The role and mechanism of protein post-translational modification in vascular calcification (Review)

DONGYAN WANG[1*], QIN LI[1*] and CAIDIE XIE[2]

[1]Department of Medical Science, Yangzhou Polytechnic College, Yangzhou, Jiangsu 225100, P.R. China;
[2]Department of Nephrology, Nanjing Second Hospital, Nanjing Hospital Affiliated to Nanjing University of Traditional Chinese Medicine, Nanjing, Jiangsu 210037, P.R. China

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Abstract. Vascular calcification is closely associated with morbidity and mortality in patients with chronic kidney disease, atherosclerosis and diabetes. In the past few decades, vascular calcification has been studied extensively and the findings have shown that the mechanism of vascular calcification is not merely a consequence of a high-phosphorus and high-calcium environment but also an active process characterized by abnormal calcium phosphate deposition on blood vessel walls that involves various molecular mechanisms. Recent advances in bioinformatics approaches have led to increasing recognition that aberrant post-translational modifications (PTMs) play important roles in vascular calcification. This review presents the latest progress in clarifying the roles of PTMs, such as ubiquitination, acetylation, carbamylation and glycosylation, as well as signaling pathways, such as the Wnt/β-catenin pathway, in vascular calcification.

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Correspondence to: Professor Caidie Xie, Department of Nephrology, Nanjing Second Hospital, Nanjing Hospital Affiliated to Nanjing University of Traditional Chinese Medicine, 1-1 Zhong Fu Road, Nanjing, Jiangsu 210037, P.R. China
E-mail: cnjxcd23@126.com

*Contributed equally

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1. Introduction

Vascular calcification (VC), which is associated with increased cardiovascular morbidity and mortality, is characterized by abnormal calcium phosphate deposition on blood vessel walls and osteogenic transdifferentiation of vascular smooth muscle cells (VSMCs) (1) VC can occur in elderly individuals and patients with chronic kidney disease (CKD), atherosclerosis, diabetes, or systemic lupus erythematosus (2). VC is a major factor associated with cardiovascular disease in patients with CKD. The Kidney Disease: Improving Global Outcomes 2017 Clinical Practice Guideline suggests that patients with CKD and with known VC have the highest risk among patients with CKD for cardiovascular events (3). VC increases in patients with either type 1 diabetes mellitus (DM) or type 2 DM (4) and the prevalence of VC is far greater in DM patients than in individuals without DM (5-7). Calcification is a hallmark of atherosclerosis (8). Coronary arterial calcification is indeed a process involved in of atherosclerosis development and occurs almost exclusively in atherosclerotic arteries (9,10). In addition, calcification can occur in the abdominal aorta and heart valves (11,12). VC induces atherosclerosis, increases vascular stiffness and decreases vascular compliance (13).

Similar to osteogenesis, VC involves diverse factors and mechanisms. Exposure to various stimuli leads to an imbalance between the promotion and inhibition of osteogenesis, ultimately leading to VC (14). VSMCs undergo a phenotypic switch during VC (15). This switch is accompanied by the upregulation of bone-related proteins, including runt-related transcription factor 2 (Runx2), bone morphogenetic protein 2 (BMP-2) (16) and osteocalcin and the downregulation of Sirtuins (SIRTs) (17,18) and contractile proteins, including smooth muscle actin α and smooth muscle 22α (14). In addition, long noncoding RNAs are involved in the occurrence of VC (19).

VC is very common and leads to increases in the incidence and mortality of cardiovascular diseases (20). Traditional pathogenic factors cannot fully explain the high prevalence of VC (21,22). Therefore, identifying new key regulators and new therapeutic targets involved in the pathogenesis of VC is a crucial requirement.

Post-translational modifications (PTMs) are chemical modifications of specific amino acid residues after protein synthesis and markedly influence the biological function of

proteins (23). Different types of PTMs, as well as the same modification at different sites, often have different effects on the biological behavior and mediate functions of the modified protein (24). In addition, PTMs may interact with each other and co-operatively regulate the function of the protein, suggesting that the effects of PTMs are quite complex. PTMs regulate the activity, stability and function of numerous proteins, including transcription factors, and have been reported to be involved in the progression of diseases such as cancer, autoimmune diseases and neurological diseases (25-27). New insights into protein modifications and its related key roles could lead to new strategies to improve management of multiple human diseases. Recent advances in proteomics and bioinformatics approaches have led to increased recognition that aberrant PTMs play important roles in VC. This review presents the latest progress in clarifying the roles of PTMs in VC.

2. Classical pathogenesis of vascular calcification

VC is an actively regulated process involving multiple calcification-related factors and signaling pathways, which remain incompletely elucidated. BMP-2, Runx2 and other calcification factors can promote VC, whereas osteoprotegerin (OPG), osteopontin (OPN) and other anticalcification factors can inhibit VC. Pathways such as the BMP signaling pathway, Wnt/β-catenin pathway and AKT pathway regulate the development of VC (28). In addition, apoptosis, mitochondrial metabolism, inflammation, oxidative stress and autophagy are involved in the occurrence and development of VC (29-31).

