Roles of vitamin K-dependent protein in biomineralization (Review)

MENG ZHANG[1], QINGQI ZHANG[1], PENGFEI DU[1], XIN CHEN[1-3] and YUMEI ZHANG[1-3]

[1]College of Veterinary Medicine, Yangzhou University; [2]Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses; [3]Joint International Research Laboratory of Agriculture and Agri-Product Safety, The Ministry of Education of China, Yangzhou University, Yangzhou, Jiangsu 225009, P.R. China

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Abstract. Vitamin K (VK), a fat-soluble vitamin, is well known as an anticoagulant in the clinic. It is essential for the post-translational activation of VK-dependent proteins (VKDPs) because hydroquinone VK is a cofactor of glutamine carboxylase. At present, 17 VKDPs are known, which are mainly involved in coagulation and calcification. When Glu residues are carboxylated to Gla residues, these proteins gain a higher calcium-binding ability, which explains why VK has an important role in blood coagulation and biomineralization. However, the current view on the role of VK and several VKDPs in biomineralization remains inconsistent. For instance, conflicting results have been reported regarding the effect of *osteocalcin* gene knockout on the bone of mice; matrix Gla protein (MGP) promotes osteoblasts mineralization but inhibits vascular smooth muscle cell mineralization. The present review aimed to summarize the existing evidence that several VKDPs, including osteocalcin, MGP, Gla-rich protein and growth arrest specific 6 are closely related to calcification, including bone health, vascular calcification and lithiasis. The current review discussed these controversies and provided suggestions for future studies on VKDPs, i.e. taking into account dietary habits, geographical environments and genetic backgrounds.

Correspondence to: Professor Yumei Zhang, College of Veterinary Medicine, Yangzhou University, 12 East Wenhui Road, Yangzhou, Jiangsu 225009, P.R. China
E-mail: ymzhnet@sina.com

Abbreviations: VK, vitamin K; VKR, VK reductase; VKOR, VK epoxide reductase; VKDPs, VK-dependent proteins; MGP, matrix Gla protein; GRP, Gla-rich protein; Gas6, growth arrest specific 6

Key words: vitamin K, vitamin K-dependent proteins, osteoporosis, vascular calcification, urolithiasis

Contents

1. Introduction

Biomineralization is a complex process in which inorganic ions are deposited on organic matter under the action of proteins, hormones and enzymes (1,2). It has a critical role in a variety of physiological and pathological processes, such as bone and cardiovascular health, and lithiasis. Bone requires mineralization to maintain its strength and function. Insufficient mineralization and calcium (Ca) loss from bone may cause osteochondrosis and osteoporosis; however, excessive mineralization may lead to sclerosteosis (3). In addition, the excessive deposition of inorganic ions in soft tissues, such as blood vessels, joints and internal organs, is pathological, resulting in gall-stones, kidney stones and vascular calcification (4). Certain studies have reported that multivitamins, such as vitamin D (VD), vitamin C (VC) and vitamin K (VK), have important roles in maintaining bone and blood vessel health and preventing pathological calcium deposition or loss (5).

VD affects mineralization mainly by influencing Ca and phosphorus (P) metabolism and osteoclast activity (6). VC promotes bone mass via anti-oxidation, promoting collagen formation and inhibiting osteoclast activity (7). VK is a pivotal factor in the biomineralization balance. VK maintains blood vessel and bone health by promoting osteoblast differentiation, anti-oxidation and inhibiting cell autophagy and ferroptosis (8-10). In addition, the partial role of VK in biomineralization is thought to be associated with VK-dependent proteins (VKDPs), including osteocalcin, matrix γ-carboxyglutamic acid (Gla) protein (MGP), Gla-rich protein (GRP) and growth arrest specific 6 (Gas6) (11). The current review introduces the role of VK and several VKDPs in physiological and pathological bone mineralization.

2. The VK family

VK, a fat-soluble vitamin, was discovered in 1929 by the Danish biochemist Henrik Dam as being essential for blood coagulation. VK is a general term for compounds with different structural forms, including VK_1, VK_2, VK_3 and VK_4 (Fig. 1A), which share the same menadione structure (the 2-methyl-1,4-naphthoquinone core) (12). These VKs contain different numbers of isoprenoid side chains at the 3-position of the naphthoquinone ring (Fig. 1A), which is the reason for their different solubilities. VK_1, consisting of a side chain containing three isoprenes (liposoluble), is exclusively synthesized by algae and green plants. The different VK_2 (lip solubility) members are called menaquinones (MKn) according to the amount of isoprene in their side chain (MK-4 to MK-13) and are mainly found in fish, chicken, Japanese natto and cheese. In addition, certain components of the intestinal flora are able to produce MK-6, MK-8 and MK-11, the form and concentration of which are influenced by the composition of the intestinal microbiota. VK_3, without a side chain, is an artificially synthesized water-soluble vitamin. VK_4, a synthetic vitamin, is an oxidized form of VK_3 (12). To date, several international institutions have published a recommended daily intake (RDI) dose because of the important role of VK in physiological functions; however, the recommended RDIs are not consistent. The National Academy of Medicine recommends an adequate intake of 120 μg per day for an adult male and 90 μg per day for an adult female. The World Health Organization and the Food and Agriculture Organization recommend a VK dose of 65 μg per day for an adult male and 55 μg per day for an adult female (13,14).

3. Absorption, distribution and metabolism of VK

The different types of VK vary in their absorption, distribution and metabolism. As indicated in Fig. 1B, VK_1 and VK_2 from food are absorbed in the small intestine by forming a mixture containing bile salts, pancreatic lipolysis products and other dietary lipids, and then spread throughout the body via chylomicrons because of their fat-soluble nature. In addition, the absorption of VK_1 is related to three protein transporters, Neimann-Pick C1 like 1, scavenger receptor class B type I and CD36 (12,15). VK_3 and VK_4 are generally used for intramuscular injection and are absorbed in the intestines, without bile water solubility. In blood transportation, VK_1 and VK_2 are different. VK_1 is transported by triglyceride-rich lipoproteins, while VK_2 is mainly transported by low-density lipoproteins. Previous research reported that VK_1 is highly enriched in the liver and VK_2 is more widely distributed in the body's extrahepatic tissues. However, recent research has demonstrated that MK-4 is the major VK form in mammalian tissues, regardless of the dietary input of VK_1 or VK_2, which results from the action of the enzyme UbiA prenyltransferase domain-containing protein 1, which converts phylloquinone to MK-4. VK_1 and VK_2 are catabolized in the liver and excreted through a common degradation pathway. The two types of VK are reduced to hydroquinone in the liver and excreted after combining with glucuronic acid and sulfuric acid (Fig. 1C). In addition, a recent study found that ATP-binding cassette protein G5 (ABCG5)/ABCG8, a heterodimer exporting cholesterol, participates in the excretion of VK from the intestines (16).

