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特 集

Medial vascular calcification: a new concept challenging the classical paradigm of dystrophic calcification

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ABSTRACT : Klotho deficient (*kl/kl*) mice are well known to develop hyperphosphatemia and resultant Mönchеberg's vascular sclerosis, which consists of elongated or fragmented elastic lamellae and abundant collagen fibrils inside the vessels. Instead of normal vascular smooth muscle cells (VSMCs), the tunica media of the *kl/kl* aorta has cells rich with abundant endoplasmic reticulum and Golgi apparatus, somewhat resembling osteoblasts. There were many matrix vesicle-like structures and calcifying nodules in the vicinity of these osteoblast-like cells in *kl/kl* aorta. The calcifying nodules seem to trigger calcification in the elastic lamellae, without promoting it in the collagen fibrils inside the *kl/kl* aorta. Also, mineral deposition was observed within the intravascular amorphous organic component, suggesting dystrophic calcification. Thus, two possible pathways for vascular calcification exist: one mediated by matrix vesicle-like structures, and another taking place after the deposition of calcium phosphates in the amorphous organic component. Compared to the latter, which consists of the classical view of intravascular calcification, the former appears to mimic osteoblastic mineralization in bone, and could be the result of trans-differentiation of VSMCs into osteoblastic cells. In this work, we will review our current findings on the process of medial vascular calcification found in *kl/kl* mice.

Key Words : *klotho, medial calcification, vascular smooth muscle cell, matrix vesicles, trans-differentiation*

Introduction

Vascular calcification is an important aging-related disease and is also a risk factor for cardiovascular morbidity and mortality. The hallmark of vascular calcification is calcium phosphate deposition, which can occur in the aorta, myocardium, and in the cardiac valves. Calcification of the tunica media is associated with vascular stiffening and arteriosclerosis, and medial calcification appears to be more common in patients with nephropathy[1~5].

The osseous tissue provides not only structurally supports the human body, but also maintains mineral ion homeostasis. Osteo-renal interplay is essential for maintaining physiological serum concentration of calcium and phosphate levels (Fig.1). Bone is a target organ for several hormones such as parathyroid hormone (PTH), 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] and calcitonin. PTH and 1α,25$(OH)_2D_3$ were shown to influence serum calcium and phosphate levels by affecting the rate of intestinal and renal absorption, as well as bone formation and resorption. Recent studies also found an important role for fibroblast growth factor 23 (FGF23) in the systemic regulation of phosphate metabolism[6~8]. The delicate functional balance between kidney and bone is, therefore, an important determinant of serum calcium-phosphate concentration; any imbalance caused by osteo-renal diseases often results in vascular calcification.

Klotho is a gene primarily expressed in the kidney crucial for calcium-phosphate homeostasis, as its protein serves as a co-receptor in the FGF23 signaling pathways linked to inhibition of phosphate reabsorption in the

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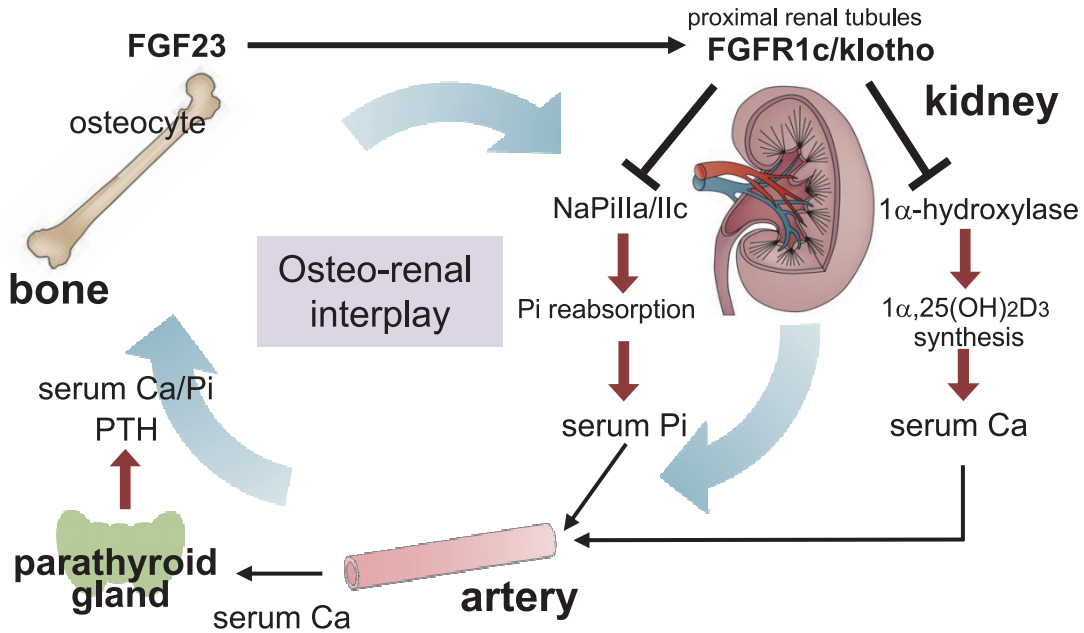

