR−/−* mice fed a high-fat diet showed elevated levels of serum tumor necrosis factor α and Pit-1 in calcified aortas. *In vitro*, several factors that have been shown to induce vascular calcification also induce Pit-1 in SMCs. Long-term treatment of human SMCs with elevated calcium levels leads to increased Pit-1 mRNA levels, phosphate uptake, and calcification.[82] Likewise, PDGF promotes calcification in cultured SMCs and strongly induces Pit-1 expression.[75,83] In addition, bone morphogenetic protein 2 (BMP-2), a potent osteogenic protein, has been shown to promote vascular

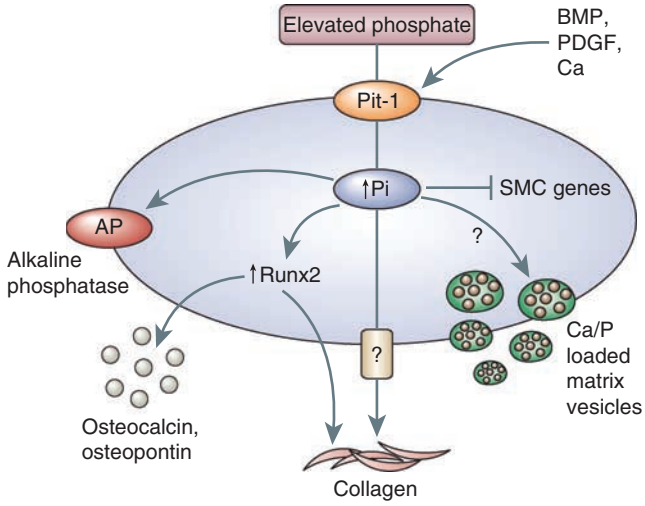

Figure 2 | Proposed role of elevated phosphate (Pi) in osteochondrogenic phenotype change and matrix mineralization in vascular SMC. Pi enters the cell through the sodium-dependent phosphate cotransporter, Pit-1, and induces an osteochondrogenic phenotype change. This stimulates matrix vesicle calcium and Pi loading, as well as matrix changes that promote calcification. Molecules involved in regulating Pi loading of matrix vesicles or Pi efflux are currently unknown.

calcification[36,84,85] and BMP-2 increased phosphate uptake in human SMC in a time- and dose-dependent manner.[86] Finally, transglutaminase (TG) appears to be an important regulator of endogenous expression, as Pit1 levels were observed in *TG+/+* SMCs but absent in *TG−/−* SMCs in the presence or absence of elevated phosphate.[87] Whether Pit-1 plays a role in vascular calcification in human disease is not yet known, but it is of interest that a single nucleotide polymorphism that was significantly associated with left ventricular mass index and relative wall thickness in hypertensive black siblings was identified in the Pit-1 gene (SLC20A1_cv9546580). The functional significance of this SNP is not yet clear as it occurs in the intronic region of the gene, but its association with outcomes that are thought to be impacted by vascular calcification is provocative.[88]

It is becoming increasingly clear that vascular calcification shares a number of similarities with osteogenesis and bone mineralization, and indeed, phosphate transport through Pit-1 has been recently implicated in regulation of bone formation and mineralization. Pit-1 mRNA levels increase during osteoblast differentiation and are correlated with mineralization.[89] Additionally, *in situ* hybridization in developing embryonic murine metatarsals showed that Pit-1 expression was first detected in 17-day-old embryos at a stage when the chondrocytes undergo hypertrophy and initiate mineralization. Moreover, Pit-1 expression was restricted to a subset of hypertrophic chondrocytes where matrix mineralization occurs.[90] In osteoblast-like cells, epinephrine, insulin-like growth factor 1, platelet-derived growth factor, and BMP2 have been shown to upregulate Pit-1,[77,91–93] when transforming growth factor β and interleukin 8 induce Pit-1 in chondrogenic cells[94,95] Importantly,

as in SMC, inhibition of Pit-1 in osteoblasts blocked mineralization *in vitro* [93,96] and *in vivo*.[96] Thus, it is likely that phosphate transport through Pit-1 is a common requirement for cell-mediated biomineralization.

CONCLUSIONS

Vascular calcification is a major contributor to CVD in ESRD patients. Susceptibility to vascular calcification is genetically determined and involves a growing number of inducers and inhibitors. Hyperphosphatemia promotes vascular calcification in part by promoting SMCs to undergo an osteochondrogenic phenotype change through a mechanism requiring sodium-dependent phosphate cotransporters. Upregulation of sodium-dependent phosphate cotransporters in SMCs by disease state and cytokines may facilitate vascular calcification even when serum phosphate levels are in the normal range.

DISCLOSURE

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