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Mechanisms of Vascular Calcification: The Pivotal Role of Pyruvate Dehydrogenase Kinase 4

Jaechan Leem[1], In-Kyu Lee[2,3]

[1]Department of Immunology, Catholic University of Daegu School of Medicine, Daegu; [2]Division of Endocrinology and Metabolism, Department of Internal Medicine, Kyungpook National University School of Medicine; [3]BK21 PLUS KNU Biomedical Convergence Program, Kyungpook National University, Daegu, Korea

Vascular calcification, abnormal mineralization of the vessel wall, is frequently associated with aging, atherosclerosis, diabetes mellitus, and chronic kidney disease. Vascular calcification is a key risk factor for many adverse clinical outcomes, including ischemic cardiac events and subsequent cardiovascular mortality. Vascular calcification was long considered to be a passive degenerative process, but it is now recognized as an active and highly regulated process similar to bone formation. However, despite numerous studies on the pathogenesis of vascular calcification, the mechanisms driving this process remain poorly understood. Pyruvate dehydrogenase kinases (PDKs) play an important role in the regulation of cellular metabolism and mitochondrial function. Recent studies show that PDK4 is an attractive therapeutic target for the treatment of various metabolic diseases. In this review, we summarize our current knowledge regarding the mechanisms of vascular calcification and describe the role of PDK4 in the osteogenic differentiation of vascular smooth muscle cells and development of vascular calcification. Further studies aimed at understanding the molecular mechanisms of vascular calcification will be critical for the development of novel therapeutic strategies.

Keywords: Vascular calcification; Vascular smooth muscle cells; Pyruvate dehydrogenase kinase 4; Bone morphogenetic proteins; Osteogenic differentiation; Mitochondria

INTRODUCTION

Vascular calcification is often observed in advanced vascular lesions and is a common consequence of aging, diabetes mellitus, and chronic kidney disease [1,2]. Patients with diabetes mellitus or chronic kidney disease often exhibit more severe atherosclerosis and a higher prevalence of vascular calcification [3,4]. Vascular calcification is closely associated with arterial stiffness and ultimately contributes to increased cardiovascular mortality [5,6]. Therefore, developing therapeutic strategies to prevent and treat vascular calcification has a great clinical importance.

Vascular calcification was long considered to be a passive degenerative process. However, accumulating evidence shows that bone-associated proteins, including osteocalcin, osteopontin, and alkaline phosphatase, are preferentially expressed in calcified atherosclerotic plaques [1,2]. In addition, bone-associated structures such as matrix vesicles, which are the initial sites of primary nucleation during the mineralization of bone, were found in calcified atherosclerotic plaques [1,2]. These

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Corresponding author: In-Kyu Lee
Department of Internal Medicine, Kyungpook National University School of Medicine, 130 Dongdeok-ro, Jung-gu, Daegu 41944, Korea
Tel: +82-53-420-5564, **Fax:** +82-53-426-2046, **E-mail:** leei@knu.ac.kr

findings suggest that vascular calcification is an active and highly regulated process that is similar to bone formation. However, although a number of studies explored the mechanism of vascular calcification, it remains poorly understood.