4. Functions of VK

Although VK is well known as an anticoagulant, the function of VK in other physiological processes is being increasingly recognized. For instance, VK is able to regulate the immune response, maintain intestinal health and inhibit cancer growth (17,18). Clinically, VK has been used to treat and prevent numerous diseases, such as osteoporosis, atherosclerosis, intestinal diseases and cholestatic liver disease (17,19,20). Some of the mechanisms by which VK can treat and prevent these diseases are partly known. For instance, MK-4, converted from VK, interacts with the cell surface VK-binding nuclear receptor steroid and xenobiotic receptor to improve bone quality. In addition, VK exerts anti-inflammatory effects by inhibiting nuclear factor κB (NF-κB) signaling and antioxidant effects by blocking the production of reactive oxygen species (21). A recent study reported that reduced forms of VK, including menadione and chloroquinone, are potent anti-ferroptosis agents (22).

The most important role of VK is associated with the VK cycle. In this cycle, VK is firstly reduced to hydroquinone VK (KH_2) under the action of VK reductase (VKR) or VK-2,3-epoxidoreductase (VKOR). Subsequently, KH_2, as a coenzyme, assists carboxyglutamyl carboxylase (GGCX) to carboxylate VKDPs [specific glutamyl (Glu) residues in VKDPs are converted to Gla], while KH_2 is oxidized to epoxide VK (KO). Finally, KO is reduced to VK under the action of VKOR (23) (see Fig. 2). Currently, 17 members of the Gla protein family of VKDPs have been identified. These include S prothrombin, factor VII, factor IX, factor X, protein C, protein S and protein Z, which are crucial in maintaining the delicate balance of blood coagulation. In addition, there are MGP, osteocalcin, Gas6, GRP, periostin and periostin-like factors, which have significant roles in biomineralization. Furthermore, the family consists of two amino acid-rich Gla proteins and two transmembrane Gla proteins (24,25). When Glu residues are carboxylated to Gla residues, these proteins gain a higher calcium-binding ability, which is why VK has an important role in blood coagulation and biomineralization. The American Health Association recommends that VK is injected into newborns to reduce the risk of bleeding because of low levels of VK in their bodies. Warfarin is used to prevent and treat coagulation by decreasing the activity of VKOR. However, the long-term use of warfarin is associated with calcification of blood vessels, heart valves and osteoporosis (26-28). Verma *et al* (29) found that VK antagonism impairs the bone marrow microenvironment and bone density, and increases osteoclast activity.

5. VK and biomineralization

Biomineralization is a physical process that includes the maintenance of bone and teeth. However, inappropriate mineralization may cause several diseases, such as osteoporosis, vascular calcification and renal calculi.

Bone is composed of organic matter (type I collagen, proteins), inorganic matter and cells (osteoblasts, osteocytes

