



# Narrative review of exosomes: novel players in vascular calcification of chronic kidney disease

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**Abstract:** Vascular calcification (VC) has been proved the main pathophysiology basis that results in the cardiovascular disease which become the leading cause of death in patients with chronic kidney disease. The disordered mineral metabolism, hyperphosphatemia, overuse of calcium binders, occurred in the setting of uremia, have become the critical risk factors of vascular calcification in chronic kidney disease, in which the vascular smooth muscle cells undergo differentiation to osteoblast-like cells. The mechanisms involved in vascular calcification are complicated and exosomes, as novel players, are proven to play a vital role in VC and function as initializers for mineral deposition. Exosomal miRNAs play an important role in the regulation of cellular functions in vascular calcification. In this review, we focused on the roles of exosomes during VC, especially on their effects on regulating vascular calcification through initializing mineral deposition as a nidus, transporting microRNAs to the recipient cells and mediating the vascular smooth muscle cells differentiation to osteoblast-like cells.

**Keywords:** Vascular calcification; exosomes; chronic kidney disease; vascular smooth muscle cells; microRNA

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## Introduction

Vascular calcification (VC) is highly prevalent in chronic kidney disease which is associated with major adverse cardiovascular events. The core mechanism of VC is that the vascular smooth muscle cells (VSMCs) undergo differentiation to osteoblast-like cells in the uremic environment, especially in the circumstances of hyperphosphatemia and hypercalcemia, and generate matrix vesicle that serves as a nidus for calcium-phosphate deposition in the vessel wall (1,2). As an important intercellular signaling molecule and a novel player, the role of exosome in vascular calcification has been paid more attention in recent years. This review will briefly describe the role of exosomes in the process of vascular calcification and analyze the potential mechanisms associated with

exosomes in VC.

We present the following article in accordance with the Narrative Review checking checklist (available at <http://dx.doi.org/10.21037/apm-20-910>).

## Vascular calcification in chronic kidney disease (CKD)

Cardiovascular disease is the dominant cause of death among patients with chronic kidney disease, in which vascular calcification has been proved the main pathophysiology basis that substantially decreases vessel compliance and increases the incidence and mortality of hypertension, aortic stenosis, myocardial hypertrophy, congestive heart failure and myocardial ischemia (3). Vascular calcification can occur both in the media and the intima and patients

with CKD can develop both types of them, but calcification of the media is more specific to CKD. It is reported that vascular calcification occurs as early as in patients with stage CKD2. In patients with stage CKD 3–4, the incidence of vascular calcification is about 25%, while that in patients undergoing maintenance hemodialysis is as high as 50–80%. VC further exacerbates the deterioration of renal function (4). The disordered mineral metabolism, hyperphosphatemia, overuse of calcium binders, occurred in the setting of uremia, have become the critical risk factors of VC in CKD, compared with the traditional risk factors such as hypertension, dyslipidemia and inflammation.

### **Differentiation of vascular smooth muscle cells to an osteoblast-like phenotype in vascular calcification**

The smooth muscle cells mainly made up for the media of the vascular in a framework of loose connective tissue (mainly elastin). Smooth muscle plays a vital role in maintaining blood pressure through contraction and relaxation. And also, VSMCs is essential in vascular calcification of CKD.

Vascular calcification occurring in CKD was initially supposed to be a passive process where the disordered metabolism of calcium and phosphorous resulted in a precipitation of hydroxyapatite that deposited in the media layer of the vessel wall, which led to increased arterial stiffness. However, in recent years, it is reported that vascular calcification is an positive, regulatable biological process which is similar to bone formation (5). The process involved the differentiation of the VSMCs to osteoblast-like cells and enacted a cellular program that mediated deposition of bone matrix in blood vessels (5). Usually, the VSMCs have a low proliferative rate, but under dysregulation of mineral in the uraemic milieu, contractile VSMCs can undergo phenotypic change to an active synthetic phenotype, demonstrating an increased proliferation rate and secretory ability (6).

It is reported that this phenotypic switch of VSMCs accompanying with elastin break is the starting point of medial calcification (7). This process is mediated by the down-regulation of mineralization inhibitors and up-regulation of the calcification stimulating molecules, in combination with loss of SMC markers (SM22a and SM a-actin) and gain of osteogenic markers [alkaline phosphatase (ALP), Runx2, bone morphogenetic protein2 (BMP2), Osterix] (1,8-10). However, the underlying

mechanism deserved deeply explored.