Metabolic flexibility is the capacity of the body to adjust fuel oxidation on the basis of nutrient availability [7]. Competition between glucose and fatty acids for fuel oxidation is primarily controlled by the pyruvate dehydrogenase complex (PDC) [8]. PDC is a mitochondrial enzyme complex that regulates the entry of glycolytic products into the tricarboxylic acid (TCA) cycle by converting pyruvate into acetyl coenzyme A (acetyl-CoA) in mammalian cells [9]. PDC is relatively active in a fed state and stimulates glucose oxidation to produce energy from glucose or convert it to fat for energy storage in peripheral tissues [10]. However, inhibition of PDC activity by pyruvate dehydrogenase kinase (PDK)-dependent phosphorylation reduces glucose oxidation and provides three-carbon substrates such as pyruvate, lactate, and alanine for gluconeogenesis in a fasted state. To date, four PDK isozymes (PDK1, 2, 3, and 4) have been identified in humans and rodents and are expressed in a tissue-specific manner [11]. Among them, PDK4 expression levels were found to be dramatically increased in several peripheral tissues, including skeletal muscle, heart, mammary glands, adipose tissue, kidneys, and liver in fasting or diabetic rodents [12-14]. In addition, PDK4 knockout mice had lower blood glucose levels in a fasted state than wild-type mice, consistent with an important role for PDK4 in maintaining glucose levels during fasting [14], and, after feeding a high-fat diet, PDK4 knockout mice exhibited improved glucose tolerance and insulin sensitivity compared with wild-type mice [15]. In diabetic mice lacking hepatic insulin receptor substrates 1 and 2, deletion of the PDK4 gene resulted in improvement in hyperglycemia and glucose tolerance [16]. Furthermore, PDK4 deficiency attenuated fat accumulation in the livers of mice fed a high-fat diet [17]. These results suggest that PDK4 plays an important role in the development of metabolic diseases, including hyperglycemia, insulin resistance, and hepatic steatosis. A more comprehensive overview of recent research findings regarding the roles of PDKs in metabolic diseases is provided by other excellent reviews [8-10,18,19].

In this review, we will summarize recent studies on the mechanism of vascular calcification and discuss our recent findings regarding the role of PDK4 in the development of vascular calcification.

MECHANISM OF VASCULAR CALCIFICATION

Osteochondrogenic phenotype change in vascular smooth muscle cells

Accumulating evidence suggests that a phenotypic change in vascular smooth muscle cells (VSMCs) plays a critical role in the development of vascular calcification [20-22]. Before the initiation of vascular calcification, VSMCs undergo a phenotypic change from a contractile to a synthetic and osteochondrogenic phenotype. This phenotype change is accompanied by downregulated expression of VSMC contractile markers such as smooth muscle α-actin and smooth muscle 22α and upregulated expression of osteochondrogenic markers such as osteocalcin, osteopontin, and alkaline phosphatase [20-22]. These osteochondrogenic cells lose their contractile properties and acquire synthetic functions.

Bone morphogenetic proteins (BMPs) provide essential signals for determining cell fate, embryonic patterning, organogenesis, and the postnatal remodeling of diverse tissues [23]. BMPs form the largest group of proteins within the transforming growth factor β superfamily, and more than 20 subtypes of BMPs have been identified [24]. Among them, BMP2 is well known to play a role in the development of vascular calcification [25]. BMP2 was found to be expressed in human calcified atherosclerotic plaques [26], and smooth muscle-specific overexpression of BMP2 in apolipoprotein E (apoE)-deficient mice accelerated vascular calcification [27]. In addition, pharmacological inhibition of BMP signaling ameliorated vascular calcification in low density lipoprotein receptor (LDLR)-deficient mice, suggesting that BMP signaling plays an important role in the development of vascular calcification [28].

Interestingly, a number of studies provide evidence that BMP2 signaling contributes significantly to the transdifferentiation of VSMCs into osteochondrogenic cells [25,28]. Under atherosclerotic calcifying conditions, BMP2 binds to type I and II receptors and triggers heteromeric complex formation [25,29]. After activation by the type II receptors, the type I receptors phosphorylate small mothers against decapentaplegic (SMAD) 1/5/8 to propagate the signal into the cell. SMAD1/5/8 form heteromeric complexes with SMAD4 and move into the nucleus, where they assemble into transcriptional machinery that regulates the expression of osteogenic genes. Recently, we found that expression of estrogen-related receptor γ (ERRγ), a member of the orphan nuclear receptor superfamily, is upregulated during *in vitro* osteogenic differentiation of VSMCs [30]. Adenovi-

rus-mediated overexpression of ERRγ in VSMCs induced BMP2 expression, leading to increased phosphorylation of SMAD1/5/8. In addition, inhibition of endogenous ERRγ expression or activity using specific siRNAs or the selective inverse agonist ameliorated vascular calcification both *in vitro* and *in vivo* [30]. Our findings suggest that ERRγ plays an important role in the development of vascular calcification by upregulating BMP2 signaling, and that inhibition of ERRγ may be a promising therapeutic strategy for preventing vascular calcification.