Procalcification factors. BMPs are members of the transforming growth factor beta (TGF-β) superfamily and have been reported to play a causal role in osteogenesis and VC (32). BMP-2 has been widely studied *in vivo* and *in vitro* for its procalcification properties. The main BMP-2 signaling axis involved in osteogenic differentiation and VC is the BMP-2/Smad1/Runx2 axis, in which BMP-2 binds to the corresponding receptor to activate the intracellular BMP effector proteins Smad1/5/8. The activated Smad complex then enters the nucleus to positively regulate Runx2, the transcription factor controlling bone formation (33). In addition, BMP-2 can activate the Wnt/β-catenin pathway, thereby inducing VC-associated processes in VSMCs (34). Multiple experiments have shown that the expression of BMP2 is upregulated in the setting of VC. When BMP2 was used to stimulate human coronary artery smooth muscle cells, the expression of Runx2 was upregulated within 24 h and intracellular calcium deposition was significantly increased (35). Treatment with a BMP antagonist (LDN-193189 or ALK3-Fc20) to inhibit BMP signaling attenuated VC in low density lipoprotein receptor-deficient ($LDLR^{-/-}$) mice (36). A report indicating that atherosclerotic intimal calcification is significantly accelerated in BMP-2-transgenic mice with high expression of BMP-2 provided further support for a role of BMP in VC (37). Runx2 is a transcription factor involved in osteoblast differentiation and bone formation. The expression level of Runx2 is low in normal blood vessels but high in calcified blood vessels (38). Runx2 can induce VSMC-mediated calcification *in vitro* and plays a key role in oxidative stress-induced VSMC-mediated calcification (39). Decreased expression of Runx2 was found to strongly inhibit intimal VC in atherosclerosis-susceptible mice and medial VC in mice with CKD, further supporting the critical role of Smooth muscle cell-specific Runx2 in the development of VC (Fig. 1) (40,41).

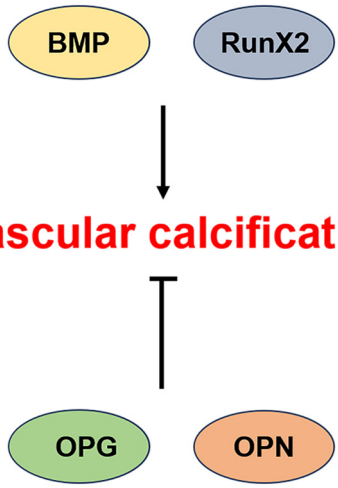

Figure 1. BMP and RunX2 promote vascular calcification, whereas OPG and OPN inhibits calcification. BMP, bone morphogenetic protein 2; Runx2, Runt-related transcription factor 2; OPG, osteoprotegerin; OPN, osteopontin.

Anticalcification factors. OPG can directly inhibit the differentiation and maturation of osteoclasts and induce the differentiation of osteoblasts, which are key inhibitors of calcification (42). OPG has been shown to be highly expressed in blood vessels, but its expression is low in calcified blood vessels in mice. OPG knockout mice were found to develop early and severe VC (43) and treatment of atherogenic mice with OPG resulted in a reduced calcified lesion area (44). However, the results of numerous clinical studies are opposite those reported in animal studies. For example, a meta-analysis involving 26,442 patients in the general population revealed that an elevated OPG level is associated with an increased risk of cardiovascular disease (45). In addition, Morena *et al* (46) reported that high expression of OPG is closely associated with coronary artery calcification.

OPN is a phosphoglycoprotein adhesion molecule found primarily in mineral-rich teeth and bone and is found to alleviate VC by inhibiting the formation and growth of mineral crystals (47). OPN expression is increased in calcified plaques, but OPN is not expressed in normal arteries (48). OPN deficiency does not cause calcification in wild-type mice but increases VC in mice deficient in the matrix protein Gla, suggesting that OPN plays an inducible inhibitory role in VC (Fig. 1) (49).

Signaling pathways related to vascular calcification. The Wnt/β-catenin pathway plays a crucial role in VC (28). When Wnt ligands bind to the frizzled (FZD)/low-density lipoprotein-related receptor (LRP) coreceptor complex, the canonical Wnt signaling pathway is activated. Dishevelled interacts with the intracellular tail of FZD and the integrated AXIN1/glycogen synthase kinase 3 (GSK3)/Adenomatous polyposis coli degradation complex binds to the phosphorylated tail of LRP, resulting in dissociation of the degradation

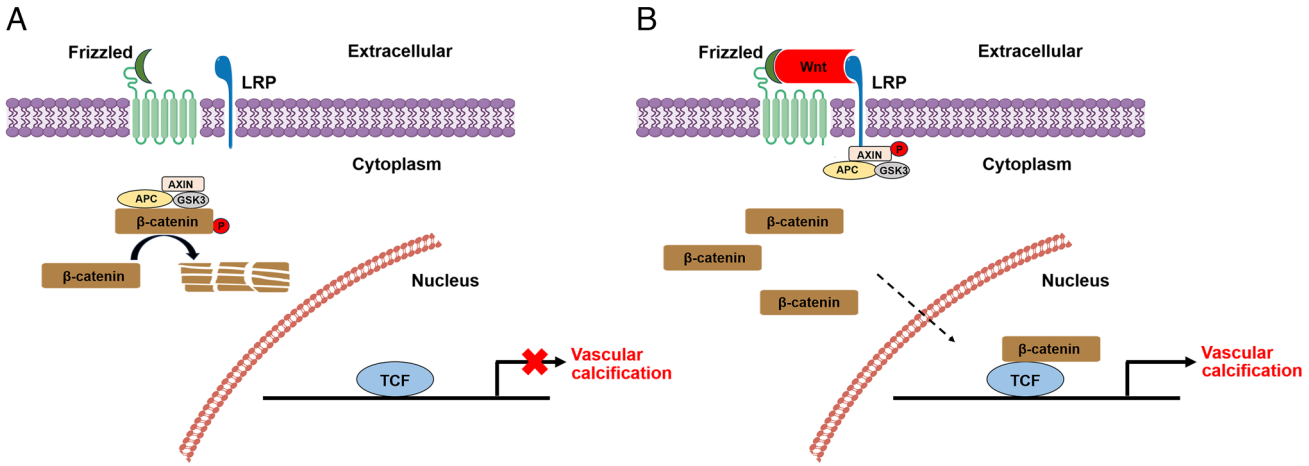

Figure 2. Wnt/β-catenin signaling pathway is involved in vascular calcification. (A) In the absence of Wnt ligand, β-catenin is phosphorylated by GSK3 and destroyed, avoiding its translocation to the nucleus to trigger the vascular calcification. (B) If Wnt ligands bind to its LRP and Frizzled co-receptors, GSK3 is inactivated, β-catenin is stabilized in the cytoplasm and translocate into the nucleus which activates target genes promoting vascular calcification. LRP, low density lipoprotein-related receptor; GSK3, glycogen synthase kinase 3; APC, adenomatous polyposis coli; TCF, T cytokine enhancer factor; P, phosphorylation.