### **Biological characters of exosome**

Exosomes, microvesicles and apoptotic bodies are all included in extracellular microvesicles (EV) which are classified basing on the mechanism of biogenesis (11). Exosome is firstly discovered as part of reticulocyte maturation, where exosome is a vehicle for removing useless proteins and membrane, as the reticulocyte transforms into a mature erythrocyte (12).

Exosomes are formed within the endosomal network, where stores kinds of intraluminal vesicles and guides them to their proper destinations, including lysosomes and cell surface membranes (13). Endosomes play their role by targeting some proteins or lipids for lysosomal degradation while targeting others for recycling or exocytosis. And the endosomes can be further divided into three sections in different stages: early endosomes, late endosomes, and recycling endosomes (13). Early endosomes fuse with endocytic vesicles and fill them with their contents for recycling, degradation, or exocytosis. The contents destined for recycling are called recycling endosomes. Subsequently, the early endosomes change to late endosomes through a series of transformations. During this transformation stage, some vesicles, about 30–100 nm, can be observed to bud into the lumen of late endosome, including the contents to be degraded or exported. These late endosomes concluding multiple small vesicles also known as multi-vesicular bodies (MVBs). The late endosomes can fuse either with lysosomes or the plasma membrane. Fusion with the former will lead to destruction of the contents of the late endosome, while, fusion with the latter gives rise to the generation of the 30–100 nm vesicles into the extra-cellular space. These excreted vesicles are exosomes (13). And there are some markers, such as CD9, CD63, CD81, which are all the members of the membrane proteins called tetraspanins, to identify the exosomes and have been targeted for selective isolation (2,14).

Additionally, exosome generation is a complex process and involves lots of molecules. The endosomal sorting complexes required for transport (ESCRT) machinery, including the ESCRT-0, I, II, III, is necessary for the exosome packing (15). All of them play important role in binding the special ubiquitinated cargos and localizing them to the endosomal membrane, or interacting with each other to recruit them to the endosomal membrane, and finally lead to the formation of Snf7 oligomers that

cause budding of the vesicle into the endosome lumen (15). The phosphatidylinositol 3-phosphate (PIP3) should be mentioned through which the ESCRT-I functions (16). And Alix, a protein that binds to the tumor susceptibility gene 101 (TSG101) component of the ESCRT-I complex, is also necessary for simultaneously recruiting the ESCRT-III to the site of ESCRT-I and II. Therefore, TSG101 and Alix are two other markers of exosome (17,18). Therefore, it is reported that exosomes are recognized as vesicles containing at least one transmembrane protein (CD9, CD63, CD81, adhesion molecules, etc.) and one cytosolic protein (TSG101, annexins, Rabs, etc.), without any endoplasmic reticulum proteins (calnexin and Golgi matrix proteins) or nuclear proteins (19). In addition, heat shock proteins (HSP60, HSP70, and HSP90), cytoskeletal proteins (actin and tubulin), and membrane transport proteins also exist in exosomes (11). It also has been found that sphingomyelin phosphodiesterase 3 (SMPD3) is implicated in exosome biogenesis and inhibition of SMPD3 blocks exosome secretion and VSMC calcification (20).

Exosome uptake is a process of internalization via endocytosis and complete in an energy-requiring manner requiring an active V-ATPase. The capacity of exosome to generate extracellular ATP may play a role in this process (21,22). However, that may not be the only mechanism of exosome uptake because inhibiting the endocytic pathways can just significantly reduce the exosome uptake but cannot completely avoid it (23,24). It indicates that other underlying mechanisms exist involved in the exosome uptake, for instance, the receptor-ligand interactions on the exosome and recipient cell membranes (11). To know what receptors are activated by exosomes in specific disease states may bring specific therapeutic targets (11). The process of exosome uptake and the merge routes are visible under the fluorescence microscopy through the PKH67 or DiI, some special lipophilic dyes to label the exosome (25,26).

