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MicroRNA in cardiovascular calcification: Focus on targets and extracellular vesicle delivery mechanisms

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Abstract

Cardiovascular calcification is a prominent feature of chronic inflammatory disorders — such as chronic kidney disease (CKD), type 2 diabetes (T2D), and atherosclerosis — that associate with significant morbidity and mortality. The concept that similar pathways control both bone remodeling and vascular calcification is widely accepted, but the precise mechanisms of calcification remain largely unknown. The central role of microRNAs (miRNA) as fine-tune regulators in the cardiovascular system and bone biology has gained acceptance and has raised the possibility for novel therapeutic targets. Additionally, circulating miRNAs have been proposed as biomarkers for a wide range of cardiovascular diseases, but knowledge of miRNA biology in cardiovascular calcification is very limited. This review focuses on the role of miRNA in cardiovascular disease, with emphasis on osteogenic processes. Herein, we discuss the current understanding of miRNAs in cardiovascular calcification. Furthermore, we identify a set of miRNAs common to diseases associated with cardiovascular calcification (CKD, T2D, and atherosclerosis), and we hypothesize that these miRNAs may provide a molecular signature for calcification. Finally, we discuss this novel hypothesis with emphasis on known biological and pathological osteogenic processes (e.g. osteogenic differentiation, release of calcifying matrix vesicles). The aim of this review is to provide an organized discussion of the known links between miRNA and calcification that provide *emerging concepts* for future studies on miRNA biology in cardiovascular calcification, which will be critical for developing new therapeutic strategies.

Keywords

miRNA; circulating miRNA; cardiovascular calcification; extracellular vesicles; cardiovascular disease; type 2 diabetes mellitus; chronic kidney disease; aortic stenosis; calcification; osteogenic; bone

Introduction

Micro-RNAs (miRNAs) are a large class of evolutionarily conserved, small, endogenous, non-coding RNAs serving as essential post-transcriptional modulators of gene expression that play a crucial role in normal physiology.[1] miRNAs can be transcribed in parallel with host transcripts through two different transcription classes, exonic and intronic.[2] Independent expression from intronic promoters could explain why host gene and miRNA expression do not always directly correlate.[3] Another level of regulatory biological control

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of miRNAs are “sponge RNAs”, which act as a decoy for miRNAs.[4] miRNAs regulate biological processes by binding to mRNA 3′-untranslated region (UTR) sequences to attenuate protein synthesis or mRNA stability.[5] Acting as genetic switches or fine-tuners, miRNAs are key regulators of diverse biological and pathological processes, including development, organogenesis, apoptosis, and cell proliferation and differentiation. In addition to these known physiological roles, miRNA dysregulation often results in impaired cellular function and disease progression.[5] The detailed regulation of miRNA biogenesis in cardiovascular disease is reviewed elsewhere.[6] The central role of miRNAs as fine-tune regulators in the cardiovascular system is still under investigation; however, miRNAs have received little attention in cardiovascular calcification—one of the most severe pathophysiological outcomes associated with cardiovascular disease.

Cardiovascular calcification is a major characteristic of chronic inflammatory disorders — such as chronic kidney disease (CKD), type 2 diabetes (T2D), and atherosclerosis — that are associated with significant morbidity and mortality. The precise mechanisms of calcification within the vessel wall or heart valve leaflets remain largely unknown, but the concept that pathways controlling bone remodeling also occur in the cardiovascular system is well accepted. Indeed, vascular calcification associates with osteoporosis in humans and animal models[7] — the so-called “calcification paradox”.[8] The physiological balance between induction and inhibition of calcification becomes dysregulated in CKD, T2D, and atherosclerosis, leading to calcification at several sites in the cardiovascular system, including the intima and media of vessels and the cardiac valves.[8] Atherosclerotic calcification occurs as a part of the atherogenic progress in the vessel intima with small hydroxyapatite mineral crystals (microcalcifications) that seem to associate with cholesterol crystals observed in early lesions.[9] Medial calcification occurs primarily in association with CKD and T2D, independently of hypercholesterolemia. Calcification of the aortic valve is associated with many of the same risk factors as intimal and medial calcification and leads to impaired movement of aortic valve leaflets, resulting in valve dysfunction. All three of these processes share risk factors and etiological factors, including inflammation and oxidative stress that appear to lead to changes in phenotype of resident or circulating progenitor cells.