Fig. 1: Schematic design for osteo-renal interplay for maintaining physiological serum concentration of calcium and phosphate levels

FGF23 secreted by osteocytes would bind to its receptor, *i.e.*, FGFR1c/klotho, and signaling linked to klotho/FGF23 inhibits phosphate reabsorption in renal proximal tubules by mediating NaPi IIa and IIc. This signaling also inhibit 1*α*-hydroxylase to reduce active form of hydroxy-vitamin D3.

proximal renal tubules[6~9]. Therefore, disruptions in the FGF23/klotho axis raise the concentration of serum phosphate and often induce vascular calcification.

In this review, we will describe our recent findings and discuss our current understanding on the process of medial vascular calcification found in *kl/kl* mice.

Ⅰ. Types of vascular calcification and its candidate triggers

Cardiovascular events are the leading cause of death in patients suffering from chronic kidney disease (CKD). Patients with metabolic bone disorder (MBD) in CKD are at highest risk for cardiovascular events independent of classical risk factors[4, 5]. Vascular calcification contributes substantially to increasing the risk of cardiovascular events, and the extent of vascular calcification is a strong predictor of cardiovascular-related and other causes of mortality.

Vascular calcification can be seen in two distinct histological sites in the arteries[10]: One is the intima, where it is commonly associated with atheromas and atherosclerosis lesions; the other is the media, where Mönchеberg's sclerosis occurs, as seen in the coronary artery. Among these types, Mönchеberg's vascular sclerosis is known to be related to CKD-MBD. Until recently, medial vascular calcification has been considered a passive, degenerative, and end-stage process of vascular disease. However, the discovery of matrix vesicles-like structures, expression of bone morphogenetic proteins (BMPs), and non-collagenous proteins such as osteopontin, osteocalcin, and matrix Gla protein (MGP) in the calcified vascular tissues may implicate a sort of biological calcification, and has thereby challenged the paradigm of dystrophic calcification[11~15].

There are many candidates for inducing medial calcification - high concentration of serum phosphate, calcitriol, diabetes, warfarin, BMPs, oxidative stress[16~20]. Despite these candidates, the molecular mechanisms regulating vascular calcification remain obscure. Phosphate balance in the body is regulated by complex cross-organ interactions that involve the kidney, intestine, bone and parathyroid gland; functional impairments in any of these organs can lead to abnormal phosphate levels. Indeed, hyperphosphatemia is highly correlated with the extent of medial calcification. Consistently, several observations on serum phosphate levels provided a clue for this process, with a tendency toward vascular calcification[21].

Ⅱ. *Kl/kl* mice as a hyperphosphatemia-induced medial calcification model

There are many animal models mimicking medial

calcification such as adenine administration[22], CKD[23~25] and klotho-deficient (*kl/kl*) mice[26, 27]. Currently, two klotho proteins are recognized: transmembrane klotho and circulating klotho. The transmembrane klotho is a main co-receptor in the FGF23 signaling pathways[6~8]. Since signaling linked to klotho/FGF23 inhibits phosphate reabsorption in renal proximal tubules by mediating sodium/phosphate cotransporter (NaPi) IIa and NaPi IIc, *kl/kl* mice exhibit hyperphosphatemia and vascular calcification[27~29]. Ectopic calcification was evident in various organs of *kl/kl* mice such as arterial walls, stomach, bronchial mucosa, alveolar cells, choroid plexuses, skin, testes and cardiac muscle[27].

