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Vascular Calcification in CKD-MBD: Roles for Phosphate, FGF23, and Klotho

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Abstract

Vascular calcification (VC) is highly prevalent in aging, diabetes mellitus, and chronic kidney disease (CKD). VC is a strong predictor of cardiovascular morbidity and mortality in the CKD population. Complex pathological mechanisms are involved in the development of VC, including osteochondrogenic differentiation and apoptosis of vascular smooth muscle cells, instability and release of extracellular vesicles loaded calcium and phosphate, and elastin degradation. Elevated serum phosphate is a late manifestation of CKD, and has been shown to accelerate mineral deposition in both the vessel wall and heart valves. α -Klotho and fibroblast growth factor 23 (FGF23) are emerging factors in CKD-mineral and bone disorder (CKD-MBD) and are thought to be involved in the pathogenesis of uremic VC. There are discordant reports regarding the biomedical effects of FGF23 on VC. In contrast, mounting evidence supports a well-supported protective role for α -Klotho on VC. Further studies are warranted to elucidate potential roles of FGF23 and α -Klotho in VC and to determine where and how they are synthesized in normal and disease conditions. A thorough systemic evaluation of the biomedical interplay of phosphate, FGF23, and α -Klotho may potentially lead to new therapeutic options for patients with CKD-MBD.

Keywords

chronic kidney disease; fibroblast growth factor 23; α -Klotho; vascular calcification

1. Cardiovascular Mortality and Vascular Calcification (VC)

Patients with chronic kidney disease (CKD) and endstage kidney disease (ESKD) have an increased risk for cardiovascular mortality and morbidity [1,2]. Cardiovascular diseases account for 30–50% of all-cause mortality in patients with CKD and ESKD worldwide. Although traditional risk factors contribute to the development of cardiovascular disorders in

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CKD, they cannot fully explain the unacceptably high incidence of cardiovascular mortality in these patients. Hence, non-traditional risk factors, including abnormal mineral metabolism, are considered to be involved in the enhanced risk of cardiovascular events [3,4]

VC, abnormal deposition of calcium-phosphate (Pi) salts in vascular tissues including blood vessels, valves, and heart, is frequently observed in aging, diabetes mellitus, CKD, calcific aortic valve disease (CAVD), and several genetic diseases [5–8]. Mounting clinical evidence has shown that VC is indeed an independent predictor of cardiovascular morbidity and mortality in CKD and ESKD [9–11]. Dialysis patients show a 5-fold to 30-fold increase in cardiovascular mortality risk compared to the general population [1]. Hence, VC is now considered to be a major contributor to increased cardiovascular mortality rates. Importantly, nontraditional risk factors including abnormal mineral metabolism appear to underlie this increased risk [3,12]. There are currently no treatments available that can halt or reverse the progression of VC. Hence, understanding how abnormal mineral metabolism leads to VC is greatly needed in order to develop earlier diagnostic tools and new therapies that prevent or regress VC in the CKD population.

2. VC in CKD-Mineral and Bone Disorder (CKD-MBD)

CKD-MBD is a newly termed systemic disorder that is characterized by abnormal serum biochemistries including hyperphosphatemia and hypercalcemia, bone disorders, and VC [13]. Among these defining characteristics, VC is the hallmark of CKD-MBD.

VC can be classified by the vascular site of abnormal mineral deposition. Deposition of calcium salts in the intimal layer and medial layer are termed as intimal calcification and medial calcification, respectively [14]. Valvular calcification, often observed in CAVD, is characterized by the deposition of calcium salts in the heart valves. These three types of VC are highly prevalent and accentuated in the CKD population [7,14].

The impact of VC on cardiovascular outcome relates to the location of mineral deposition. Intimal calcification reflects atherosclerotic plaque burden and may influence plaque rupture, and is a strong predictor of cardiovascular events and mortality [15]. On the other hand, medial calcification induces stiffening of the vessel, increased pulse wave velocity, and left ventricular hypertrophy, and can result in heart failure [16]. Valvular calcification causes valve stenosis, and can lead to cardiac hypertrophy, valve and heart failure, and sudden cardiac death [17]. All forms of calcification contribute to increased cardiovascular mortality in CKD-MBD [13,18]. Deeper understanding of the pathological calcification process is required to reduce the risk of VC, create novel therapeutic options, improve quality of life, and extend the life expectancy in CKD population.