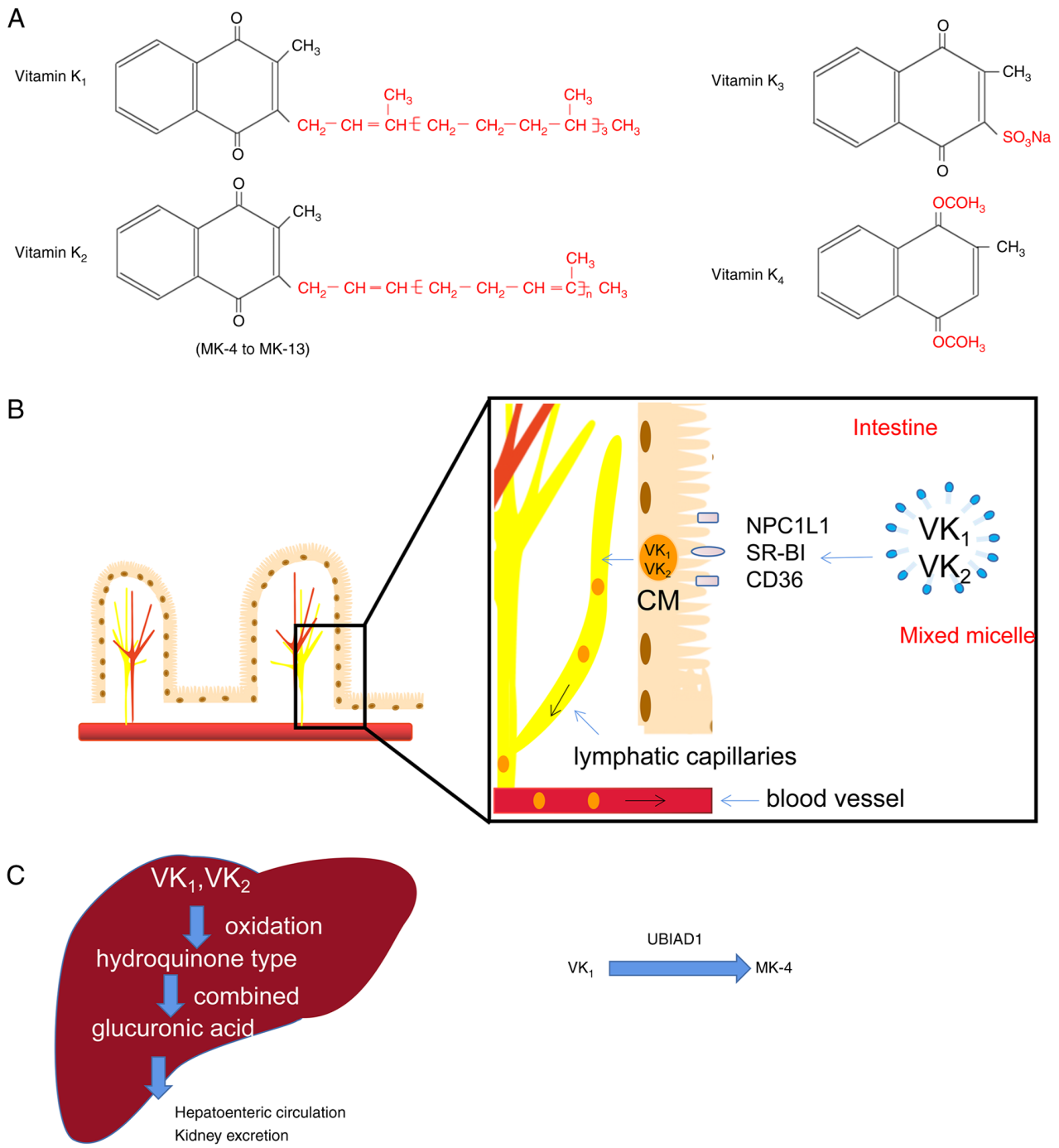

Figure 1. Structure, absorption and metabolism of VK. (A) Structure of VK. VK is a general term for a group of compounds including VK_{1-4}. VK_2 is divided into MK3-14 based on the amount of isoprene in the side chain. (B) Absorption of VK. VK_1 and VK_2 are absorbed in the small intestine through mixed micelles with the assistance of NPC1L1, SR-BI and CD36. Chylomicrons containing VK_1 and VK_2 in the small intestine then enter blood vessels through lymphatic capillaries and are transferred to tissues. (C) Metabolism of VK. VK, vitamin K; MK, menaquinone; NPC1L1, Neimann-Pick C1 like 1; SR-BI, scavenger receptor class B type I; UBIAD1, UbiA prenyltransferase domain-containing protein 1; CM, chylomicrons.

and osteoclasts). Phosphate-calcium (crystalline hydroxyapatite) combines with type I collagen to enhance bone strength through proteins (including VKDPs) and enzymes (30,31). Loss of Ca and the insufficient ability of Ca to bind collagen may cause osteoporosis and fracture. Excessive deposition of Ca/P may cause osteosclerosis, which increases bone fragility. At present, it is inconclusive whether VK can improve osteoporosis and reduce the fracture rate. A clinical study showed that VK deficiency is associated with osteoporosis and vascular calcification (25). A meta-analysis by Ma *et al* (32) showed that VK increased the bone mineral density (BMD) in the lumbar spine. In addition, VK can reduce bone loss by increasing osteoprotegerin levels (33). However, a meta-analysis by Salma *et al* (34) reported that VK supplementation reduced the fracture rate but did not improve the BMD in the femur and tibia. The notion that VK has a positive effect on bone mineralization is dominant, which is recognized by the European Food Safety Authority Association (35).

Vascular calcification, common in the elderly, and in patients with diabetes and chronic kidney disease (CKD), is

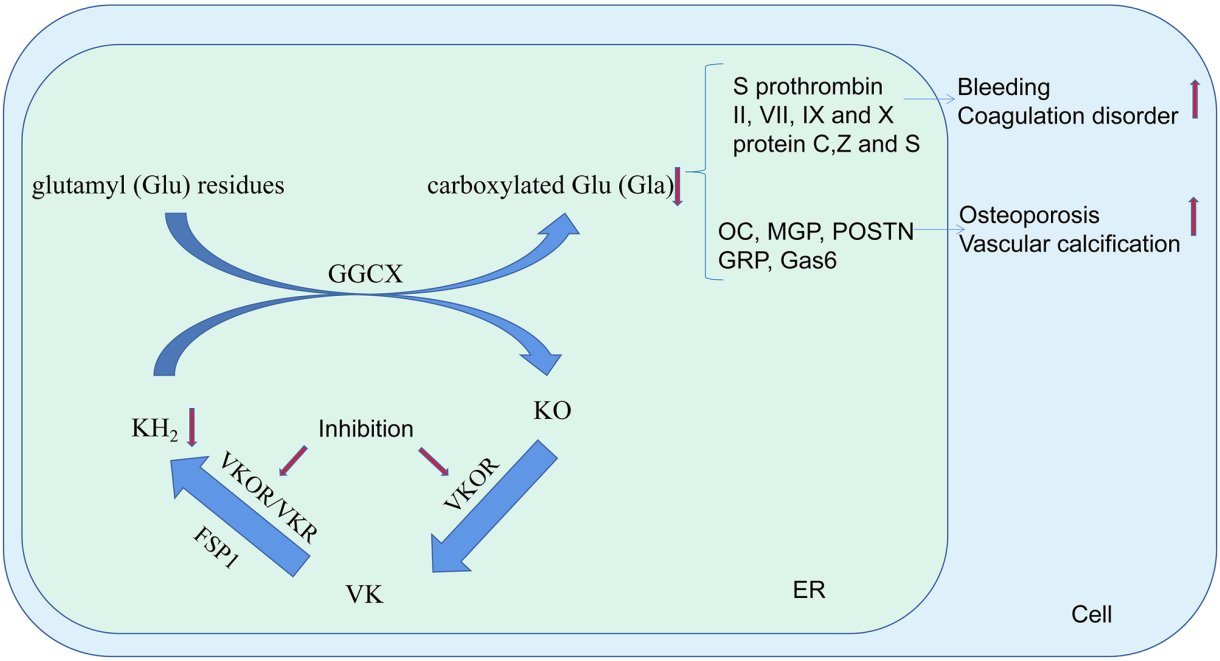

Figure 2. The VK cycle. The inhibition of VKOR and/or VKR decreases the level of KH_2, which is a cofactor of GGCX. This results in a decrease in carboxylation (Glu to Gla) of VK-dependent proteins and a subsequent increase in the risk of bleeding, osteoporosis and vascular calcification. ER, endoplasmic reticulum; VK, vitamin K; VKR, VK reductase; VKOR; VK epoxide reductase; MGP, matrix Gla protein; GRP, Gla-rich protein; Gas6, growth arrest specific 6; POSTN, Periostin; GGCX, carboxyglutamyl carboxylase; KO, epoxide VK; FSP1, ferroptosis suppressor protein-1.

caused by excessive Ca/P deposition in aortic elastin, which is the main component of the intermediate elastic fibers (4). Vascular calcification is affected by numerous factors. High expression of bone-related genes such as RUNX family transcription factor 2 (RUNX2) and bone morphogenetic protein-2 (BMP2) in vascular smooth muscle cells leads to the phenotypic transformation to osteoblasts (36). After phenotypic transformation, the high expression of alkaline phosphatase (ALP), osteopontin, osteocalcin and MGP induced by RUNX2 result in extracellular deposition of hydroxyapatite in a blood vessel, leading to atherosclerosis. Endoplasmic reticulum stress, mitochondrial damage and hyperphosphate blood damage vascular smooth muscle cells during vascular calcification (37,38). In addition, the lack of mineralization inhibitors (VKDPs) results in excessive deposition of hydroxyapatite crystals on blood vessels, which is also a key factor in vascular calcification (39). Treatment with VK antagonists for anticoagulation carries the risk of concomitant vascular or valvular calcification for patients (40,41). MK-7 supplementation may reduce the progression of vascular calcification in patients with coronary artery disease. VK may also reduce vascular calcification by decreasing the production of inflammatory cytokines. VK inhibits the production of tumor necrosis factor (TNF) in macrophages. TNF can promote the osteogenic differentiation of vascular smooth muscle cells under stimulation by high phosphate, oxidative stress and high glucose (42).