Alternatively, *kl/kl* mice showed a phenotype consisting of osteoporosis, skin atrophy, pulmonary emphysema, gonadal dysplasia, and defective hearing, which appear to be, at least in part, involved in senescence[27]. Therefore, it is necessary to verify whether medial calcification is caused by suppression of anti-aging mechanisms or by imbalances in the concentration of serum phosphate. It is known that overexpression of klotho rescues the klotho-deficient phenotype, including the ectopic calcifications[27]. In the rescue experiments, the *kl/kl* thymus and genital organs were restored to nearly normal weights. The serum levels of calcium, phosphorus and glucose were also restored to almost normal values, and no residual calcium persisted in the fundic gland cells. In addition, mice with null-deleted genes encoding klotho and NaPiIIa, a major sodium/phosphate co-transporter in rodents, failed to induce vascular calcification despite the absence of klotho[30]. Thereby, it seems likely that vascular calcification seen in the *kl/kl* mice is due to hyperphosphatemia, rather than being a consequence of aging suppression. Thus, many reports have attempted to elucidate the histological abnormalities found in the calcified aorta of *kl/kl* mice as a hyperphosphatemia-induced medial calcification model[26, 27, 31].

Ⅲ. Histopathology of medial calcification in the *kl/kl* aorta

Although the wild-type counterparts revealed no medial calcification (Fig. 2A, B), we have shown broad calcification in the tunica media of the *kl/kl* aorta (Fig. 2C-E). Calcification was seen mainly along with straightly-elongated and fragmented elastic lamellae in the *kl/kl* tunica media (Fig. 2D). Interestingly, the elastic lamellae, but not the abundant type I collagen in the area, were preferentially calcified in *kl/kl* media. This finding