The best-studied transcription factors regulated by BMP2 signaling are runt-related transcription factor 2 (Runx2) and muscle segment homeobox 2 (Msx2) [20,25]. Runx2 is a member of the runt-related transcription factor family and plays an essential role in osteoblast differentiation and bone formation [31,32]. Multiple signaling pathways, including the BMP2 pathway, converge on Runx2 to induce osteoblast differentiation [33]. Runx2 regulates the expression of osteochondrogenic markers, including osteocalcin, osteopontin, and alkaline phosphatase [34]. Although Runx2 is not expressed in normal vessels, it is abundantly expressed in calcified human vessels and calcified VSMCs in mice [35-37]. Previous studies demonstrated that functional inactivation of Runx2 by dominant-negative mutations or knockdown prevents calcification in VSMCs, while its overexpression stimulates calcification, suggesting that Runx2 is essential for the osteochondrogenic phenotype change in VSMCs [38,39]. Furthermore, smooth muscle-specific deficiency of Runx2 markedly inhibited vascular calcification in mice [40].

Msx2 is also a key transcription factor involved in vascular calcification induced by BMP2 signaling [20,25]. Msx2 is a member of the homeodomain transcription factor family and plays an important role in osteoblast differentiation and bone formation [41,42]. Expression of Msx2 was also detected in calcified human vessels [36,43]. Previous studies show that BMP2-dependent activation of Msx2 promotes the osteogenic differentiation of VSMCs and vascular myofibroblasts [43,44]. In LDLR-deficient mice, a high-fat diet stimulated vascular calcification, and this was accompanied by upregulation of Msx2 expression in vessel walls [45]. In addition, transgenic overexpression of Msx2 in the vessel wall promoted vascular calcification via activation of canonical Wnt signaling [46]. Furthermore, smooth muscle-specific deficiency of Msx1 and Msx2 attenuated vascular calcification and aortic stiffness in LDLR-deficient mice fed high-fat diets [47].

The main pathological stimuli that induce the osteochondrogenic phenotype change in VSMCs are oxidative stress, oxylipids, and phosphates [48]. Among them, oxidative stress plays a critical role in the pathogenesis of atherosclerosis and other cardiovascular diseases [49]. In addition, increased oxidative stress is closely associated with several medical conditions that are linked to an elevated prevalence of vascular calcification, including diabetes mellitus and chronic kidney disease [50,51]. Several *in vitro* studies show that oxidative stress can induce an osteochondrogenic phenotype change in VSMCs [39,52,53]. Expression of Runx2 was found to be involved in oxidative stress-induced osteogenic differentiation and calcification of VSMCs [39]. Furthermore, a recent study showed that antioxidant treatment inhibited osteogenic differentiation of VSMCs and vascular calcification in uremic rats, supporting the idea that antioxidants may represent promising therapeutic agents for the treatment and prevention of vascular calcification [54].

The transcription factor nuclear factor E2-related factor 2 (Nrf2) plays a critical role in cellular antioxidant defenses by activating a wide range of antioxidant genes [55]. A recent *in vitro* study demonstrated that Nrf2 inhibits osteoblast differentiation through the inhibition of Runx2-dependent transcriptional activity [56]. Recently, we found that dimethyl fumarate, a potent synthetic Nrf2 activator, inhibits *in vitro* osteogenic differentiation and calcification of VSMCs, *ex vivo* calcification of vessel rings, and vitamin D-induced *in vivo* vascular calcification, suggesting that Nrf2 is a potential therapeutic target for the treatment of vascular calcification [57].