Urolithiasis is a common disease including stones of the kidney, bladder, ureter and urethra. It is affected by numerous factors, including hypercalcemia, dietary habits, obesity, diabetes and kidney disease. The most common types of stones are calcium oxalate, calcium phosphate and uric acid. Certain studies have demonstrated that the VK cycle and its dependent proteins are related to the formation of various urinary stones (43,44).

In general, there is a close relationship between VK and biomineralization. The effects of VKDPs on biomineralization are mainly described in the following sections.

6. VKDPs are essential in biomineralization

Role of osteocalcin in biomineralization. Osteocalcin, secreted by osteoblasts and osteocytes, was found in cattle (45). Osteocalcin is encoded by the BGLAP gene on chromosome 1 at 1q25-q31, is highly conserved among species and regulated by 1,25-dihydroxyvitamin-D3 [1,25(OH)D3] at transcription (46). It is the most abundant non-collagen protein (constituting ~20% of non-collagen proteins) in bone and has an important role in bone mineralization. After its synthesis, osteocalcin is targeted by a signaling peptide in primary structures to the extracellular matrix. It is then cleaved by a furin-like proteolytic enzyme at the RxxR site to produce a prodomain that only contains the γ-glutamyl carboxylase recognition site (47). Mature osteocalcin contains three Glu residues (at positions 17, 21 and 24), which endow different Ca-binding abilities depending on their degree of carboxylation (48). After carboxylation under the action of GGCX and VK, osteocalcin with a high binding ability to the bone matrix is secreted into the bone matrix. Undercarboxylated osteocalcin (uc-osteocalcin) is secreted into the blood because of its low binding ability (Figs. 3 and 4). In addition, osteocalcin in bone is released into the blood by decarboxylation of osteoclasts.

The levels of uc-osteocalcin in the blood are used as a maker for the clinical detection of osteoporosis and VK deficiency. However, clinical trials concerning the relationship

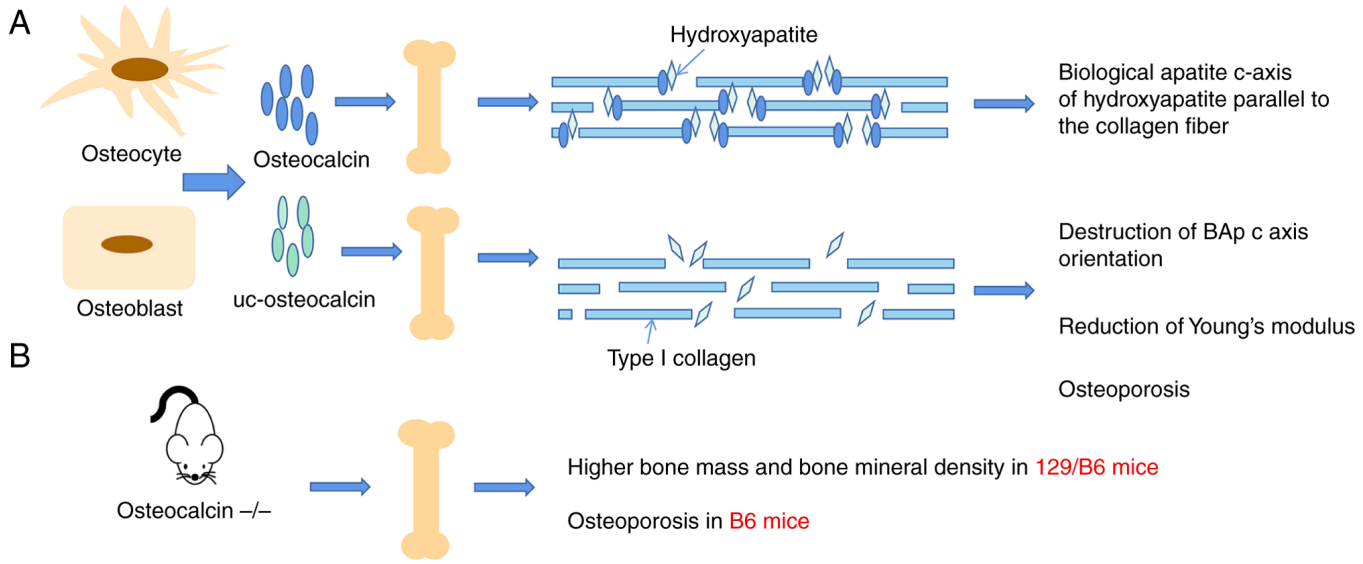

Figure 3. Effect of osteocalcin on bone in mice. Osteocalcin is secreted by osteoblasts and osteocytes. (A) Osteocalcin is secreted into bone to maintain the arrangement of collagen and hydroxyapatite. Lack of osteocalcin in bone leads to a disturbed crystal arrangement and osteoporosis. (B) 129/B6 mice with osteocalcin deficiency exhibit higher bone mass and bone mineral density; however, B6 mice with osteocalcin deficiency exhibit osteoporosis. Uc, undercarboxylated; BAp, biological apatite.