3. Mechanisms of VC

VC was once considered to be a passive deposition of calcium-Pi salts from supersaturated fluids related to aging and degenerative process in the vasculature. It is now clear that VC is an actively cell-regulated pathology [19]. Advances in this field have unveiled the complex molecular mechanisms regulating VC [20]. Under normal conditions, vessels and valves are protected from supersaturated concentrations of serum calcium and Pi by a number of active

inhibitors that protect against abnormal mineral deposition in soft tissues [21–24]. Several calcification inhibitors have been identified, including: pyrophosphate, adenosine, matrix Gla protein, osteopontin, fetuin-A, osteoprotegerin, and bone morphogenetic protein-7. However, once the balance between the total capacity of active inhibitors and active inducers is tipped, VC can occur in the vessel walls and valves (Fig. 1). In the CKD population, active inducers of calcification include hypercalcemia, increased levels of parathyroid hormone (PTH), inflammatory cytokines, oxidative stress, uremic toxins, advanced glycation end products, and perhaps most importantly, Pi [20]. A number of these calcification inducers are increased and, simultaneously, active inhibitors are decreased, likely explaining the extremely high prevalence of vascular intimal, medial, and valvular calcification [25–27].

4. Elevated Pi as a Major Inducer of VC

Among various inducers of calcification in CKD, hyperphosphatemia is most strongly associated with VC and a typical manifestation of CKD-MBD [3,28]. Serum Pi level is maintained in the normal range by the balance among intestinal Pi absorption, renal tubular Pi reabsorption, and equilibrium of extracellular Pi with Pi in intracellular fluid or bone [29]. Among them, renal Pi filtration and reabsorption are believed to be the main determinants of serum Pi level at steady state.

As kidney function declines and nephron mass decreases, phosphaturic hormones such as PTH and fibroblast growth factor 23 (FGF23) are synthesized and secreted in response to relative Pi overload as early as CKD stages 2 and 3 [30]. These hormones act on the renal proximal tubules and down-regulate sodium-Pi co-transporter type IIa and IIc, important transporters that regulate Pi resorption in the renal tubules, thereby increasing renal Pi excretion and maintaining serum Pi level within normal range [31]. However, as CKD reaches advanced stages, kidneys can no longer filter as much Pi as dietary Pi intake, finally leading to overt hyperphosphatemia at CKD stages 4 and 5.

Clinical studies have shown that elevated serum Pi is a risk factor for VC and cardiovascular mortality and morbidity in the CKD population and particularly, patients with ESKD on dialysis [3,32,33]. More recently, even Pi levels at the high end of the normal range have been correlated with increased risk of cardiovascular mortality in the general population, indicating the potential toxicity of Pi [34,35]. Clinical studies have shown that hyperphosphatemia is closely associated with advanced VC in CKD and multiple *in vivo* studies have now shown that Pi loading promotes VC in uremic rodents [36–42].

A growing amount of evidence has begun to reveal the mechanisms by which Pi promotes VC (Fig. 2). Vascular smooth muscle cells (SMCs) express type III sodium-dependent Pi co-transporters; PiT-1 and PiT-2, encoded by SLC20A1 and SLC20A2, respectively [43]. In vascular SMCs, PiT-1 promotes and PiT-2 inhibits matrix mineralization induced by elevated Pi [44,45]. PiT-1 utilizes both Pi uptake-dependent and -independent mechanisms to promote osteochondrogenic phenotype change, synthesis of bone-related proteins, and calcification of the extracellular matrices [46–48]. In contrast, PiT-2 protects against Pi-induced vascular SMCs calcification, though the precise mechanism for this effect is still under investigation [44]. In addition, elevated Pi regulates vascular SMCs extracellular

matrix stability, apoptosis, and extracellular vesicle release, though the receptors mediating these effects are not yet known [19,20]. Finally, Pi is a major component of hydroxyapatite, and thus increases in calcium Pi product may also contribute directly to crystal precipitation in the vasculature when concentrations exceed the solubility product [48].