Loss of anticalcific molecules

Several anticalcific molecules, including matrix Gla protein (MGP), fetuin-A, and osteoprotegerin (OPG), have been identified and these anticalcific molecules play an important role in suppressing vascular calcification under normal conditions [22]. In patients with chronic kidney disease, dysregulation of anticalcific molecules may contribute to the development and progression of vascular calcification [58].

MGP is an extracellular matrix protein that binds to calcium ions with high affinity and acts as an inhibitor of vascular mineralization [22]. In addition, MGP can bind to BMP2 and inhibit its activity [59]. MGP-deficient mice exhibited vascular calcification [60,61], while overexpression of MGP in apoE-deficient mice reduced the amount of vascular calcification [62]. Vitamin K-dependent γ-carboxylation of glutamate residues is required to convert MGP into its active form [22,61]. Recent studies show that, in animal models, treatment with therapeutic doses of warfarin, a vitamin K antagonist, stimulates the development of vascular calcification, while treatment with high di-

etary vitamin K1 inhibits it. In addition, treatment with warfarin is associated with coronary artery plaque calcification in patients with suspected coronary artery disease [63,64].

Fetuin-A is a glycoprotein that is secreted from the liver and adipose tissue and is present at high concentrations in human blood, where it binds calcium ions and hydroxyapatite [22]. Fetuin-A inhibited VSMC calcification *in vitro* [65], and fetuin-A deficiency in apoE-deficient mice promoted vascular calcification [66]. Low serum levels of fetuin-A are associated with increased vascular calcification and cardiovascular mortality in patients on dialysis [67,68].

OPG is a phosphoprotein that regulates bone formation by inhibiting apatite crystal growth and osteoclast differentiation [22]. OPG was found to inhibit osteogenic differentiation and calcification in VSMCs [69], and OPG-deficient mice developed osteoporosis and vascular calcification [70]. In addition, treatment with OPG ameliorated warfarin or vitamin D-induced vascular calcification in animal models [71].

Clinical studies show that serum OPG levels are significantly higher in patients with chronic kidney disease than in controls, indicating that this increase might be a compensatory response to the disease, rather than a risk factor [72,73].

Matrix vesicle formation, apoptosis, and mitochondrial dysfunction

Accumulating evidence suggests that matrix vesicles play an important role in the development of vascular calcification [74]. Apoptosis of VSMCs also contributes to the development of phosphate-induced VSMC calcification [75,76]. Vascular calcification is initiated both by matrix vesicles released from viable VSMCs and by apoptotic bodies from dying cells [75,77]. These extracellular vesicles provide nucleation sites for mineral deposition in the extracellular matrix. In addition, several studies show that the growth arrest-specific gene 6 (Gas6)-mediated survival pathway plays a central role in preventing phosphate-induced VSMC apoptosis and calcification [76,78]. We also found that α-lipoic acid, a naturally occurring antioxidant with anti-apoptotic properties [79], reduced phosphate-induced VSMC apoptosis and calcification through inhibiting phosphate-induced downregulation of cell survival signals via the binding of Gas6 to its cognate receptor Axl and subsequent Akt activation [80].