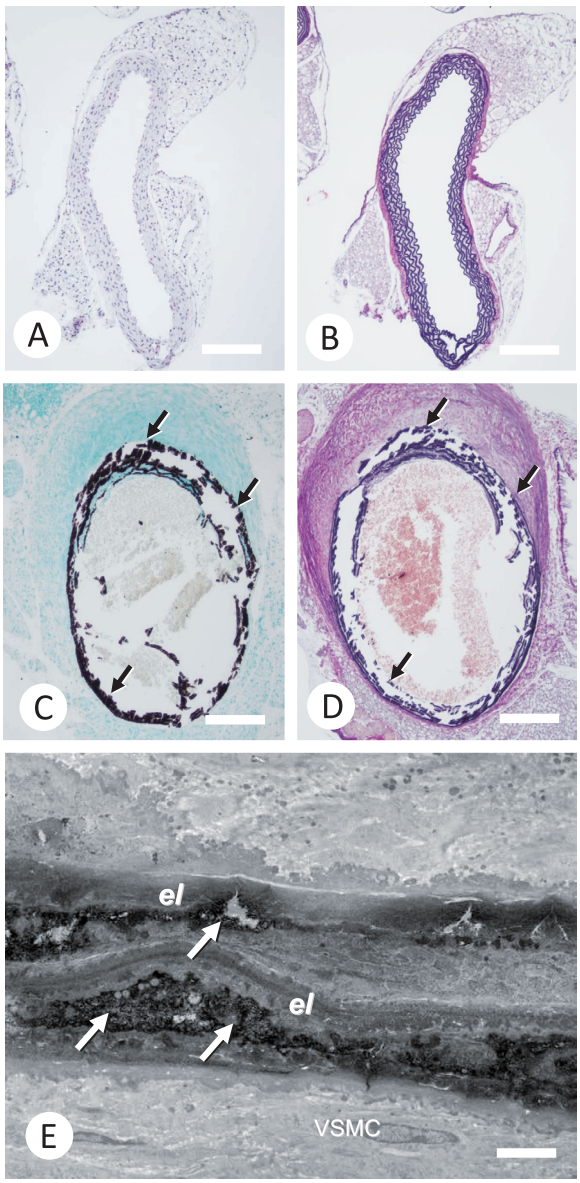

Fig. 2: Histology of medial calcification in *kl/kl* aorta

Panels A and C are von Kossa staining (detection of calcification), while B and D show van Gieson staining (visualization of elastic fiber). The wild-type aorta showed no medial calcification (A) with featuring wary prolife of tunica lamella (B). In contrast, *kl/kl* aorta had developed broad medial calcification (C) with fragmented elastic lamellae (D). Transmission electron microscopy demonstrated calcification (arrows) associated with elastic lamellae (el) in tunica media.

VSMC: vascular smooth muscle cell, el : elastic lamellae

Bars, A-D; 0.3 mm, E; 10μm

is consistent with the report that vascular calcification in patients with CKD differs from atherosclerotic calcification and affects the structure of the arterial elastic lamellae[32].

Under transmission electron microscopy (TEM), elastic lamellae was shown to be well calcified (Fig. 2E),

and there were vascular smooth muscle cell (VSMC) -like cells which developed cisterns of rough endoplasmic reticulum and Golgi apparatus (Fig. 3). These cells resemble matrix-synthesizing osteoblasts. In the vicinity of such VSMC-like cells, there were numerous collagen fibrils and amorphous organic materials similar in electron density to the elastic lamellae. Thus, it is interesting to verify whether vascular calcification is mediated by dystrophic calcification, or whether biological calcification occurs. The latter indicates trans-differentiation of VSMCs into the osteoblastic phenotype[16, 33, 34]. The presence of "matrix vesicles" has been very important for verifying the occurrence of biological calcification in bone and cartilage[35]. In agreement with the recent reports on medial calcification[36, 37], our observations demonstrated the presence of matrix vesicle-like structures that included fine mineral crystals. The mineral crystals appeared to grow out of the vesicle, forming calcifying nodules. But, the calcifying nodules preferentially calcified the elastic lamellae, rather than abundant collagen fibrils surrounding them within the *kl/kl* aorta. Notwithstanding, our findings also supported the classical idea of dystrophic calcification in media: mineral deposition, which was not related to matrix vesicle-like strictures, could also be seen inside the amorphous materials. Therefore, it seems that two possible pathways for vascular calcification exist: one mediated by matrix vesicle-like structures, and other being occurring via deposition of calcium phosphates inside the amorphous materials (Fig. 3).