5. Emerging Players in Pi Homeostasis: α -Klotho and FGF23

Two new players recently identified in the field of CKD-MBD related to Pi homeostasis are FGF23 and α -Klotho [49]. FGF23 is a phosphaturic hormone mainly produced by osteocytes in the bone [50]. Although regulation of FGF23 synthesis and secretion have not been fully elucidated, Pi, calcium, vitamin D derivatives, PTH, and other factors appear to influence FGF23 levels [51]. FGF23 binds to fibroblast growth factor receptors (FGFRs) 1c, 3c, and 4 and plays a major role in directly regulating serum Pi levels. It does this by down-regulating the sodium dependent Pi cotransporters, sodium-Pi IIa and IIc, in the proximal tubule, thereby increasing renal Pi excretion [52]. In addition, FGF23 inhibits 1 α -hydroxylase and increases 24-hydroxylase activities, thereby decreasing 1,25-dihydroxyvitamin D (calcitriol), which also favors serum Pi normalization. Furthermore, FGF23 negatively regulates PTH synthesis in the parathyroid gland [53]. Combined, the functions of FGF23 act together to maintain normal serum Pi levels.

FGF23 binding to FGFRs requires the type I transmembrane protein, α -Klotho, as an obligatory co-receptor [54]. Because FGFRs are ubiquitously expressed, the presence of α -Klotho on a cell is thought to confer the tissue specificity for FGF23 action. As α -Klotho is mainly expressed in kidney, parathyroid gland, and choroid plexus, the function of FGF23 was historically thought to be restricted to those organs, though this paradigm is shifting with growing evidence that FGF23 may have other receptors and target tissues, including the heart [55–58].

6. Roles of FGF23 in VC

It is well accepted that FGF23 levels are elevated in CKD and correlated with renal dysfunction and abnormal mineral metabolism [30,52]. However, the potential effects of FGF23 on VC are controversial [59–64]. A major question that remains unresolved is whether FGF23 can directly act on vascular cells to promote or inhibit matrix calcification. As shown in Table 1, there is evidence both for and against this possibility. Scialla *et al* showed that addition of FGF23 to human vascular SMCs did not promote matrix calcification *in vitro* under normal or high Pi conditions. Furthermore, no effect on mouse aortic ring calcification was observed either in the presence or absence of soluble α -Klotho [60]. Likewise, Lindberg *et al* showed that FGF23 did not affect β -glycerophosphate-induced calcification of bovine vascular SMCs *in vitro* [61]. On the other hand, Zhu D *et al* reported that FGF23 had a protective effect on VC in cultured SMCs [62]. Similarly, Lim *et al* showed that FGF23 decreased human aortic smooth muscle cell calcification, and this effect was dependent on the induction of α -Klotho [63]. In contrast, Jimbo *et al* showed that FGF23 enhanced Pi-induced calcification in cultured human vascular SMCs overexpressing α -Klotho [64]. Hence, further studies in this field are required to address the roles of FGF23

on vascular SMCs matrix calcification and answer whether increased FGF23 has α -Klotho-dependent and -independent effects on VC in CKD.

7. Roles of Klotho in VC

α -Klotho gene and protein were first discovered in 1997 and subsequently shown to regulate longevity in mice [65–67]. Interestingly, α -Klotho deficient mice show high circulating levels of Pi and calcitriol and develop arterial medial calcification, almost identical to findings observed in FGF23 knockout mice [68]. These results confirm that both FGF23 and α -Klotho coordinate mineral homeostasis. Indeed, α -Klotho levels decline in people and animal models of CKD concomitant with renal insufficiency, and are thought to contribute to CKD-MBD progression [69,70].

Two forms of α -Klotho have been identified; membrane bound α -Klotho and soluble α -Klotho. Soluble α -Klotho is created by shedding of the extracellular domain of membrane-bound α -Klotho into the circulation, which could allow it to act at distant sites like a hormone. Membrane α -Klotho is involved in FGFR signaling, whereas soluble α -Klotho exerts its function by its glycosidase activity and regulates transporter function [71,72]. α -Klotho is mainly expressed in kidney, parathyroid gland, and choroid plexus, but a major unresolved question is whether α -Klotho is expressed in the vascular cells.

There are conflicting results regarding the presence of α -Klotho in the vasculature. As shown in Table 2, Lau *et al* found no α -Klotho mRNA in mouse aortas either under normal or CKD conditions [69]. Likewise, no α -Klotho or FGF23 mRNA was found in human aortic SMCs [60]. Table 2 also shows a number of other studies that failed to find α -Klotho expression in vascular SMCs or tissues [60,61,64,69,73–77]. On the other hand, there are almost an equal number of studies where α -Klotho was detected in vascular tissues or SMCs [62–64,78–86,88,89] (Table 2).