complex. After phosphorylated β-catenin saturates the degradation complex, newly synthesized β-catenin translocates to the nucleus after attaining a certain concentration in the cytoplasm. β-Catenin subsequently forms a complex with T cytokine/Lymphoid enhancer factor (TCF/LEF), which activates downstream calcification genes such as Runx2. When the Wnt pathway is inactive, β-catenin is phosphorylated by the degradation complex and subsequently undergoes ubiquitination and proteasomal degradation (Fig. 2A and B) (50). In addition, previous cellular and animal studies have shown that the activation of AKT signaling plays an important role in upregulating Runx2 and promoting VC (39,51,52). Byon *et al* (39) reported that AKT phosphorylation can upregulate Runx2 expression and promote H_2O_2-induced calcification of VSMCs. Moreover, Heath *et al* (51) reported that AKT O-linked N-acetylglucosaminylation (O-GlcNAcylation) at two newly identified sites-threonine (T) 430 and T479-promoted the phosphorylation of AKT, thereby inducing the VMSC-mediated calcification. Deng *et al* (52) confirmed that phosphorylation of AKT promotes the loss of FOXO1/3 in the nucleus, inhibits Runx2 ubiquitination and promotes VSMC-mediated calcification (Fig. 3).

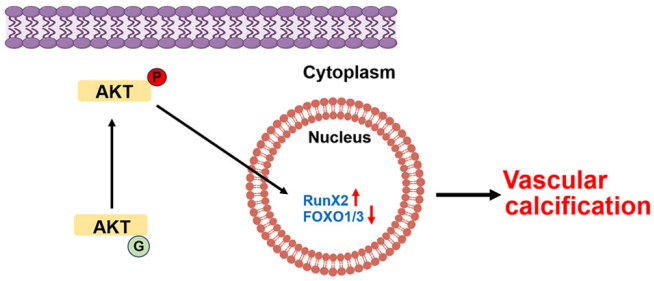

Figure 3. O-GlcNAcylation and phosphorylation of AKT is involved in vascular calcification. Runx2, Runt-related transcription factor 2; FOXO1/3, forkhead box O1/3; G, O-GlcNAcylation, P, phosphorylation.

Other common risk factors for vascular calcification. Apoptosis is another common factor that affects VC. Reports have indicated that initial calcium phosphate precipitation could be associated with apoptosis via the release of phosphorous via intracellular metabolism and calcification-promoting membrane phospholipid-rich microparticles (53,54). Further studies revealed that after cell death, cells can release free DNA to precipitate calcium phosphate crystals on blood vessel walls, leading to VC (55,56). In addition, *in vitro* experiments have suggested that calcification can be blocked by inhibiting apoptosis and that apoptosis induction can increase the incidence of calcification by 10-fold (53).

Mitochondria are important bioenergetic powerhouses and biosynthetic centers in the cell and various stimuli, such as hyperphosphataemia, hyperglycaemia and increased mitochondrial outer membrane permeability (57), can induce mitochondrial dysfunction. Mitochondrial dysfunction results in reduced production of adenosine triphosphate (ATP), abnormal production of active oxides, abnormal regulation of apoptosis and changes in the autophagic ability of cells, all of which are involved in the occurrence of VC (58). Previous studies have shown that inhibiting mitochondrial fission with melatonin can abrogate β-GP-induced VSMC-mediated calcification (59,60).

3. Post-translational modifications and vascular calcification

Various PTMs, such as ubiquitination, acetylation, carbamylation and glycosylation, play different roles in VC. In addition, different PTMs may interact with each other to co-operatively control the occurrence and development of VC (Table I).

Ubiquitination and vascular calcification. Ubiquitination is a process by which target proteins are degraded through the ubiquitin-proteasome pathway via a process regulated by a cascade comprising an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme and an E3

Table I. Targets for posttranslational modification related to VC.