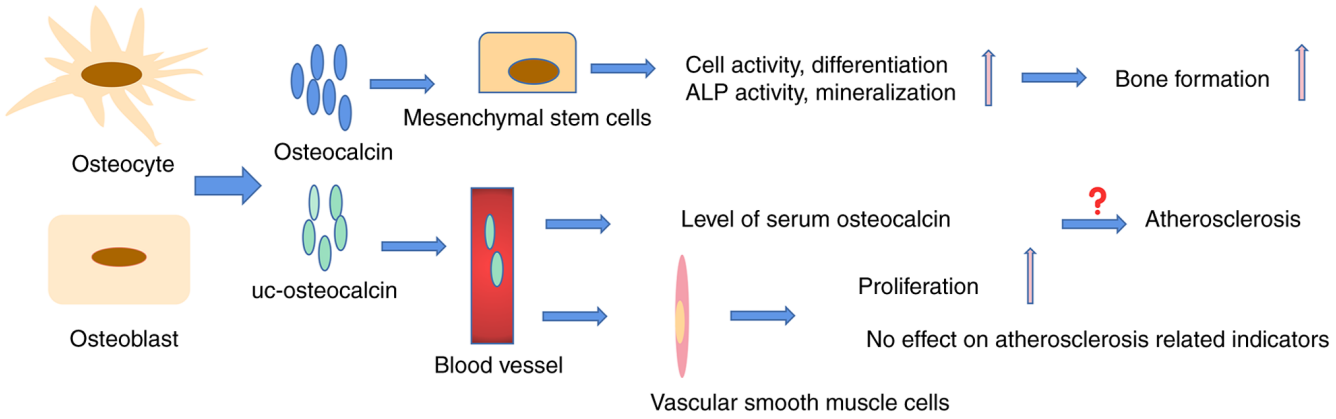

Figure 4. Effect of osteocalcin on human MSCs and human vascular smooth muscle cells. Osteocalcin can promote the transformation of preosteoblasts and MSCs into osteoblasts by promoting cell activity and ALP expression. The association between osteocalcin, undercarboxylated osteocalcin and vascular atherosclerosis requires further validation. ALP, alkaline phosphatase.

between osteocalcin and osteoporosis are not consistent. Most clinical research reported that high serum osteocalcin (uc-osteocalcin and osteocalcin) levels are closely associated with a lower BMD and higher fracture risk (49,50). However, other studies determined that the serum osteocalcin level was not associated with BMD (32,51). A reasonable explanation is that the reagents for detecting serum osteocalcin are not uniform, i.e., antibodies cannot distinguish uc-osteocalcin from osteocalcin. However, the effect of osteocalcin on bone has also been controversial in animal studies. It was reported that the bone biophysical properties in osteocalcin-deficient mice were altered, with fewer hydroxyapatite crystals, resulting in increased bone fragility and fracture rates. A recent study reported increased bone fragility and altered energy dissipation mechanisms in osteocalcin-deficient mice and highlighted the role of glycation of osteocalcin in bone (52). The biological apatite (BAp) c-axis of hydroxyapatite parallel to the collagen fiber is necessary for optimal bone strength. Microbeam X-ray diffraction system analysis showed that osteocalcin-knockout mice have a disrupted BAp c-axis orientation, which demonstrated that osteocalcin is necessary for the alignment of the BAp c-axis parallel to collagen fibers. The results of a nanoindentation test showed that the Young's modulus of the femur in osteocalcin-knockout mice was significantly lower than that of wild-type mice (Fig. 3) (53). However, the osteocalcin-deficient mice were found have a higher bone mass and BMD, which resulted in osteocalcin being identified as a negative regulator of bone formation (54,55). Of note, in subsequent studies, the same research group reported that knockdown of osteocalcin in B6 mice resulted in a lower BMD and bone strength (Fig. 3) (56). This opposite effect was caused by different genetic backgrounds. Although warfarin (a VK circulation inhibitor) has a controversial role in BMD, certain studies suggest that it has a negative effect on bone. Impaired

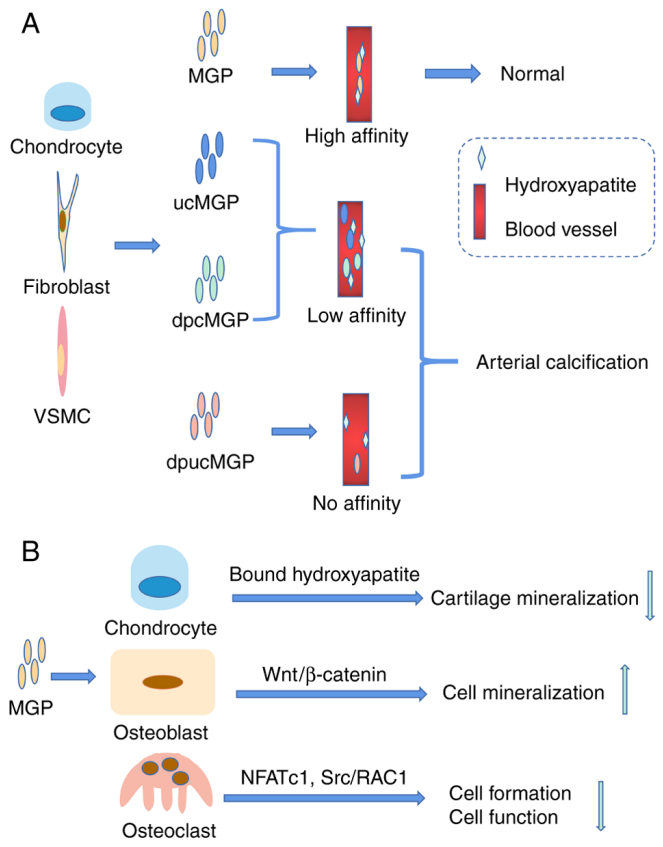

Figure 5. Effects of MGP on animals' bone and blood vessels. (A) The four forms of MGP and their affinity for calcium and phosphorus. Low affinity of MGP for hydroxyapatite results in arterial calcification. (B) The effect of MGP on chondrocytes, osteoblasts and osteoclasts. ucMGP, undercarboxylated matrix Gla protein; dpcMGP, carboxylated but unphosphorylated MGP; dpucMGP, undercarboxylated and dephosphorylated MGP; VSMC, vascular smooth muscle cell; NFATc1, nuclear factor of activated T cells, cytoplasmic 1; Src, steroid receptor coactivator; RAC1, ras-related C3 botulinum toxin substrate 1.

osteoblast function and osteoporosis have been described in mice receiving warfarin (29). In addition, warfarin was able to further reduce the bone calcium loss caused by 1,25(OH) D3, which is thought to be related to osteocalcin. In an *in vitro* study, osteocalcin promoted the proliferation, ALP activity and mineral deposition of human osteoblast-like MG63 cells (57). Similarly, Tsao *et al* (58) also confirmed that osteocalcin has an important role in osteogenic differentiation and mineralization processes of mesenchymal stem cells. Overall, osteocalcin has a positive effect on bone and osteoblasts, but the opposite result may occur in different genetic backgrounds.