There is increasing evidence suggesting that mitochondrial dysfunction can be an important contributor to the development of atherosclerosis [81]. Mitochondrial DNA damage results in decreased mitochondrial function, including impaired respiratory chain function and reduced adenosine triphosphate (ATP) production, and eventually compromises cellular function. In animal studies, it was shown that mitochondrial DNA damage and mitochondrial dysfunction are early events in the development of atherosclerotic lesions and promote progression of atherosclerosis [82-84]. Furthermore, mitochondrial DNA damage was observed in blood cells and atherosclerotic lesions of patients with coronary artery disease [82,84,85]. These findings raise the prospect that mitochondrial dysfunction may induce vascular calcification, because atherosclerosis is a progressive disease that can lead to vascular calcification, which is often found in advanced atherosclerotic lesions [86]. Interestingly, we observed that functional and structural mitochondrial defects, as evidenced by reduced mitochondrial membrane potential, decreased intracellular ATP content, increased production of mitochondrial reactive oxygen species, and disruption of mitochondrial structural integrity, in calcifying VSMCs treated with inorganic phosphate [80]. These defects were accompanied by mitochondria-dependent apoptotic events. These results suggest a potential role for mitochondrial dysfunction in VSMC apoptosis and calcification. Indeed, mitochondria play an essential role in the regulation of intrinsic apoptotic pathways [87]. Mitochondria-dependent intrinsic apoptosis involves the release of cytochrome c from the inner membrane space to the cytosol, which in turn triggers the activation of caspase-9 and effector caspases, leading to nuclear DNA fragmentation and other changes that culminate in apoptotic death. In line with this, we showed that the protective effect of α-lipoic acid against phosphate-induced VSMC apoptosis and calcification can be attributed to the restoration of mitochondrial function as well as to the activation of the Gas6/Axl/Akt survival pathway [80]. Finally, administration of α-lipoic acid ameliorated vitamin D-induced vascular calcification and mitochondrial dysfunction in mice.

The role of PDK4 in the development of vascular calcification

Normal resting cells generate energy by converting glucose into pyruvate via the glycolysis pathway, which does not require oxygen, followed by oxidation reactions in the mitochondria [88]. Under normoxic conditions, pyruvate produced by glycolysis is transported primarily into the mitochondria and then decarboxylated by PDC into acetyl-CoA, which enters the TCA cycle. However, under hypoxic conditions, inhibition of PDC prevents the conversion of pyruvate into acetyl-CoA, leading to decreased TCA cycle activity in the mitochondria