Vascular calcification is an active, cell-regulated process (Figure 1). Various studies provide evidence of phenotypic transition/dedifferentiation of mature smooth muscle cells (SMCs) into an osteogenic phenotype — a key feature in vascular calcification. Osteogenic transition of vascular SMCs or stem cells is induced by bone morphogenetic proteins, inflammation, oxidative stress, or high phosphate levels, and leads to a unique molecular pattern marked by osteogenic transcription factors.[10] In medial calcification, SMCs undergo dedifferentiation from a contractile to a pro-atherogenic synthetic phenotype, lose the expression of their marker genes, acquire osteogenic markers, and deposit a mineralized bone-like matrix. The major lineage source of osteogenic cells within the calcified atherosclerotic intimal lesion are SMCs; however, circulating bone marrow-derived cells also contribute to early osteochondrogenic differentiation in atherosclerotic vessels.[11] Recently, a novel concept emerged that circulating cells harboring osteogenic potential can home to atherosclerotic lesions and contribute to intimal calcification.[12, 13] Independent of the source cell, it seems that master transcription factors, including Runx2/Cbfa1, Msx2, and osterix (SP7), designate cells for osteoblast lineages through the induction of downstream genes such as alkaline phosphatase (ALP), and osteocalcin. Runx2 acts as a critical regulator of osteogenic lineage and a modulator of bone-related genes[14] and is essential and sufficient for driving SMC calcification.[15,16, 17]

Discovered in the bone biology field, a program of miRNAs controls Runx2 expression to prevent skeletal disorders.[18] Three of these miRNAs (miR-133a, miR-135a, and miR-218)

are altered in the circulation of patients with cardiovascular diseases.[19, 2021, 22] Additionally, circulating miRNAs have been proposed as biomarkers for a wide range of cardiovascular diseases, but knowledge of circulating miRNA in cardiovascular calcification is scant. No pattern of miRNA has been reported for vascular calcification; however, as new studies emerge, it may be helpful to understand the role of miRNAs in diseases and cellular signaling processes known to be associated with calcification. Therefore, in this review we begin by discussing the current, limited understanding of miRNA in cardiovascular calcification. We then document a set of circulating miRNAs, which is dysregulated in certain cardiovascular diseases (e.g., CKD, T2D, atherosclerosis), and we propose a potential circulating miRNA signature for vascular calcification. We discuss this novel hypothesis with emphasis on known biological and pathological osteogenic processes (e.g. osteogenic differentiation, release of calcifying matrix vesicles). Furthermore, we summarize and discuss miRNAs that control bone osteogenesis and link them to potential regulation of cardiovascular calcification.

miRNA in Cardiovascular Calcification and Osteogenesis

Evidence for the role of miRNA in cardiovascular calcification is very limited (Table 1). However, we recently provided the first miRNA-dependent mechanism in the progression of vascular calcification by demonstrating that miR-125b dysregulation leads to the transition of human coronary arterial SMCs into osteoblast-like cells partially by targeting the transcription factor osterix.[23] Inhibition of miR-125b promoted ALP activity and matrix mineralization *in vitro*. Correspondingly, *in vivo* observations indicate that miR-125b decreased in calcified aortas of apolipoprotein-deficient (*Apoe*) mice fed a high fat diet for 26 weeks compared to those sacrificed after 10 weeks.[23] Additionally, miRNA-processing enzymes DROSHA and DICER — essential for SMC function[24] — were reduced in calcified SMCs.[23]