More than a decade ago, a strong association between osteocalcin and atherosclerosis was reported (59). This finding was subsequently confirmed in clinical investigations (60,61). For instance, in a five-year study of 9,413 patients with type 2 diabetes, patients with lower serum osteocalcin levels had a higher risk of all-cause and cardiovascular mortality (62). A study of 59 patients with atherosclerosis identified osteocalcin in any form as a biomarker of vascular risk (63). Guo *et al* (64) demonstrated that higher serum osteocalcin levels were associated with severe arteriosclerosis in patients with kidney disease. *In vitro*, osteocalcin is also used as an important indicator to detect the transformation of vascular smooth muscle cells into osteoblasts, as it is highly expressed in the early differentiation of osteoblasts (65). However, recently, certain clinical investigations found that there was no association between osteocalcin and atherosclerosis (Fig. 4) (66-68). Research by Millar *et al* (66) demonstrated that osteocalcin has no effect on the calcification of vascular smooth muscle cells, suggesting that osteocalcin is not directly involved in atherosclerosis or vascular disease. Certain researchers even proposed that osteocalcin has a good protective effect on vascular calcification in animal experiments (70,71). A long-term high-sugar and -fat diet is a factor in the development of atherosclerosis. Huang *et al* (70) found that osteocalcin improved the protective effect against the induction of arteriosclerosis in diabetic rat models by affecting glucose levels, insulin sensitivity and lipid metabolites. Osteocalcin had an endothelial-protective effect in mouse thoracic aortic atherosclerosis caused by apolipoprotein E (ApoE) knockout (71). Therefore, it may be speculated that the high serum level of osteocalcin in patients with atherosclerosis may be the body's attempt to produce more osteocalcin to prevent the process of atherosclerosis.

Role of MGP in biomineralization. MGP (a 14 kDa protein) is a calcification inhibitor that was first isolated from bovine bone matrix in 1985 (72). The MGP gene consists of four exons separated by three large intervening sequences; in addition, the typical TATA and CAT boxes, and the binding sites of retinoic acid and VD, were identified at regions of the MGP gene promoter (73). It is expressed in chondrocytes, fibroblasts and vascular smooth muscle cells. The structural features of MGP are similar to those of osteocalcin, both sharing a common historic ancestor. As for osteocalcin, MGP contains a signaling peptide in primary structures; in addition, it contains five Glu residues and three serine phosphorylation sites at its N-terminus (72,74). MGP Gla residues provide binding sites for Ca and hydroxyapatite. In addition, phosphorylation is important for its function and influences its protein structure (74). Therefore, there are four forms of MGP in the body, including MGP, carboxylated but underphosphorylated MGP, phosphorylated but undercarboxylated MGP, and completely inactivated dephosphorylated and undercarboxylated MGP (dpucMGP) (Fig. 5A).

MGP has an important role in the activity of bone cells, bone calcification and osteoarthritis (OA). MGP gene mutations and the long-term use of VK antagonists are associated with an increased risk of OA and a decrease in BMD (75,76). Osteoporosis was found in MGP knockout mice because of osteoblast dysfunction. Recently, Laurent *et al* (77) demonstrated that the femur size of MGP-deficient mice was smaller in the early stages of life. The Wnt/β-Catenin signaling pathway is important in osteoblast differentiation, promoting the expression of osteogenic differentiation genes through RUNX2. Zhang *et al* (78) demonstrated that MGP promotes the proliferation and mineralization of MG63 osteoblasts and improves osteoporosis caused by ovariectomy through the Wnt by/β-Catenin signaling pathway. The effect of MGP on bone cells is shown in Fig. 5B. A high-fat diet of pregnant mice resulted in a decrease in bone structure of their offspring 6 weeks after birth, which is thought to be related to the level of MGP gene expression (79). Further experiments in MGP knockout mice confirmed the role of MGP in high-fat

diet-induced bone mass loss (80). Conversely, another view is that MGP is a bone calcification inhibitor. MGP inhibits bone matrix formation and hydroxyapatite deposition (81). MGP inhibits the formation and function of osteoclasts through nuclear factor of activated T cells, cytoplasmic 1 and steroid receptor coactivator/ras-related C3 botulinum toxin substrate 1 signaling (82). Certain studies have indicated that overexpression of MGP inhibits cartilage mineralization and endochondral ossification. MGP-deficient mice present with inappropriate calcification of cartilage, short stature, osteopenia and fractures (83). In addition, the expression of MGP in chondrocytes is regulated by extracellular inorganic phosphorus, which may represent a feedback regulatory mechanism to inhibit inappropriate calcification in cartilage (84). Of note, ectopic expression of MGP in human osteosarcoma (OS) cells significantly increased cancer cell metastasis to the lung in patients with OS, which may lead to poor prognosis (88). In general, MGP maintains bone health by affecting cartilage mineralization, osteoblast function and osteoclast activity.

MGP was the first identified vascular calcification inhibitor, which combines with Ca/P to prevent its deposition in blood vessels. Several studies have indicated that the serum level of MGP is associated with vascular diseases. A prospective study reported that the levels of plasma dpucMGP were strongly associated with vascular death, from an average of 15.5 years of follow-up among 684 elderly patients aged 50-89 years (86). In an observational study of 7,066 adults, a significant association between high levels of ucMGP and arterial stiffness was observed. In a subsequent experimental study, it was reported that MGP heterozygous mice showed arterial stiffness (87). In animal studies, MGP-deficient mice showed severe vascular calcification and premature bone mineralization, and died within two months because of vascular rupture caused by arterial calcification (83). In addition, rats treated with warfarin for a long time developed extensive vascular calcification, indicating that carboxylation of MGP has a key role in inhibiting vascular calcification. However, a recent study suggested something different. Parashar *et al* (88) constructed MGP mutant mice and found that the serine residues at the MGP N-terminus and glutamate at the C-terminus had a synergistic effect to inhibit vascular tissue calcification; however, the serine residues had a more critical role. In addition, a study used immunohistochemistry to confirm that cMGP and pMGP have anti-calcification effects in veins (89). Based on its high anti-calcification ability, MGP has been considered an agent with great potential in preventing calcic aortic valve disease (90).

The polymorphism of the MGP gene is related to kidney stones in Chinese Han and Japanese populations (91,92). Li *et al* (43) confirmed that VK_1 reduced the crystal deposition in HK2 cells by promoting MGP expression. In rats with hyperoxaluria induced by 0.75% ethylene glycol, crystals were only deposited in the damaged renal tubules lacking MGP expression, indicating that MGP has a protective role in maintaining cell survival and inhibiting crystal retention (93). Goiko *et al* (94) detected the effect of MGP on hydroxyapatite formation and calcium oxalate crystallization using dynamic light scattering and scanning electron microscopy. The results showed that MGP inhibited the formation of calcium oxalate monohydrate, whether the polypeptide was in the modified or unmodified form after translation. In addition, high concentrations of Ca significantly inhibited the expression of MGP and then promoted the mineralization of NRK-52E cells. However, Castiglione *et al* (95) found no significant difference in serum dpucMGP levels among 498 cases of calculous formers and 395 cases of non-calculous formers after the evaluation of symptomatic patients with recurrent kidney stones within 5 years, indicating that the increase in serum dpucMGP was not related to recurrent renal stone events.