First author/s, year	Enzyme	Target protein	Effect on VC	(Refs.)
A, Ubiquitination				
Deng *et al*, 2015	PTEN	Runx2	Decrease	(52)
Ouyang *et al*, 2022	Kynurenine	Runx2	Decrease	(68)
Kim *et al*, 2011	Nedd4	Smad1	Decrease	(69)
Kwon *et al*, 2016	MDM2	HDAC1	Increase	(70)
Al-Huseini *et al*, 2018	IKKβ	β-catenin	Decrease	(72)
Ishiwata *et al*, 2022	Unknown	TFEB	Increase	(73)
B, Acetylation/deacetylation				
Gu *et al*, 2019 Li *et al*, 2017	P300	Histones 3 and 4	Increase	(76,77)
Bouras *et al*, 2005 Hecht *et al*, 2000	P300	β-catenin	Increase	(78,79)
Jeon *et al*, 2006 Jun *et al*, 2010	P300	Runx2	Increase	(80,81)
Al-Huseini *et al*, 2018 Zhang *et al*, 2012	HDACs	Runx2	Decrease	(72,82)
Bartoli-Leonard *et al*, 2019	SIRT1	Runx2	Decrease	(87)
Rabadi *et al*, 2015 Hwang *et al*, 2015	SIRT1	HMGB1	Decrease	(88,89)
Zhang *et al*, 2021	SIRT1	PERK	Decrease	(90)
C, Carbamylation				
Mori *et al*, 2018	N/A	Several mitochondrial, cytoskeletal proteins (ATP synthase)	Increase	(94)
Alesutan *et al*, 2021	N/A	Uromodulin	Increase	(95)
Jankowski *et al*, 2022	N/A	Sortilin	Increase	(97)
D, Glycosylation				
Xu *et al*, 2020	OGT	Keap1	Increase	(101)
Xu *et al*, 2020	OGT	YAP	Increase	(102)
Heath *et al*, 2014	OGT	AKT	Increase	(51)
Xu *et al*, 2020	N/A	IGFR	Decrease	(101)
Watanabe *et al*, 2011 Wen *et al*, 2016	N/A	TGFβR	Increase	(107,108)

VC, vascular calcification; PTEN, phosphatase and tensin homologue; Nedd4, neural precursor cell expressed, developmentally down-regulated 4; IKKβ, IκB-kinase β; P300, histone acetyl transferase P300; HDACs, histone deacetylases; SIRT1, Sirtuin1; OGT, β-N-acetylglucosaminyl-transferase; Runx2, Runt-related transcription factor 2; Smad1, mothers against decapentaplegic homologue 1; HDAC1, histone deacetylase 1; TFEB, transcription factor EB; HMGB1, high mobility group box1; PERK, protein kinase RNA-like ER kinase; ATP, adenosine triphosphate; Keap1, Kelch like ECH associated protein 1; YAP, Yes-associated protein; IGFR, insulin-like growth factor receptor; TGFβR, transforming growth factor β receptor; N/A, not applicable.

ubiquitin ligase that binds to specific target proteins (61,62). Ubiquitination is widely involved in physiological processes such as DNA damage repair, transcriptional regulation, cell cycle progression, apoptosis and vesicle transport by regulating the stability, localization, activity and interactions of proteins (63,64).

The osteogenic transcription factor Runx2 plays a key role in regulating VC. Studies in osteocytes have shown that the degradation and stability of the Runx2 protein are regulated via the ubiquitin-proteasome pathway. The molecular mechanisms that promote Runx2 protein degradation involve Smad ubiquitination regulatory factor 1 (Smurf1) and cyclin D1. Smurf1 binds to Runx2 and mediates the conjugation of ubiquitin to Runx2, leading to its degradation (65,66). Cyclin D1 induces the C-terminal phosphorylation of Runx2 through cyclin D1/Cdk4 and promotes its proteasome-dependent degradation (67). Deng *et al* (52) demonstrated *in vitro* and *in vivo* that the phosphatase and tensin homologue (PTEN)/AKT/forkhead box O1/3 (FOXO1/3) signaling axes regulate VC by regulating Runx2 ubiquitination. Specifically, PTEN deficiency activates AKT, phosphorylates FOXO1/3, inhibits Runx2 ubiquitination, upregulates Runx2 and promotes VC without affecting the expression of common regulators of Runx2 ubiquitination, such as Smurf1 and cyclin-D1. Knockdown of FOXO1/3 in VSMCs mimics the aforementioned effects of PTEN deficiency, including the inhibition of Runx2 ubiquitination, the upregulation of Runx2 and the promotion of VC. In addition, a recent study revealed that kynurenine, a major product of indoleamine 2,3-dioxygenase 1 (IDO1)-mediated tryptophan metabolism, promoted Runx2 ubiquitination and delayed the progression of intimal calcification in transgenic apolipoprotein E-/- mice. Moreover, ubiquitination-mediated proteasomal degradation of Runx2 was found to be associated with the kynurenine-mediated aryl hydrocarbon receptor-dependent nongenomic pathway (68). Therefore, the molecular mechanisms regulating Runx2 ubiquitination in VSMCs may be novel and worthy of further exploration.

In cell models, the neuronal precursor cell-expressed developmentally downregulated 4 (Nedd4) E3 ubiquitin ligase was found to negatively regulate VC induced by a high-phosphorus environment through the ubiquitination of Smad1 and the development of VC is induced by the specific inhibition of Nedd4 (69). Paradoxically, another study showed that Murine double minute 2 (MDM2) can promote VC through the ubiquitination of histone deacetylase 1 (HDAC1) via its E3 ligase activity. In both *in vitro* and *in vivo* models of VC, the HDAC1 protein level is significantly reduced, a phenomenon related to the ubiquitination of HDAC1 at lysine (K) 73. Coimmunoprecipitation assays revealed that the E3 ubiquitin ligase MDM2 induces the polyubiquitination of HDAC1. The overexpression of MDM2 significantly increases VC in a dose-dependent manner, whereas the knockdown of MDM2 inhibits VC (70). In addition, that study confirms that blocking proteasomal degradation by treatment with various ubiquitination inhibitors ameliorates VC in both *in vitro* and *in vivo* models (70).