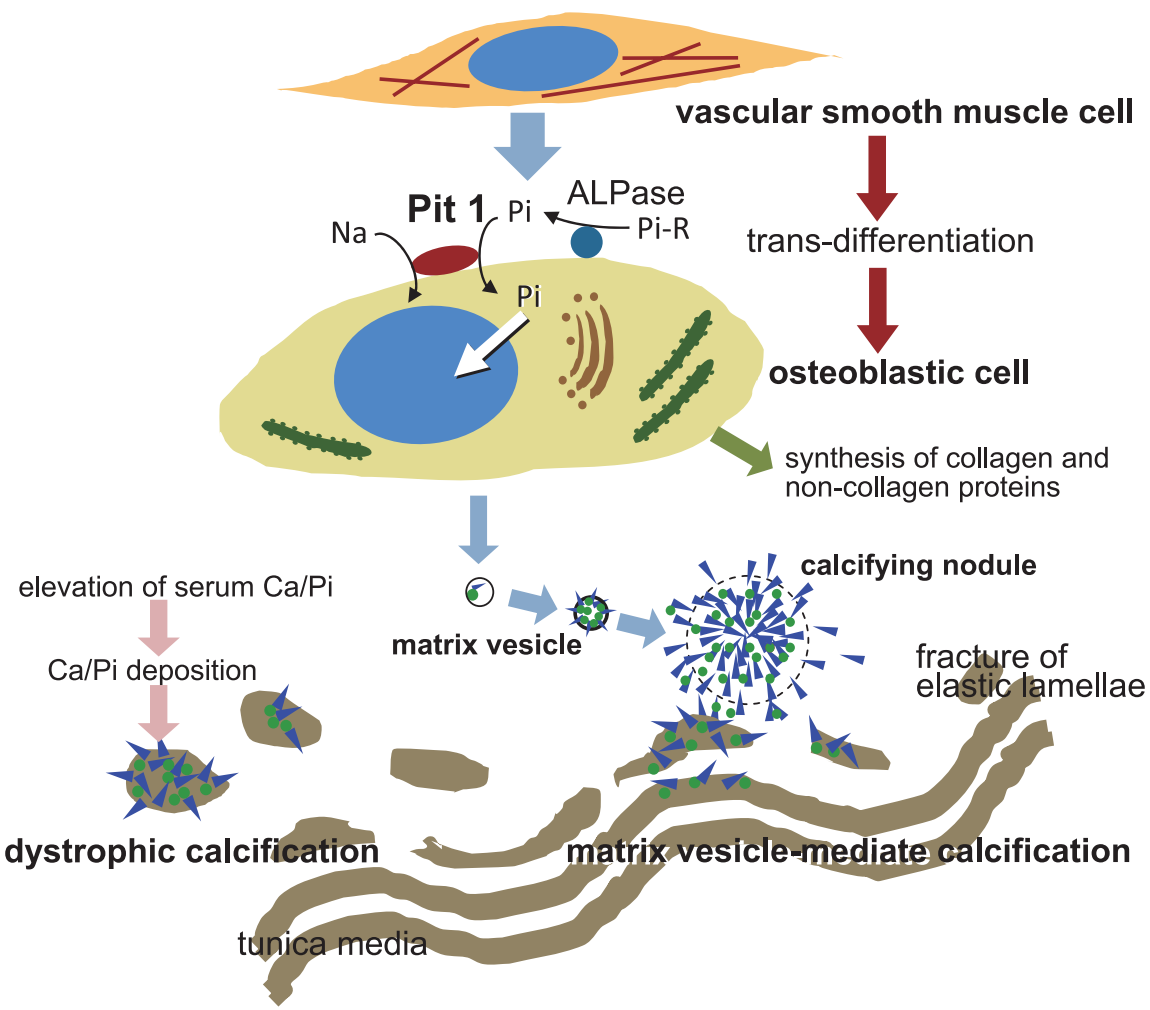

Fig. 3: Schematic design for trans-differentiation from vascular smooth muscle cells into osteoblastic phenotypes
Under circumstance of disrupted FGF23/klotho axis, vascular smooth muscle cells may differentiate into osteoblastic cells, which then, secret matrix vesicles and calcify fragmented elastic lamellae. Alternatively, the classical idea of dystrophic calcification in media was also seen inside the fragmented elastic lamellae. Therefore, it seems that two possible pathways for vascular calcification exist: one mediated by matrix vesicle-like structures, and other being occurring via deposition of calcium phosphates inside the amorphous materials.

Ⅳ. Trans-differentiation of vascular smooth muscle cells into the osteoblastic phenotype

One may ponder the origin of the matrix vesicle-like structures. Vascular calcification has previously been considered to be a passive dystrophic calcification resulting from oversaturation of serum calcium and phosphate. But, recently it has been reported to involve trans-differentiation, *i.e.,* dedifferentiation and reprogramming of VSMCs into the osteoblastic phenotype, which then initiates vascular calcification[34, 36, 38]. Thus, vascular calcification appears to be an active, biological process. VSMCs were reported to express runt-related transcription factor 2 (Runx2), osteopontin, osteocalcin, tissue nonspecific alkaline phosphatase (TNAPase), and ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1)[39~41]. It seems feasible that VSMCs differentiate into the osteoblastic lineage as many *in vitro* studies suggest that myoblasts have the potential to differentiate into osteoblasts after BMP administration[42~44]. In our observations on the *kl/kl* aorta, we verified immunoreactivity for TNAPase and ENPP1 instead of absence of α-smooth muscle actin, a hallmark of VSMCs. Yet, TNAPase and ENPP1 were weak, and the tunica media was calcified. Since klotho deficient circumstance induces high concentration of inorganic phosphate and calcium in serum, even without intense activity of TNAPase and ENPP1, there may be abundant inorganic phosphate available to easily induce medial calcification.