Briefly, exosomes have been identified as novel players mediating the mechanism of intercellular signaling through the delivery of proteins and nucleotides, which bring corresponding phenotypic changes to the recipient cells and series of cascade reactions. Different kinds of cells secrete exosomes into body fluids and various microenvironments by paracrine and other methods, and play a role in adjacent regions or distant cells.

### **The role of exosomes in VC**

Exosome, as an important intercellular signaling molecule

and a novel player, has attracted wide attention with its role in vascular calcification in recent years. It is proved that exosomes derived from stromal cells, and macrophages are play important role in plaque calcification in heart valves and atherosclerosis (AS) (27). Similarly, exosomes released by VSMCs are the smallest molecules to form microcalcification serving as a nidus for the aggregation of the extracellular matrix and then gradually formed the mature minerals in CKD, and exosomes are also found to deliver intracellular contents such as microRNAs (miRNAs) and proteins, functioning as message transporters to promote VC (28,29).

### *Exosomes regulate vascular calcification through initializing mineral deposition as a nidus*

Imbalance of calcium-phosphorus is a typical manifestation in CKD and hyperphosphatemia and hypercalcemia aggravate the precipitation of calcium and phosphate in the form of hydroxyapatite. Exosomes released by smooth muscle cells in the vessel wall interact with fibrillar collagen and may thus serve as nucleating foci for calcific mineral crystallization in early calcification (30). In normal condition, calcification do not occur in these VSMC-derived vesicles, for they are loaded with calcification inhibitors such as fetuin-A and matrix Gla protein (MGP), which act to block mineral nucleation (31). However, VSMCs incubated in a calcifying media can stimulate the production of calcifying vesicles, mainly in the form of exosomes, that are filled with preformed apatite, a hallmark of mineralization competent MVs (31). And these exosomes are related with the reduction of calcification inhibitors and membrane association of annexin (Anx) and phosphatidylserine to form a complex that enable to nucleate hydroxyapatite, which initialize mineral deposition as microcalcification (30). Exosomes with minimal fetuin-A content and increased Anx II and VI can mineralize on an extracellular matrix (ECM), while exosomes with reduced content of Anx II and VI and rising amount of fetuin-A do not (32). Therefore, exosomes released by the calcifying VSMCs are the smallest molecules to form microcalcification. Study has shown that these exosomes are more prone to accelerate and form microcalcifications in areas with sparse collagen when released into the ECM and subsequently formed mature minerals (28). And at the same time the collagen fibrils can effectively slow down the diffusion of oxygen and induce oxidative stress, thus leading to elevated level of Pi and Ca<sup>2+</sup> (33). When the Ca<sup>2+</sup> release from the exosomes, they

can interplay with Pi and phosphatidylserine to form PS-Ca<sup>2+</sup>-P<sub>i</sub> complexes, and with PS and Anx existing on the surface of the exosomes to form PS-Ca<sup>2+</sup>-Anx complexes to nucleate hydroxyapatite and osteogenic proteins, thus triggering VC (34). Therefore, exosomes regulate vascular calcification through initializing mineral deposition as a nidus.

#### ***Exosomes regulate vascular calcification by transporting miRNAs to the recipient cells***

Cell interaction is an important mechanism in the occurrence of VC. Studies have shown that exosomes, as message transporters among cells, functioned by delivering intracellular molecules, such as mRNAs, miRNAs or proteins, to recipient cells to trigger some reactions in the process of promoting vascular calcification (35).

MiRNAs are a vital kind of endogenous, single stranded, non-coding RNAs, which are involved in regulating the gene expression and translation. MiRNAs mainly suppress gene expression through imperfect base pairing to the 3'untranslated region of target mRNAs leading to repression of protein production or mRNA degradation. And the exosomes appear to protect encapsulated miRNAs from the degradative enzymes that are replete within the serum. Therefore, exosomal miRNAs play an vital role in the regulation of cellular functions (35). It is reported that Pri-miRNA is initially transcribed from the genome, processed by Drosha to pre-miRNA, and is then transported into cytoplasm through Exportin-5 where mature miRNA is formed. Mature miRNA is integrated into the miRNA-induced silencing complex (miRISC) and therefore targets mRNA. The P-body fuses with late endosomes and releases miRNA-containing exosomes. Exosomes are further taken up by the recipient cells, regulating a series of gene expression (35).