Although the presence of α -Klotho in the vascular walls remains unclear, several studies have examined the roles of α -Klotho on VC *in vitro* and *in vivo* [70, 87–89]. Notably, all the experiments to date suggest that α -Klotho is protective against VC. Hu *et al* reported that α -Klotho deficiency in CKD mice caused VC, and soluble α -Klotho could suppress sodium-dependent uptake of Pi and Pi-induced calcification of rat vascular SMCs [70]. Zhang *et al* showed that soluble α -Klotho suppressed Pi-induced calcification of human bone marrow derived mesenchymal stem cells via inactivation of the FGFR1/ERK signaling pathway [87]. Zhao *et al* reported that inhibition of mammalian target of rapamycin signaling suppressed VC in CKD via up-regulation of membrane bound vascular α -Klotho [88]. Chang *et al* reported that intermedin_{1–53} attenuates VC in CKD rats via upregulating of membrane-bound α -Klotho in the vessel wall [89]. The former two groups demonstrated the protective roles of the soluble form, whereas the latter two groups showed the anti-calcific effects of membrane-bound form. These results suggest that both soluble and membrane-bound forms of α -Klotho are the potential therapeutic targets of VC in CKD.

8. Candidate Reasons For Discordant Findings

What explains the conflicting findings regarding the effects of FGF23 and α -Klotho on VC? Types and origins of cultured cells as well as culture conditions may partially account for those discrepancies. In addition, as we discussed above, FGF23 requires α -Klotho as an obligatory co-receptor, therefore the presence or absence of membrane bound α -Klotho in vascular cells may also affect FGF23's ability to interact with these cells. As has been highlighted recently, it still remains unknown whether or not α -Klotho is expressed in the vessel wall [90]. Here again, methodological drawbacks may partially account for the conflicting results regarding the presence of vascular α -Klotho. Notably, previous studies employed a variety of species, antibodies with different sensitivity and specificity, polymerase chain reaction methods, disease state, vascular beds, cell isolation/culture methods for the detection of vascular α -Klotho. Furthermore, vascular components include endothelial cells, SMCs, adventitial fibroblasts, neural cells, inflammatory cells, and extracellular matrix, and conflicting results may be due to complicated cellular interactions. Currently, it is still an open question to be addressed in the future.

9. Implication for Treatment of VC in CKD-MBD

To date, there is no definitive treatment for VC. However, increasing body of evidence has begun to point to Pi, FGF23, and α -Klotho as novel therapeutic targets against uremic VC.

It is now widely accepted that elevated Pi in CKD directly induces VC [19]. Therefore, it is reasonable to think that lowering serum Pi level with dietary Pi restriction in combination with Pi-binders can prevent VC in CKD. Indeed, increasing evidence has suggested that non-calcium based Pi-binders retard the progression of VC in CKD population compared to calcium-based Pi-binders [40–42,91]. These results are partly explained by the observation that calcium overload aggravates VC by inducing positive calcium balance and directly activating the multiple calcification process of VSMCs [48,92]. Interestingly, Finch *et al* reported that Pi-restriction reversed VC in uremic rats, indicating that Pi control can reverse established VC [93]. At present, since few clinical studies have shown the effects of Pi-binders on the reversibility of established VC [42], controlling serum Pi level within target ranges is a clinically relevant approach to retard the progression of uremic VC. However, given that not all CKD patients, especially those on dialysis, can achieve the recommended range of serum Pi level with the use of Pi-binders, it remains a challenging issue to prevent the progression of VC by this treatment modality alone in the CKD population. In addition, some clinical studies suggest that sodium-Pi co-transporter type IIb at the luminal side of the small intestine may be up-regulated and weaken the efficacy of Pi-binders in CKD patients [94]. In this regard, inhibitors of sodium-Pi co-transporter type IIb such as nicotinamide, which blocks Pi absorption from the intestine, are expected to improve P control in the CKD population, especially when they are used in combination with conventional Pi-binders [95,96]. The ongoing COMBINE clinical trial that assesses the impact of combination use of conventional Pi-binders and nicotinamide on various outcomes such as serum levels of Pi, FGF23, and biomarkers, renal fibrosis, and left ventricular hypertrophy is particularly of interest in this regard [97]. Although that study does not target VC, combination of the conventional Pi-binders and inhibitors of type IIb sodium-Pi co-transporter may be a

promising treatment strategy that prevents VC and decreases the risk of cardiovascular mortality in patients with CKD.