Role of GRP in biomineralization. GRP, a newly identified VKDP, was isolated from an Adriatic sturgeon by Viegas *et al* (96) in 2008. In the same year, two other independent groups identified this gene and named it 'upper zone of growth plate and cartilage matrix associated protein' (UCMA) while searching for chondrocyte-specific genes (97,98). Later, GRP was found in the skin, vasculature and mammary gland and it has been described to be conserved among different vertebrate species. The GRP gene contains 5 coding exons, which are separated by phase-1 introns. After removal of the transmembrane signaling peptide, pro-GRP containing 135 amino acids is cleaved into a propeptide (38-39 amino acids) and a mature peptide (67-74 amino acids) (99). At present, only a small number of studies on regulating GRP gene expression are available. Le Jeune *et al* (97) reported that E-twenty six-related gene transcription factors was indicated to regulate GRP gene transcription by combining ETS binding site. Runx2 and osterix (OSX) were identified as direct transcriptional promoters by DNA microarray analysis of heterozygous embryos. In addition, transcription factor E2-α (E47), myocyte enhancer factor 2 and the signal transducer and activator of transcription 1 were predicted as the human GRP gene promoter (100). GRP got its name because it contains highest content of Gla residues (15 or 16 Gla sites in sturgeon and human) among VKDPs, so it is considered to have the highest Ca binding affinity (96,101). The accumulation of GRP in pathological calcification sites seems to confirm its Ca-binding ability (99). In addition, ucGRP also has the ability to bind Ca/P (102).

The role of GRP in bone is controversial in different species. GRP gene deficiency experiments in zebrafish showed that a lack of GRP and inhibition of GRP carboxylation resulted in severe growth retardation and bone development disorder, which indicated the important role of GRP in bone development (103). However, this was not observed in mice. GRP-deficient mice did not show any obvious defects in bone and cartilage, indicating that GRP was not necessary for mouse bone development (Fig. 6A). In cells, previous studies reported that GRP is an inhibitor of osteogenic differentiation; however, two studies by Lee *et al* (104,105) found the opposite result, as they suggested that GRP is regulated by RUNX2 and OSX (osteogenic differentiation proteins) and recombinant GRP promotes MC3T3 cell osteogenic differentiation and mineralization, suggesting that it is a candidate bone mineralization promoter (Fig. 6A). OA is a movement-limiting joint disease and is characterized by loss of articular cartilage, tissue inflammation, abnormal bone formation and extracellular matrix mineralization. Studies have reported that the serum GRP concentration is positively correlated with OA; however, it may be a compensatory response mechanism. ucGRP/GRP

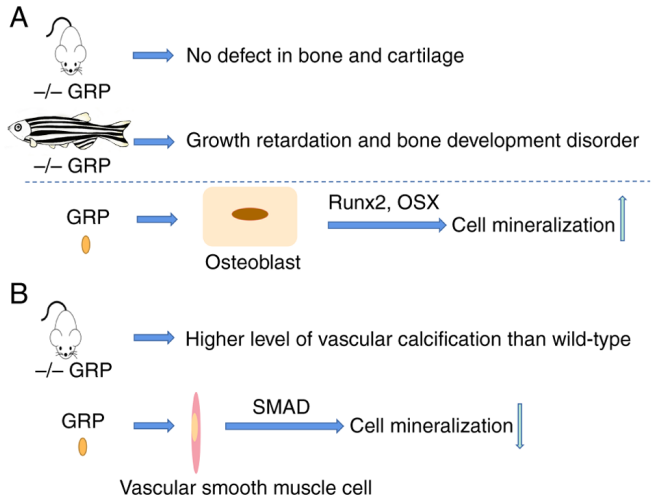

Figure 6. Effect of GRP on animals' bone and blood vessels. (A) GRP deficiency inhibits bone development in zebrafish but not in mice. GRP promotes osteoblast mineralization via Runx2 and OSX. (B) GRP inhibits vascular smooth muscle cell mineralization through inhibition of SMAD. GRP, Gla-rich protein; OSX, osterix; Runx2, RUNX family transcription factor 2.

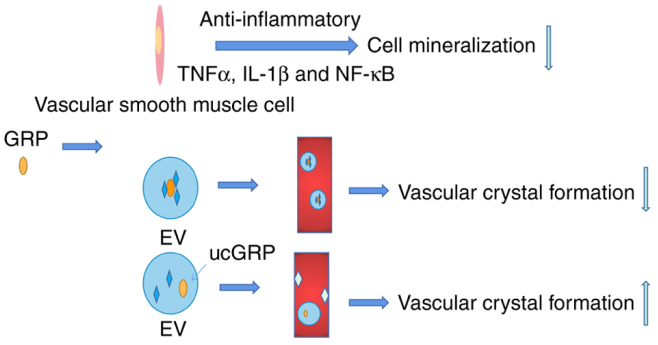

Figure 7. Effect of GRP on human vascular calcification. GRP inhibits vessel mineralization through anti-inflammatory and binding calcium and phosphorus in EVs. ucGRP, undercarboxylated Gla-rich protein; EV, extracellular vesicle.

reduced the mineral deposition of chondrocytes and synovial cells derived from OA, which may be associated with inhibition of aggregase activity and the anti-inflammatory effect of GRP with or without γ-carboxylation (106). Thus, GRP has been proposed as a potential therapeutic candidate in OA (106).