Exosomes derived from mineralizing pre-osteoblast MC3T3-E1 cells can accelerate bone marrow stromal cell (ST2) differentiation to osteoblasts, which is mediated by an intricate network formed by exosomal miRs (25). And other researches show that some miRNAs are proven to be transported by exosomes to the recipient VSMCs to promote VC in CKD. It is reported that someone aims to differentially present miRNA in calcifying VSMCs and the exosomes they produce, using miRNA array and bioinformatics analyses to identify dysregulated miRNA in CKD animals that may provide insight into the cellular regulation of exosomes packaging of miRNA and to

determine what post-transcriptional networks are involved as VSMCs initiating calcification. The results demonstrate that percent of miRNA of total RNA in exosomes is 4 times greater than that in VSMCs, confirming that miRNA are concentrated in exosomes. And there are 14 miRNAs increased and 19 miRNAs decreased in exosomes compared to VSMCs, and miR-702, miR-667, miR-3562, miR-3584 and miR-3568 are the significantly higher expression genes in exosomes (36). Exosomal miR-223 is found significantly upregulated in Pi-treated VSMCs and increase proliferation and markedly enhance VSMC migration, which display vascular calcification (37). MiR-155 (38) is also proved to be important in VC and it is reported that exosomal miR-155 derived from Treg cells might work as an additional source of miRs during VC (39). Some other miRs, including miR-133b, miR-29b, miR-211, are also revealed to be transported by exosomes to modulate the biological behaviour in various kinds of recipient cells in vascular calcification (40-44). A network of exosomal miRNA-mRNA associations can be observed in which these dysregulated miRNAs function by targeting many genes that regulating cellular response to extracellular stimulus, apoptosis, regulation of extracellular matrix and cell proliferation, formation of exosomes in VC (36). Some signaling pathways such as MAPK signaling pathway may be involved in these exosomal miRNAs mediating calcification process (36,45). The underlying mechanism of exosomal miRNA controlling network in vascular calcification deserve further exploration.

#### ***Exosomes regulate VSMCs differentiation to osteoblast-like cells to accelerate vascular calcification***

Osteogenic phenotype transition of the VSMCs, switching from contractile phenotype to osteoblast-like cells phenotype, represents as a crucial characteristic of VC (46). Cellular-derived exosomes from calcifying VSMC can promote phenotypic alteration of recipient VSMC in vascular calcification by inducing cell signaling changes. It demonstrates that the mRNA expression of the VSMC marker (mainly sm22a) is downregulated, and the osteoblastic differentiation genes [mainly bone morphogenic protein-2 (BMP-2)] upregulated under the calcifying VSMC-derived exosomes, accompanying the increase in intracellular calcium ion concentration (47). And the relative mechanism may involve the activation of both NADPH oxidase and mitogen-activated protein kinase (MEK1 and Erk1/2) signaling in recipient VSMC (47). Other study revealed that tumor necrosis

factor- $\alpha$  and platelet derived growth factor-BB increase exosome production and downregulate the VSMC markers, resulting in increased calcification of VSMCs in response to calcifying conditions (20). Exosomal miRNAs regulating vascular endothelial growth factor (VEGF), an essential regulator of vascular smooth muscle cell function and indicator of de-differentiation from VSMCs to osteoblast-like cells, are also reported (36). And exosomes from hyperglycemia-stimulated vascular endothelial cells can regulate calcification in VSMCs via regulating mitochondrial function with an increased expression of alkaline phosphatase, an important osteogenic marker (48). The formation mechanism of calcified exosomes has also attracted widespread interest among researchers and there are still many things about the exosomes that are not yet known.

### Future perspectives

Exosomes play an important role in the initiation and development of vascular calcification in CKD. Exosomes may provide new insight into the mechanism of the VSMCs differentiation to an osteoblast-like phenotype in vascular calcification. Therapeutic strategies to control the vascular calcification in CKD will require a better understanding of the mechanisms that related with exosomal miRNAs and pathways and prevent or reverse exosomes-driven calcification. Alteration of the properties and biogenesis of VSMC-derived vesicles may be great useful for alleviating vascular calcification. And also, exosomes can be used as biomarkers for diagnosis and prognosis of VC and can play a therapeutic role as a drug or drug carrier.

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