Extracellular matrix proteins also appear to play an important role in medial calcification. The presence of non-collagenous proteins was observed in medial calcification: MGP[15, 45], osteopontin[13, 14] and BMP-2[12] appear to be associated with earlier stages of vascular calcification. In our study, MGP, a pivotal inhibitor of calcification[15, 45], was abundantly expressed surrounding the calcified elastic lamellae and calcifying nodules in our *kl/kl* model. MGP-deficient mice developed calcification in the aorta and its branches, and typically develop aortic rupture as a direct consequence of vascular calcification[45]. Therefore, deficiency of MGP may be a critical factor for delaying vascular calcification[46].

Recently, the widely accepted idea that the initiation of vascular calcification is dependent on type III sodium-dependent phosphate co-transporter., *i.e.,* Pit-1 (also known as SLC20A1), which is mediated Runx2 expression[47, 48]. Highly elevated extracellular phosphate appears to induce the expression of Pit-1 in VSMCs[41, 49]. More recently, *in vitro* models have been developed to determine factors specific to CKD that might induce medial calcification[50]. Exposure of VSMCs into media containing elevated levels of calcium and phosphate rapidly induced calcification, with synergistic effects when both ions' levels were elevated[36]. In response to extracellular calcium and phosphate, viable VSMCs were induced to release matrix vesicle-like structures in a manner analogous for their induced calcification capacity *in vitro*, which indicates trans-differentiation of VSMCs into the osteoblastic phenotype[34, 37].

It therefore appears that a highly elevated serum concentration of phosphate serves as a trigger for trans-differentiation of VSMCs into the osteoblastic lineage.

Ⅴ. Comparison of calcification in calcified tunica media and in bone matrix

In our study, there were abundant calcified areas, as well as type I collagen fibrils, osteopontin, osteocalcin surrounding VSMC/osteoblastic cells in the *kl/kl* aorta. In addition, the presence of matrix vesicle-like structures indicates the similarity between the histological events found in medial calcification and in the bone matrix (Fig. 4). However, calcification in the *kl/kl* aorta was associated with elastic lamellae, rather than type I collagen, in spite of the large amount of existing collagen. The extensive vascular and soft tissue calcification paralleled by induction of a procalcification programming in *kl/kl* mice seems to mimic the excessive calcifications seen in patients with CKD, and both may occur via a similar osteoinductive signaling[51]. Zhu *et al.* have demonstrated the up-regulation of key osteocytic molecules during vascular calcification. Osteocyte formation and specifically sclerostin and E11 expression in vascular calcification may suggest not vascular calcification, but rather vascular ossification[52]. Consistently, our examination verified the existence of osteocyte-like cells and osteoclast-like cells in the *kl/kl* aorta. Considering the possibility of trans-differentiation of VSMC into osteoblasts during medial calcification, it seems likely that trans-differentiated osteoblasts would further progress into osteocytes.

The discovery of matrix vesicle-like structures was a breakthrough for further investigations in the research of medial calcification[53]. Authentic matrix vesicles are rich in acidic phospholipids such as phosphatidylserine and phosphatidylinositol, which have high affinity for calcium[54]. Several enzymes and proteins are involved in