The nuclear factor-κB (NF-κB) family plays important roles in inflammation and atherosclerosis. In unstimulated cells, NF-kB is present in the cytoplasm and binds to inhibitor of κB (IκB). In response to various stimuli, including inflammation, specific kinases, such as IκB-kinase β (IκKβ), mediate the phosphorylation of IκB. This leads to its ubiquitination and degradation, which are followed by the translocation of NF-κB to the nucleus and the activation of transcription (71). Knockdown of IκKβ in VSMCs reduces the ubiquitination of β-catenin, upregulates β-catenin and Runx2 signaling and accelerates calcification. By contrast, persistent activation of IκKβ inhibits calcification by increasing the ubiquitination of β-catenin (72). Dysregulation of the autophagy-lysosome pathway in VSMCs mediates VC induced by a high-phosphorus environment and this pathway has become a new target for VC therapy. Transcription factor EB (TFEB) is considered the master regulator of lysosomal biogenesis. A high-phosphorus environment can result in the degradation of TFEB through the ubiquitin-proteasome system and subsequently promote VC *in vitro* and *in vivo (*73).

The results of studies on the effects of ubiquitination on VC are inconsistent. Moreover, the related mechanisms are complex and need further study. Identifying the ubiquitination sites in calcification-related factors and targeting these sites is expected to lead to approaches for suppressing VC (Fig. 4).

Protein acetylation and vascular calcification. Protein acetylation is a reversible modification that is catalyzed by acetyltransferases (N-terminal acetyltransferases/lysine acetyltransferases, NATs/KATs), which transfer acetyl groups from acetyl-CoA to amino acid residues in proteins such as histones and transcription factors (74,75). The reverse process, deacetylation, is catalyzed by lysine deacetylases. Histone acetylation is the most common type of acetylation. The main histone acetyltransferases are the members of the P300/CBP, Gcn5-related N-acetyltransferase, Steroid receptor coactivator and MYST (MOZ-YBF2/SAS3-SAS2-TIP60) families. There are four subfamilies of histone deacetylases (HDACs): Classes I, II and IV, which are Zn^{2+} dependent and class III (for example, SIRTs), which are NAD+ dependent. In addition, acetyltransferases catalyze the acetylation of nonhistone proteins, such as transcription factors and regulate transcription factor stability and DNA binding (31). In some cases, ubiquitination competes with acetylation for the same lysine residues (75).

The levels of histone 3 and 4 (H3 and H4) acetylation are elevated in the aortic valve calcification model. However, in this model, treatment with an inhibitor of the histone acetyltransferase P300, C646, was found to attenuate calcification *in vivo* and *in vitro* by significantly decreasing the acetylation levels of H3 and H4 (76,77). Studies have shown that β-catenin and Runx2 are also targets of P300-mediated acetylation and that acetylation of β-catenin by P300 can enhance the activation of β-catenin signaling (78,79). The acetylation of Runx2 can increase its stability and its osteogenic transcriptional activity, as well as osteoblast differentiation (80,81). By contrast, HDACs can directly bind to Runx2 and act as core inhibitors of its transcriptional activity. HDACs catalyze the deacetylation of Runx2, mediate its ubiquitination and degradation and inhibit osteoblast differentiation (80,82). HDAC inhibitors have been shown to promote Runx2 acetylation, thereby promoting osteoblast differentiation *in vitro* and osteogenesis *in vivo* (80,83). However, no evidence indicates that the aforementioned acetylation events are directly related to VC.

The class III HDAC sirtuin 1 (SIRT1) is an important factor in the regulation of VC, with three main related functions. i) SIRT1 can regulate Wnt/β-catenin signaling. Under physiological conditions, SIRT1 binds to p300 and the acetylation and nuclear translocation of β-catenin are reduced,