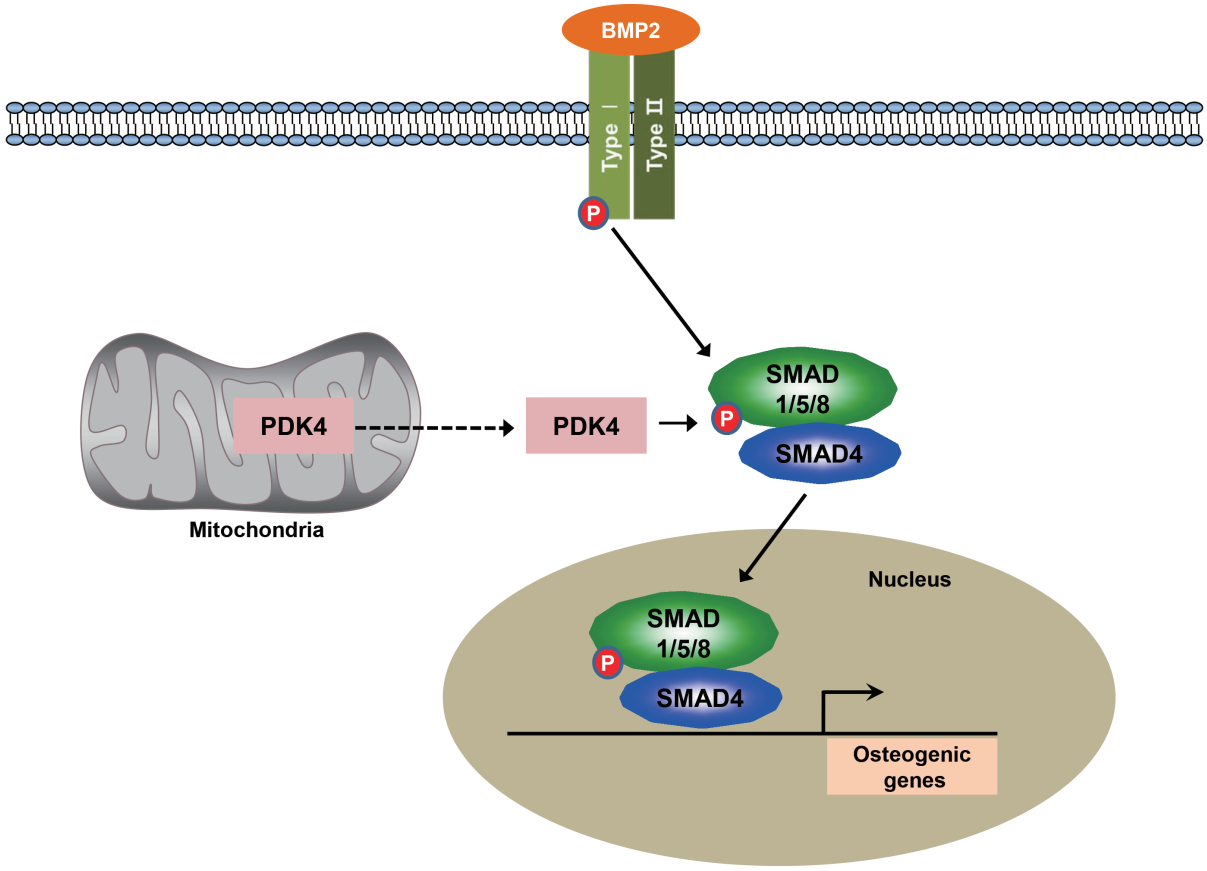

Fig. 1. The regulatory action of pyruvate dehydrogenase kinase 4 (PDK4) on the signaling pathway downstream of bone morphogenetic protein 2 (BMP2) during vascular calcification. Under calcifying conditions, BMP2 binds to type I and II receptors and triggers formation of a heteromeric complex. After activation by the type II receptors, the type I receptors phosphorylate small mothers against decapentaplegic (SMAD) 1/5/8 to propagate the signal into the cell. SMAD1/5/8 form heteromeric complexes with SMAD4 and move into the nucleus, where they assemble into transcriptional machinery that regulates the expression of osteogenic genes. Under normal conditions, PDK4 is located in the mitochondrial matrix. However, under calcifying conditions, PDK4 may be transported into the cytosol and activate SMAD1/5/8 by direct phosphorylation, leading to the translocation of phosphorylated SMADs into the nucleus for transcriptional regulation of osteogenic genes, thus enhancing BMP2 signaling pathway activity.

and increased conversion of pyruvate into lactate in the cytosol [10]. Interestingly, cancer cells depend largely on glycolysis for energy, even though sufficient oxygen is available. This phenomenon is called aerobic glycolysis or the Warburg effect [88]. Cancer cells meet the requirement for energy and biosynthetic precursors for proliferation and metastasis through aerobic glycolysis. PDK-dependent phosphorylation is essential for inhibition of PDC activity [10]. Dichloroacetate, an inhibitor of PDK, blocks aerobic glycolysis and causes cancer cell apoptosis and tumor regression, suggesting that PDK is a novel therapeutic target for cancer treatment [89]. Recently, accumulating evidence suggests that aerobic glycolysis also plays a critical role in meeting the demand for energy and biosynthetic precursors during proliferation and differentiation of other types of cells, including immune cells [90,91]. Given that osteogenic differentiation of VSMCs is critical for the development of vascular calcification [20,22], and that this process may require glucose metabolism similar to the Warburg effect to produce energy and the necessary biosynthetic precursors [92,93], it is reasonable to hypothesize that PDK plays an important role in metabolic regulation of the osteogenic switch in VSMCs. Indeed, our unpublished observations indicate that glucose consumption and lactate production are increased in phosphate-induced VSMC calcification. Furthermore, because it is suggested that mitochondrial dysfunction is a metabolic feature that controls the VSMC phenotype [94], PDK may regulate the osteogenic switch in VSMCs by controlling mitochondrial function.