As discussed above, it remains unclear whether or not increased circulating FGF23 has protective or detrimental effects on VC [60–64]. Notably, emerging evidence strongly suggests that FGF23 directly induces cardiac hypertrophy independently of α -Klotho, making it a potential therapeutic target in patients with CKD-MBD [55,56]. However, Shalhoub V *et al* showed that anti-FGF23 antibody decreased urinary Pi excretion, promoted VC, and increased mortality in α -Klotho deficient mice, though the treatment ameliorated uremic hyperparathyroidism [98]. Considering these results, even if FGF23 is confirmed as a potential therapeutic target against VC in future studies, application of anti-FGF23 antibodies to CKD population warrants caution and targeting vascular FGFR instead may be more beneficial for the treatment of uremic VC.

As for α -Klotho, there have been no clinical studies testing the protective effects of soluble α -Klotho either in the general population and CKD patients. It may be biologically plausible to replenish soluble α -Klotho or increase the expression of membrane-bound α -Klotho in patients with CKD, as CKD is a state of α -Klotho deficiency and α -Klotho exerts its multiple organ-protective function in the kidney, vessel wall, and heart [52,99]. A better understanding of the pathophysiological roles of soluble α -Klotho and the safety of administering soluble α -Klotho in human will drive us to perform clinical studies that focus on the protective effect of α -Klotho on VC in CKD.

10. Future Perspective

VC is a hallmark and major risk factor for cardiovascular morbidity and mortality in CKD. Abnormalities in serum Pi, FGF23, and α -Klotho play critical roles in the development of VC in CKD-MBD patients. Hyperphosphatemia is highly correlated with VC *in vivo* and directly promotes vascular SMCs calcification *in vitro*. More data are required to determine whether FGF23 or α -Klotho have direct effects on vascular SMCs functions related to VC, and the specific context in which this occurs. Hence, increased understanding of potential direct effects of FGF23 and α -Klotho on vascular cells may lead to development of novel therapies to treat VC in CKD-MBD.

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Abbreviations

CAVD	calcific aortic valve disease
CKD	chronic kidney disease
CKD-MBD	CKD-mineral and bone disorder

ESKD	endstage kidney disease
FGF23	fibroblast growth factor 23
FGFR	fibroblast growth factor receptors
Pi	phosphate
PTH	parathyroid hormone
SMCs	smooth muscle cells
VC	vascular calcification

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Highlights

- Elevated serum phosphate in CKD has been shown to accelerate mineral deposition in both the vessel wall and heart valves. α -Klotho and fibroblast growth factor 23 (FGF23) are emerging players thought to be involved in the pathogenesis of uremic vascular calcification. While the role of FGF23 in vascular calcification is controversial, mounting evidence supports a protective role for α -Klotho.

Fig. 1. Imbalance between active inducers and inhibitors of vascular calcification

Vessels and valves mineralize when active inducers exceed the capacity of active inhibitors.

Active inducers are increased and active inhibitors are decreased in aging, diabetes mellitus, and CKD. AGEs, advanced glycation end products; BMP, bone morphogenetic protein; CKD, chronic kidney disease; LDL, low density lipoprotein; MGP, matrix Gla protein; OPG, osteoprotegerin; OPN, osteopontin; PTH, parathyroid hormone.

Fig. 2. Molecular mechanisms of phosphate-induced vascular calcification

PiT-1 and PiT-2 are involved in the pathogenesis of phosphate-induced vascular calcification. PiT-1 promotes vascular calcification by osteochondrogenic differentiation and apoptosis of vascular SMCs and release and instability of extracellular vesicles, whereas PiT-2 protects against vascular calcification via unknown mechanisms. ALP, alkaline phosphatase; BMP, bone morphogenetic protein; Ca, calcium; ECM, extracellular matrix; Runx2, runt-related transcription factor 2; PDGF, platelet derived growth factor; Pi, phosphate; PPi, pyrophosphate; SMCs, smooth muscle cells.