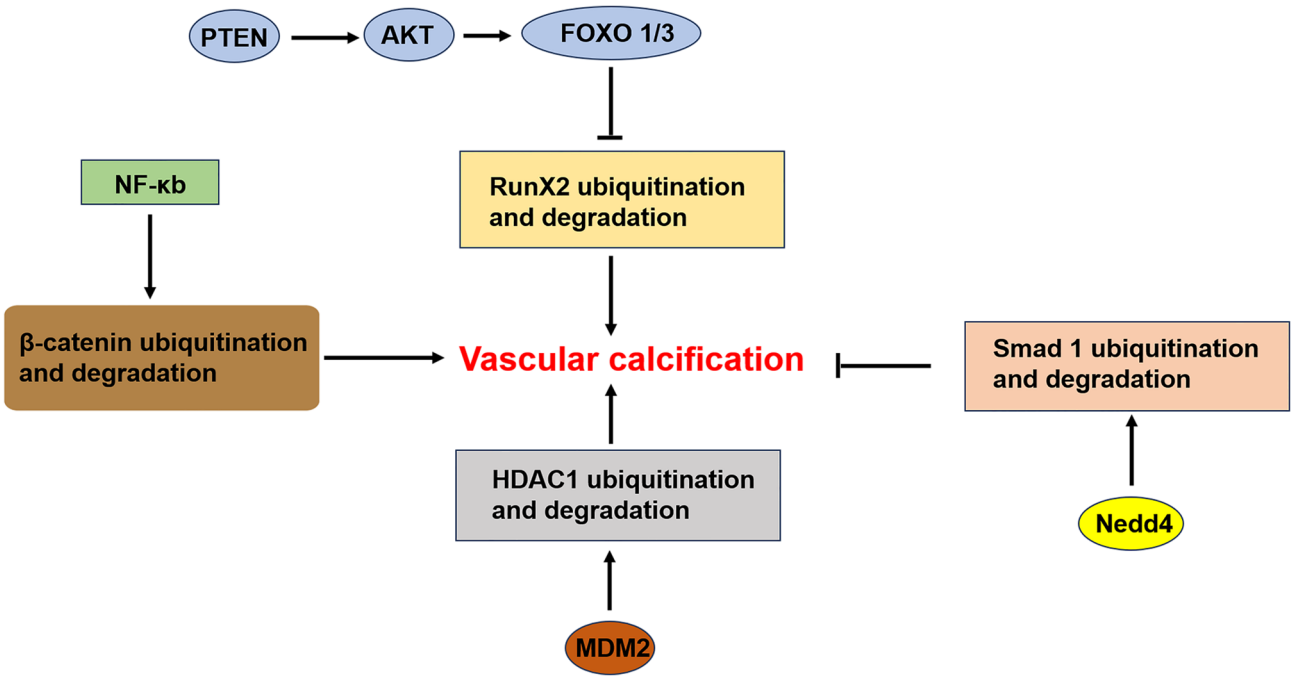

Figure 4. Ubiquitination is involved in vascular calcification. PTEN, phosphatase and tensin homologue; FOXO1/3, forkhead box O1/3; Runx2, Runt-related transcription factor 2; NF-κB, nuclear factor-κB; Nedd4, neuronal precursor cell-expressed developmentally downregulated 4; HDAC1, histone deacetylase 1; MDM2, murine double minute 2.

thereby inhibiting osteogenic activity and VC. However, in a high-glucose environment, the low abundance of SIRT1 cannot prevent P300-mediated acetylation of β-catenin, thus promoting VC (84). Similarly, hyperphosphataemia can downregulate SIRT1 expression (85), allow constitutive acetylation of downstream proteins and accelerate calcification by promoting hyperacetylation of β-catenin and Runx2 via P300 (80,86). ii) In a high-glucose environment, the decreased expression of SIRT1 results in an increase in Runx2 expression and the promotion of VC via a direct increase in the acetylation level of the Runx2 promoter (87). iii) Other studies have shown that high mobility group box 1 (HMGB1), an activator of BMP-2 and protein kinase RNA-like ER kinase (PERK) are targets of SIRT1 for deacetylation. SIRT1 can inhibit the inflammatory response through the deacetylation of HMGB1, thereby inhibiting VC (88,89). Moreover, in both *in vitro* and *in vivo* models, SIRT1 was found to ameliorate endoplasmic reticulum (ER) stress-induced VC by deacetylating K889 in PERK (90). In addition to SIRT1, SIRT3 also plays an important role in VC. Sun *et al* (91) report that SIRT3 inhibits VC by regulating Nε-carboxymethyl-lysine (CML).

SIRT1 and SIRT3 play important roles in VC. Identifying their targets will help us to understand the mechanism of VC and develop protective agents against VC that will benefit the majority of patients (Fig. 5).

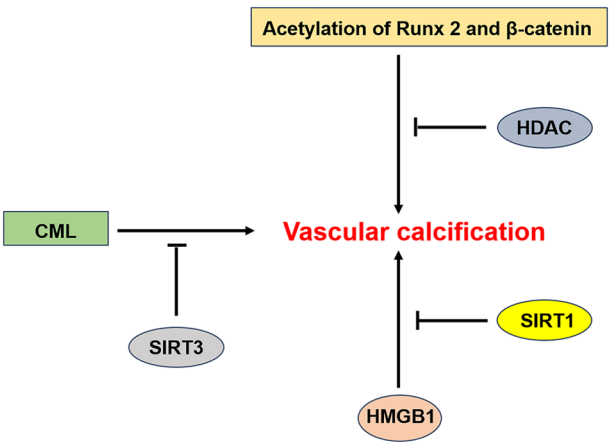

Figure 5. Acetylation is involved in vascular calcification. Runx2, Runt-related transcription factor 2; HDAC, histone deacetylase; SIRT, Sirtuin; CML, Nε-carboxymethyl-lysine; HMGB1, high mobility group box 1.

Protein carbamylation and vascular calcification. Carbamylation is a stable PTM in which a carbamoyl moiety (-$CONH_2$) binds to a functional group in a proteins, peptide and or free amino acid (92). Carbamylation is common in patients with CKD and can occur nonenzymatically in the presence of high concentrations of urea. Medial VC is common in patients with CKD with increased protein carbamylation (93). Mori *et al* (94) established a VC model of CKD *in vitro* and *in vivo* and confirmed that protein carbamylation aggravated VC. Specifically, carbamylation of ATP synthase aggravated VC in human vascular smooth muscle cells (hVSMCs) by impairing mitochondrial function and inhibiting the expression of ectonucleotide pyrophosphate/phosphodiesterase 1 (ENPP1). ENPP1 catalyzes the production of pyrophosphate, which is a potent inhibitor of ectopic calcification. Proteins sensitive to carbamylation were identified via proteomic mass spectrometry and various mitochondrial and cytoskeletal proteins were found to be carbamylated in hVSMCs exposed to uremic conditions. *In vivo* studies further confirmed the presence of evident protein carbamylation and mitochondrial dysfunction in the