First, Bordoloi *et al* (107) reported that GRP accumulates in pathological calcification sites of the skin and blood vessels. This may be because of the lack of antibodies to distinguish whether the protein is carboxylated at that time. A subsequent study pointed out that ucGRP occupies a dominant position in the area of vascular mineralization (108). Vascular smooth muscle cells from GRP-deficient mice had a higher level of calcification than wild-type cells, suggesting that GRP is an inhibitor of vascular calcification, as is MGP (Fig. 6B) (109). A five-year cross-sectional interview site study of patients with diabetic nephropathy indicated that the decrease in serum GRP levels was closely related to an increased risk of aortic and mitral valve calcification. Although the complex mechanisms of action of calciprotein particles (CPPs) and extracellular vehicles (EVs) in vascular calcification remain to be fully elucidated, CPPs and EVs have been suggested as protectors that prevent vascular calcification in healthy individuals. The change in the contents of EVs and CPPs are key factors that increased mineral deposition in patients with CKD. A study by Viegas *et al* (110) demonstrated that decreased levels of GRP in circulating CPPs and EVs are a main factor for CPP and EV pathogenicity. Of note, the addition of ucGRP to CCPs and EVs from the serum of patients with CKD did not reduce the mineralization of vascular smooth muscle cells induced by the serum of patients with CKD, suggesting the importance of carboxylation in preventing mineralization (Fig. 7) (110). In addition to combining with calcium phosphate, GRP inhibits crystal formation by inhibiting downstream signaling pathways. GRP was shown to inhibit vascular smooth muscle cell calcification and osteogenic differentiation through upregulation of actin and downregulation of osteopontin (108). The P imbalance increased systemic inflammatory response, and local microinflammation are major inducers of vascular calcification. Willems *et al* (109) pointed out that GRP inhibits phosphate-induced vascular smooth muscle cell calcification by inhibiting SMAD-dependent BMP signaling. Whether GPR is carboxylated or not, it may inhibit inflammation by downregulation of a variety of proinflammatory factors, including TNF-α, IL-1β and NF-κB (Fig. 7) (111).

Role of Gas6 in biomineralization. Gas6, a secreted protein with a high degree of amino acid conservation, was found in mouse fibroblasts (112), and was then successively identified in myeloid progenitor cells, endothelial cells, vascular smooth muscle cells and macrophages. The Gas6 gene, containing 15 exons, was isolated from the human chromosomal location 13q34 (113). Gas6 has high homology (44%) with anticoagulant protein S, including an extensively γ-carboxylated amino-terminal, four epidermal growth factor-like motifs and a large carboxy-terminal region, known as the D domain; however, it has no direct effect on blood coagulation (112). Gas6 has a Gla amino acid domain at its N-terminus and is a member of the VKDPs. Although Gla residues are important for Gas6 binding to its receptor, Gas6 does not act as a promoter or inhibitor of calcification by combining with Ca/P, as would have been expected. Gas6 is a ligand for tyrosine kinase receptors, including tyrosine-protein kinase receptor 3 (Tyro3), AXL receptor tyrosine kinase (Axl) and c-mer proto-oncogene tyrosine kinase (Mer). Axl has the highest affinity for Gas6 among the three receptors (112). Gas6 is mainly involved in the pathogenesis of inflammation, atherosclerosis and cancer through the Axl receptor. In addition, Gas6 is also considered a potential target for the treatment of malignant tumors because of the role of the Axl receptor in a variety of human malignant tumors (114).

Gas6 promotes the absorptive activity of osteoclasts by promoting the autophosphorylation of the Tyro3 receptor on osteoclasts (115). The GAS6 mRNA level in bone marrow increased after ovariectomy, suggesting that it may be related to bone loss caused by estrogen deficiency (115). However, this is inconsistent with the treatment of osteoporosis in postmenopausal women with VK. Gas6 inhibited the mRNA expression of collagen 2 and aggrecan, which are the building blocks of cartilage. In addition, Gas6 inhibits chondrocyte differentiation through the Erk1/2 and serine/threonine kinase 39/JNK

pathways (115). However, Hutchison *et al* (116) reported the opposite result: Collagen type II α1 chain (Col2a1) was upregulated after long-term Gas6 treatment, whereas short-term Gas6 treatment decreased the Col2a1 level.

Pericytes, a component of the microvasculature, have a potential role in osteogenic differentiation (117), and are similar to bone marrow stromal cells. An *in vitro* study found that inhibition of the Gas6/Axl signaling pathway increased the rate of pericytes, which could be alleviated using recombinant Gas6 (118). Inflammation, age, hyperphosphatemia and CKD are the main factors of vascular calcification. Gas6 has a role in the antagonism of adiponectin toward TNF, thereby inducing vascular calcification (119). Postmenopausal women have a higher prevalence of vascular calcification, indicating the protective effect of estrogen. Nanao-Hamai *et al* (120) reported that estradiol inhibits vascular smooth muscle cell calcification through estrogen receptor α-mediated transactivation of Gas6. In older men, testosterone levels decrease with age. There is an association between testosterone levels and atherosclerosis (120). Testosterone delays the progression of vascular smooth muscle cells through the Gas6/Axl axis (122). The apoptosis of aortic smooth muscle cells induced by high phosphorus is a major inducer of vascular calcification. The level of Gas6 decreased in apoptotic cells and restoring the Gas6/Axl signaling axis could alleviate calcification through an anti-apoptotic effect, which is the mechanism by which VK2 inhibits vascular smooth muscle cell calcification (123). In addition, studies have shown that several drugs inhibit vascular smooth muscle cell calcification via this mechanism, such as statins (124), and α-lipoic acid and taurine (125). In general, Gas6 can resist vascular mineralization complications mainly by activating Axl downstream signals. However, the Ca contents of wild-type and Gas6-/-mouse aortas were similar (125). This indicated that Gas6/Axl may have other, as-yet-unknown mechanisms in pathological conditions, which require further investigation.

7. Conclusion

In general, VK and VKDPs have an important role in biomineralization; however, their involvement and mechanisms are complex and controversial. Clinical investigations of the effect of VK on bone quality and cardiovascular health in different regions reported inconsistent results, which may be related to different dietary habits, geographical environments and genetic backgrounds (20,34,126). In addition, different VKDPs have opposite effects on the same tissue, which may also be one of the reasons for the different results of clinical investigations of VK on bone quality (VK deficiency or supplementation affects the carboxylation of total VKDPs). Similarly, in animal studies on the effects of VKDPs on bone quality, a VKDP may have different or even opposite results for the same tissue under different genetic backgrounds. Therefore, when studying the effect of VKDPs on bone quality, it is necessary to select experimental animals with an appropriate genetic background. In addition, warfarin exhibits wide inter-individual differences in its pharmacodynamic effects, resulting from polymorphisms in genes involved in the uptake of VK, including ApoE, VKORC1 and GGCX (127,128). Without any doubt, these gene polymorphisms also result in individual differences in the quality of bone and blood vessels under circumstances such as VK deficiency or warfarin treatment.

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Authors' contributions

MZ collected the data and wrote the manuscript. QZ and PD selected and classified studies in the literature search. YZ was involved in the study design. YZ and XC gave important suggestions and revised the manuscript during the writing process. All authors have read and approved the final manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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