Table 1

Evidence for the roles of FGF23 on VC

Authors	Effect of FGF23 on VC	Study type	Species	Tissue sample	Findings on role of FGF23 in VC	Reference
Scialla JJ <i>et al</i>	No effect	in vitro, ex vivo	human mouse	aortic ring, VSMCs	Recombinant FGF23 had no effects on Pi-induced calcification of the VSMCs and aortic ring	[60]
Lindberg K <i>et al</i>	No effect	in vitro	bovine	VSMCs	Recombinant FGF23 had no effects on β -GP-induced calcification of the VSMCs.	[61]
Zhu D <i>et al</i>	Anti-calcific	in vitro	mouse	VSMCs	Recombinant FGF23 inhibited Pi-induced calcification of the VSMCs.	[62]
Lim K <i>et al</i>	Anti-calcific	in vitro	human	VSMCs	Recombinant FGF23 inhibited Pi-induced calcification of the VSMCs under high calcitriol condition.	[63]
Jimbo R <i>et al</i>	Pro-calcific	in vitro, ex vivo	rat	aortic ring, VSMCs	Recombinant FGF23 accelerated Pi-induced calcification of the VSMCs and aortic ring.	[64]

FGF23, fibroblast growth factor 23; GP, glycerophosphate; Pi, inorganic phosphate; VSMCs, vascular smooth muscle cells; VC, vascular calcification.

Table 2

Evidence for α -Klotho expression in vascular tissues or SMCs

Authors	Expression of α -Klotho	Species	Samples Examined	Method of α -Klotho detection	Reference
Scialla JJ <i>et al</i>	Not detected	human	vascular SMCs	RT-PCR	[60]
Mencke R <i>et al</i>	Not detected	human	renal artery, aorta, aortic SMCs	IHC, WB, qPCR	[77]
Lindberg K <i>et al</i>	Not detected	mouse	mesenteric/femoral /lung arteries, aorta	qPCR, IHC, WB	[61]
Lau WL <i>et al</i>	Not detected	mouse	aorta	RT-PCR, WB	[69]
Shiraki-Iida <i>et al</i>	Not detected	mouse	aorta	RT-PCR	[73]
Wang Y <i>et al</i>	Not detected	mouse	aorta	IHC	[75]
Jimbo R <i>et al</i>	Not detected	rat	vascular SMCs	WB	[64]
Mitani H <i>et al</i>	Not detected	rat	aorta	WB	[74]
Wang Y <i>et al</i>	Not detected	rat	aortic SMCs	qPCR, WB	[76]
Lim K <i>et al</i>	Detected	human	epigastric/renal arteries, aortic SMCs	IHC, WB	[63]
Nakano-Kurimoto R <i>et al</i>	Detected	human	coronary artery, SMCs	RT-PCR	[78]
Donate-Correa J <i>et al</i>	Detected	human	aortic SMCs	RT-PCR, qPCR	[79]
Voelkl J <i>et al</i>	Detected	human	aortic SMCs	qPCR	[80]
Navarro-González JF <i>et al</i>	Detected	human	thoracic aorta	RT-PCR, qPCR	[81]
Van Veenrooij NA <i>et al</i>	Detected	human	coronary artery	qPCR, IHC	[84]

Authors	Expression of α -Klotho	Species	Samples Examined	Method of α -Klotho detection	Reference
Lim K <i>et al</i>	Detected	human	epigastric/renal artery, aorta	IHC, WB, mass spectrometry	[86]
Zhao Y <i>et al</i>	Detected	human, bovine	aortic SMCs	qPCR, WB	[88]
Zhu D <i>et al</i>	Detected	mouse	aorta, aortic SMCs	qPCR, IHC, WB	[62]
Fang Y <i>et al</i>	Detected	mouse	aorta	qPCR, IHC, WB	[82]
Fang Y <i>et al</i>	Detected	mouse	aorta	qPCR, IHC, WB	[83]
Jimbo R <i>et al</i>	Detected	rat	aorta	RT-PCR, IHC, WB	[64]
Ritter CS <i>et al</i>	Detected	rat	aorta	IHC	[85]
Chang JR <i>et al</i>	Detected	rat	aortic SMCs	WB, siRNA	[89]

IHC, immunohistochemistry; qPCR, quantitative polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; SMCs, smooth muscle cells; WB, western blotting.