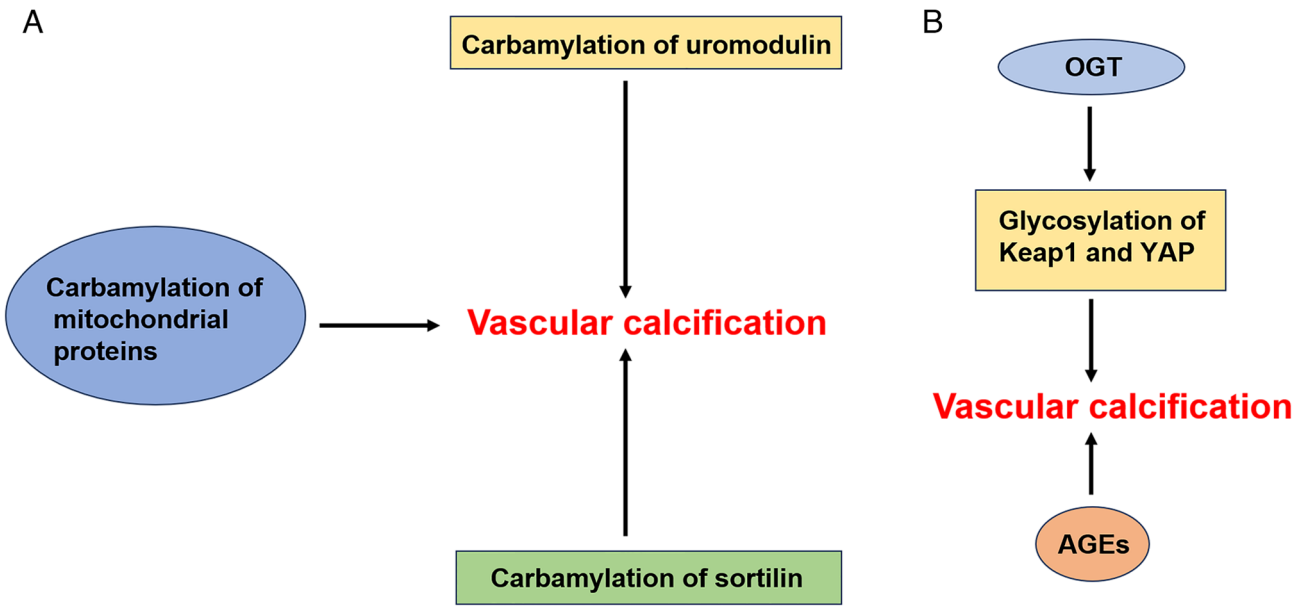

Figure 6. (A) Carbamylation and (B) glycosylation are involved in vascular calcification. OGT, O-GlcNAc transferase; Keap1, Kelch-like ECH-associated protein 1; YAP, Yes-associated protein; AGEs, advanced glycation end products.

aortas of heminephrectomized rats fed a urea-rich diet. In addition, carbamylation of ATP synthase is found in the tunica media of patients with end-stage renal disease, supporting the existence of this mechanism in human VC.

Alesutan *et al* (95) reported that uromodulin can inhibit VC by inhibiting cytokine-dependent procalcification signaling. However, carbamylation of uromodulin interferes with its ability to bind to proinflammatory cytokines, resulting in the loss of its anticalcification properties *in vitro*. Carbamylated uromodulin can be detected in the serum of dialysis patients and the levels of proinflammatory cytokines that interact with uromodulin are reduced by uromodulin carbamylation, suggesting that the function of the cytokine trap is impaired (95).

Sortilin is an intracellular sorting receptor that has been identified as a cardiovascular risk factor in humans (96). Compared with control individuals with normal renal function, patients with CKD have increased serum levels of sortilin and harbor carbamylated sortilin in the circulation (93), localized to calcified areas. By generating hVSMCs and establishing an isolated rat aortic ring model of calcification, Jankowski *et al* (97) found that carbamylated sortilin can promote VC. Further studies revealed that sortilin carbamylation promotes VC mediated by hVSMCs, possibly due to an increase in their binding affinity for leukocytes. Similarly, sortilin carbamylation was found to be associated with the volume and progression of coronary artery calcification in patients with CKD (97,98).

Carbamylation may play a role in VC by affecting mitochondrial function (94) or the inflammatory response (95). The aforementioned studies provide valuable new insights into the pathogenesis of VC and the findings have implications for other complications associated with elevated concentrations of circulating uremic toxins (Fig. 6A).

Protein glycosylation and vascular calcification. Glycosylation is one of the most important PTMs *in vivo* and can occur on 50-70% of proteins in cells (99). There are four major types of glycosylation: O-glycosylation, N-glycosylation, C-glycosylation and glycosylphosphatidylinositol anchoring (99).

O-GlcNAcylation, in which a single GlcNAc monosaccharide is transferred to serine and threonine residues in the target intracellular protein, is the most commonly studied type of O-glycosylation. This modification is catalyzed by O-GlcNAc transferase (OGT) and studies have reported that abnormal OGT activity can lead to cardiovascular complications (100). For instance, Xu *et al* (101) reported that OGT expression is significantly increased in both high-phosphorus diet-induced 5/6 nephrectomized rats and VSMC calcification models and that OGT knockdown inhibits VC induced by a high-phosphorus environment. Further study of the mechanism by which OGT promotes VC in CKD revealed that OGT overexpression increases the glycosylation of Kelch-like ECH-associated protein 1 (Keap1), which leads to the degradation of nuclear factor erythroid 2-related factor 2 and inhibits VSMC autophagy, in turn promoting VC *in vitro* and *in vivo*. Xu *et al* (102) also report that OGT promotes the glycosylation of Yes-associated protein (YAP) to increase its stability and upregulates the expression of YAP to inhibit autophagy, thus accelerating VC induced by a high-phosphorus environment. Therefore, OGT knockdown is expected to inhibit VC in CKD by reducing the glycosylation of target proteins and activating autophagy. In addition, Heath *et al* (51) report that activating the O-GlcNAcylation of AKT increases Runx2 activity and promotes the development of VC in diabetic mice.