Recently, we observed that expression of PDK4 and phos-

phorylation of PDC are increased in calcifying VSMCs and calcified vessels of patients with atherosclerosis [95]. Interestingly, the mRNA and protein levels of PDK4 were markedly increased in a time- and dose-dependent manner, whereas expression of other PDK isozymes was not significantly changed in phosphate-treated human VSMCs, suggesting that PDK4 plays a specific role in phosphate-induced osteogenic differentiation and calcification of VSMCs. We also demonstrated that both genetic and pharmacological inhibition of PDK4 ameliorated *in vitro* calcification of VSMCs, *ex vivo* calcification of aortic rings, and vitamin D-induced *in vivo* calcification [95]. To gain mechanistic insight into how PDK4 modulates vascular calcification, we examined the role of PDK4 in regulation of the osteogenic switch in VSMCs. Adenovirus-mediated overexpression of PDK4 increased, while PDK4 deficiency decreased, the expression of osteogenic genes in human VSMCs. We previously reported that α-lipoic acid, which also inhibits PDK4 activity [96], ameliorates vascular calcification by improving mitochondrial function [80]. Consistent with this, overexpression of PDK4 induced mitochondrial dysfunction *in vitro*, as evidenced by decreased ATP content, oxygen consumption rate, and maximal respiration capacity, and this was reversed by treatment with an inhibitor of PDK. These results suggest that PDK4 induces mitochondrial dysfunction, which may contribute to the development of vascular calcification [95].

Since PDK4 increased the expression of several osteogenic genes that are induced by BMP2 with no change in BMP2 expression, we explored the possibility that PDK4 may regulate the signaling pathway downstream of BMP2 without affecting the expression of BMP2 itself. Interestingly, we found that, under calcifying conditions, PDK4 phosphorylates and activates SMAD1/5/8, which leads to translocation of phosphorylated SMADs into the nucleus for the transcriptional regulation of osteogenic markers, thus enhancing BMP2 signaling pathway activity (Fig. 1) [95]. A direct interaction between PDK4 and SMAD seems unlikely, because PDK4 is located in the mitochondrial matrix [9,10]. However, using various methods, including a binding prediction model, confocal imaging analysis, immunoblots of subcellular fractions, co-immunoprecipitation, glutathione S-transferase pull-down assay, and *in vitro* kinase assay, we demonstrated that, after being transported from mitochondria into cytosol in response to calcifying stimuli, PDK4 can directly interact with and phosphorylate SMAD1/5/8, [95]. This finding is consistent with a previous report showing that the mammalian PDK4 ortholog in *Caenorhabditis elegans* is located in the cytosol as well as the mitochondria [97]. Finally, we evaluated whether the inhibitory effect of vascular calcification adversely affects bone remodeling, since vascular calcification shares many similarities with physiological bone formation [22]. Bone remodeling and osteoblastic differentiation in preosteoblasts were found to not be adversely affected by PDK4 deficiency, even though PDK4 promotes osteogenic differentiation of VSMCs in response to calcifying stimuli [95]. These results indicate that deletion of PDK4 effectively attenuates vascular calcification without adverse effects on bone remodeling.

CONCLUSIONS

The osteogenic phenotypic change in VSMCs plays a key role in the development of vascular calcification. Accumulating evidence suggests that mitochondrial dysfunction is a characteristic feature of osteogenic differentiation in VSMCs. In this review, we summarized our knowledge of the main mechanisms underlying vascular calcification, and discussed the role of PDK4 in the molecular and metabolic processes that contribute to the osteogenic switch in VSMCs during the development of vascular calcification. Although further studies are required to ascertain the metabolic changes that occur in VSMCs during osteogenic differentiation and vascular calcification, and their relationship with PDK4, the current evidence indicates that PDK4 may be a promising therapeutic target for the treatment of vascular calcification. Understanding the mechanisms of vascular calcification will be crucial for the development of novel therapeutic strategies against vascular calcification.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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ORCID

Jaechan Leem *http://orcid.org/0000-0003-2329-4374*
In-Kyu Lee *http://orcid.org/0000-0001-7908-8129*

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