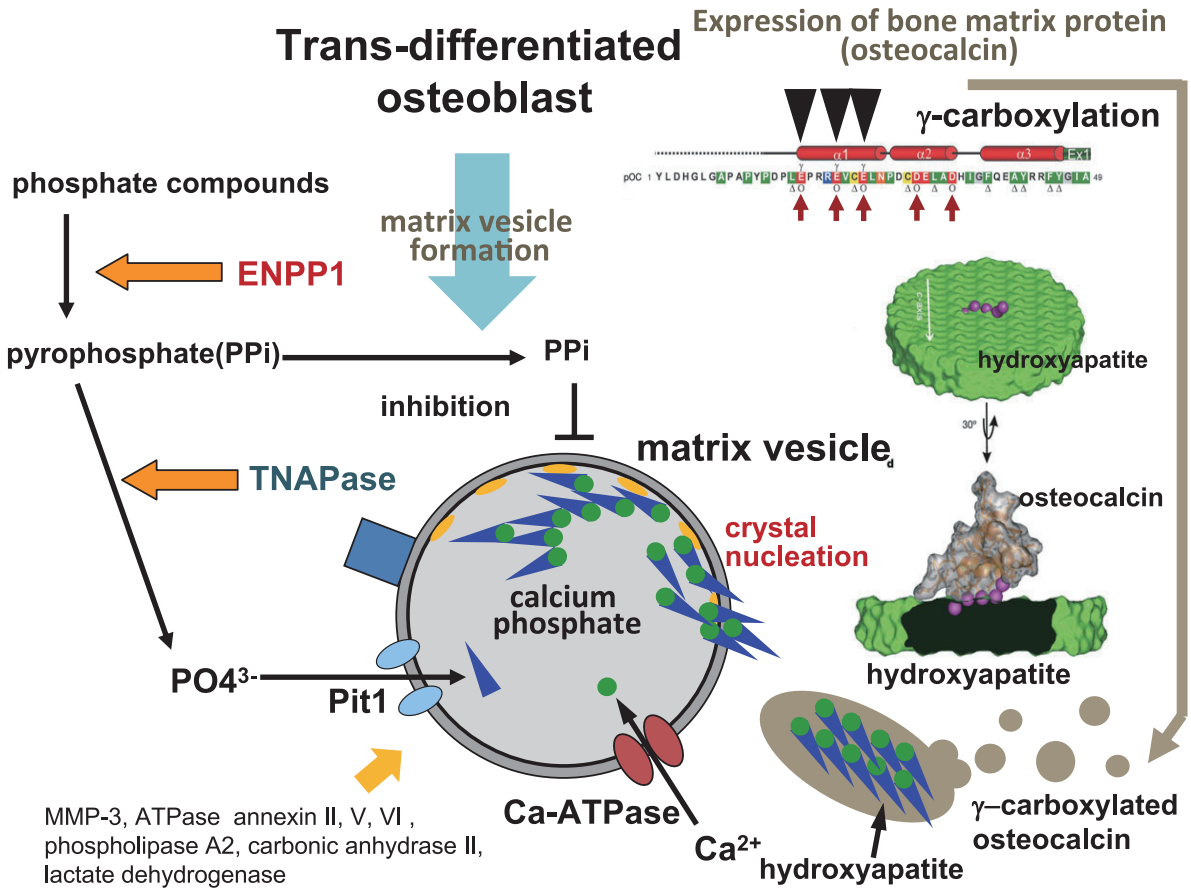

Fig. 4: Schematic design for putative action of matrix vesicles secreted by osteoblastic cells trans-differentiated from vascular smooth muscle cells

Authentic matrix vesicles acquire many enzyme-activities for mineral nucleation, adequate intake of calcium and phosphate ions, regulation of the crystal growth. It is necessary to investigate whether these enzymes are present within the matrix vesicle-like structures and how do they function in the process of medial calcification.

the metabolism of proteoglycans and pyrophosphate, and are also found in matrix vesicles. In addition to alkaline phosphatase activity[55~58], matrix metalloproteinase-3[59], ATPase[60], annexin II, V and VI[61], phospholipase A2, carbonic anhydrase II and lactate dehydrogenase[62] are believed to regulate crystal precipitation inside the matrix vesicles. In future studies, it is necessary to investigate whether these enzymes are present within the matrix vesicle-like structures and how do they function in the process of medial calcification (Fig. 4).

Concluding Remarks

Kl/kl mice developed medial vascular calcification, which seem to be due to hyperphosphatemia rather than being a consequence of faulty aging suppression mechanisms. Vascular calcification has previously been considered to be a passive dystrophic calcification resulting from the oversaturation of serum calcium and phosphate. However, recent studies have demonstrated the trans-differentiation, *i.e.*, dedifferentiation and reprogramming of VSMCs into the osteoblastic phenotype, thereby triggering vascular calcification. However, further studies are necessary to clarify the biological characteristics of the matrix vesicle-like structures, and to make clear how VSMCs can differentiate into the osteoblastic phenotype.

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