The relationship between N-glycosylation and VC is another research hotspot. Insulin-like growth factor-I (IGF-I) has been identified as a major inhibitor of VC. Siddals *et al* (103) used statins to deplete the substrates required for N-glycosylation and used tunicamycin to inhibit N-glycosylation. They found that statins and tunicamycin disrupt the inhibitory effect of IGF-I on β-glycerophosphate-induced VC by altering the glycosylation of the IGF receptor (IGFR). TGF-β is

a multifunctional cytokine that has been shown to regulate VC and the differentiation of VSMCs *in vivo* (104,105). Studies have shown that TGF-β1 is a target of N-glycosylation. Sha *et al* (106) reported that tunicamycin inhibits TGF-β1 secretion and leads to an increase in the level of the cell-associated non-glycosylated form of TGF-β1. In addition, the TGF-β receptor TGF-βR is regulated by N-glycosylation. The removal of glycosylation from TGF-βR affects its interaction with its ligand, resulting in failure to activate downstream signaling pathways (107). Although a few reports have addressed TGF-β or TGF-β receptor N-glycosylation, no relevant research has been conducted on the direct relationship between TGF-β or TGF-β receptor N-glycosylation and VC. Notably, blocking core fucosylation, a specific form of N-glycosylation, suppresses VC-associated processes in VSMCs. Inhibition of TGFβR fucosylation significantly dysregulates downstream TGFβ/Smad2/3 signaling (108).

Accumulated studies have shown that advanced glycation end products (AGEs), which are predictors of cardiovascular disease mortality, may play an important role in VC. Previous studies have shown that the local and circulating levels of AGEs in patients with diabetic nephropathy and nondialysis CKD are significantly elevated (109,110). Moreover, recent clinical studies show that the measured levels of AGEs in the skin of 122 patients with type 2 diabetes and in the radial arteries of 54 patients with CKD are positively correlated with the degree of arterial calcification (111,112). In addition, several animal studies show that the knockdown of the receptor for AGEs (RAGE) has therapeutic benefits in inhibiting the development of VC (113-115). B4GALNT3-mediated LacdiNAc (LDN) glycosylation of sclerostin may be a bone-specific osteoporosis target (116), suggesting that glycosylated sclerostin may have potential research value in VC.

In general, glycosylation contributes to the development of VC. Predicting glycosylation events and sites through machine learning models (117) and identifying inhibitors of glycosylation may lead to the development of a therapeutic strategy to target VC in patients with CKD and diabetes (Fig. 6B).

4. Diagnostic evaluations, therapeutic interventions and prospects in vascular calcification

Several auxiliary tests are currently used for the diagnosis of VC. The ankle-brachial index test is the preferred first-line screening method for VC in patients with peripheral artery disease (118). Recently, a simple VC score on the basis of a planar X-ray of the foot in two projections was proposed. Via evaluation of five vascular sites and the length of the 'pipe-steam', patients can be divided into three VC categories: Absent, moderate and severe (119). Computed tomography (CT) exquisitely visualizes peripheral and coronary artery calcifications as high-density signals and provides a whole-body estimate of the VC burden (120). Recently, several novel biological markers, such as Fibroblast growth factor-23 and Klotho, have also been used to predict VC (120).

Studies show that mutation or deletion of certain genes is closely related to VC. Mutations in the ABCC6 and ENPP1 genes are responsible for generalized arterial calcification in 14 of 28 children and patients with idiopathic infantile arterial calcification, respectively (121,122). Loss of the RAGE gene attenuated atherosclerosis in apolipoprotein-deficient mice (123). Moreover, Klotho gene deficiency is widely recognized to be responsible for VC in CKD (124). Therefore, genetic testing is valuable for predicting VC.

Numerous preclinical and clinical studies have been conducted to find further approaches for the management of VC. Potentially effective therapeutic drugs include calcium channel blockers, renin-angiotensin system inhibitors, statins, bisphosphonates, denosumab and myoinositol hexaphosphate (125). However, the therapeutic effects of these drugs are still unsatisfactory. Therefore, more effective drugs to delay or treat VC are needed.

In the present review, the important role of PTMs in the occurrence and development of VC were reviewed. In general, acetylation, carbamylation and glycosylation can promote VC, whereas deacetylation suppresses VC. Therefore, future inhibitors of acetylation, carbamylation and glycosylation and agonists of SIRT family deacetylases are expected to ameliorate VC. However, the results of studies reporting the effect of ubiquitination on VC are inconsistent. More clearly, increasing the ubiquitination-mediated degradation of the procalcification factors Runx2 and β-catenin is expected to delay the development of VC.

5. Conclusion

VC is a common complication of atherosclerotic cardiovascular disease, DM and CKD and there is still no effective treatment. PTMs have recently become a popular topic across medical research. Studies have shown that PTMs play important roles in the development of VC by participating in the regulation of calcification-related pathways, such as the Wnt/β-catenin and PTEN/AKT pathways; calcification-related factors, such as Runx2 and BMP2; mitochondrial function; inflammation; oxidative stress; and autophagy (29-31,34,52,68). In addition, various PTMs interact with each other and co-operatively control the occurrence and development of VC. However, few studies have been conducted on PTMs in VC and the results of the existing studies are inconsistent. In addition, the role of PTMs in the transdifferentiation of VSMCs to osteoblasts remains to be elucidated.

It is well known that PTMs involve the chemical modification of certain amino acids in target proteins, which can alter the biological function of the modified proteins. Therefore, the modification sites on target proteins play a crucial role in PTMs. Currently, a large number of protein databases have been established, such as the UniProt database, which contains 189 million protein sequence records (126). Given the vast amount of data, using traditional molecular biology experimental techniques to identify specific PTMs and their modification sites requires significant human resources and time. Recently, machine learning has been increasingly applied in the fields of medicine and life sciences. Computational methods based on machine learning offer an alternative strategy for predicting PTMs and their modification sites in a cost-effective and efficient manner. Moreover, identifying the specific types of PTMs and the amino acid modification sites on target proteins can provide novel insights into disease treatment strategies (127). Further studies on PTMs will help us understand

the mechanism underlying the development and progression of VC to support the development of new strategies and targets for VC prevention and treatment.

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Competing interests

The authors declare that they have no competing interests.

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