Using the Exiqon mercury Locked Nucleic Acids (LNA) microRNA array, Gui *et al.* found 20 altered miRNAs in the aortic media of klotho mutant (*kl/kl)* mice.[25] *Kl/kl* mice display vascular calcification due to hyperphosphatemia and through a Runx2-dependent mechanism, presenting the symptoms of CKD-associated bone and mineral disorders.[26] 17 miRNAs were increased and 3 decreased (miR-1, miR-93, miR-302b) in *kl/kl* mice compared to wild type mice. An increased expression of miR-135a*, miR-762, miR-714, and miR-712* was detected in Ca/Pi-stimulated vascular SMC isolated from WT mice and within the aortic media of *kl/kl* mice and is correlated with decreased expression of their potential target genes NCX1, PMCA1, and NCKX4 – all Ca^{2+} efflux proteins. This disruption of Ca^{2+} transporters and the resultant increase in intracellular Ca^{2+} concentrations could be involved in medial SMC calcification.[25] It is important, however, to mention that functional studies altering one single miRNA (miR-135a*, miR-762, miR-714, or miR-712*) by mimics or inhibitors failed to show an effect on SMC calcification; whereas inhibition of all 4 miRNAs together significantly reduce calcium content of SMC by 30%. miR-680 served as control, because it was the most highly up-regulated miRNA in the aortic media of *kl/kl* mice. The failure of miR-680 specific inhibitor to reduce SMC calcification resulted in the conclusion, that miR-135a*, miR-762, miR-714, and miR-712* are specifically involved in calcification. Indeed it has been shown that miRNAs often function in clusters; however, it is still unknown whether the cluster is limited to these 4 miRNAs. Additionally, the role of the other 15 highly regulated miRNAs in *kl/kl* mice needs to be determined.

miR-204, a known inhibitor of osteoblastogenesis,[27] and one candidate of the Runx2-miRNA-cluster in osteoblasts[18] was also recently found to contribute to SMC calcification *in vitro* and *in vivo*.[28] *In vitro*, miR-204 was identified to be a negative regulator of SMC

calcification through direct targeting the 3′UTR of the Runx2 gene in mouse SMCs cultured in calcifying media consisting of 10 mM b-glycerolphosphate.[28] Similarly, in a mouse model of vitamin D3-induced vascular calcification, overexpression of miR-204 by agomiRs decreased medial calcification and Runx2 expression mostly to control level within 3 days of treatment.[28]

As mentioned above, osteogenic differentiation of SMCs is also characterized by a loss of traditional SMC markers. Changes in the expression levels of the miR-143/145 cluster promotes differentiation and represses proliferation of SMCs thereby maintaining the SMC phenotype; [29–31] and have also been linked to vascular calcification. Exposure of human primary SMC to pathophysiological levels of inorganic phosphate (Pi) decreased miR-143 and miR-145 expression. Similarly, these miRNAs are down-regulated in the aorta of 20 week old *Apoe*-deficient mice.[32] Even though studies have yet to establish a direct functional role of miR-143 and miR-145 in vascular calcification, this hypothesis is supported by findings noted in literature of diseases associated with calcification. miR-145 promotes SMC differentiation by targeting Krüppel-like factor (KLF) 4, [30] and KLF4 mediates high phosphate-induced transition of SMCs into osteogenic cells.[33] Inhibition of miR-143/145 promotes a phenotypic switch to the synthetic, pro-atherogenic SMC state,[29] including the inhibition of SMC marker-like alpha-smooth muscle actin and smooth muscle myosin heavy chain[34] — both diminished in osteogenic SMCs.[35] Additionally, circulating miR-145 levels are reduced in patients with coronary artery disease (CAD).[19] Moreover, miR-145 was identified as part of the specific miRNA profile of destabilized human plaques,[36] a biomechanical failure of the plaque that may involve microcalcification.[37, 38]

In mediating these cellular changes, the paracrine osteogenic signals facilitated by morphogens of the bone morphogenetic protein (BMP) and wingless-type MMTV integration site family member (Wnt) superfamilies, are effective regulators of vascular and valvular calcification, but also necessary in controling skeletal osteogenesis.[39] Two members of the BMP signaling pathway (reviewed in detail elsewhere),[39] BMP-2 and BMP-4 are potent osteogenic differentiation factors detected in calcified areas of atherosclerotic lesions.[40, 41] BMPs elicit their effects through activation of a receptor complex composed of type I and type II receptors and activate receptor-type–dependent and ligand-dependent Smad transcription factors, which modulate the expression of Runx2.[39]

A recent study indicates that BMP-2 promotes SMC calcification by decreasing the expression of miR-30b by 6.2-fold and miR-30e by 5.5-fold though an Smad-independent pathway that leads to a direct increase in Runx2.[42] Using antagomirs to block these miRNAs it was found that downregulation of miR-30b and miR-30e *in vitro* is sufficient to increase Runx2 expression, even in the absence of BMP-2. Accordingly, calcified human coronary arteries demonstrate higher BMP-2 levels and lower levels of miR-30b than non-calcified coronary arteries. Similar miRNA modulations of BMP-2 signaling have been observed in studies of heart valve calcification. Nigam *et al.* recently identified a miRNA pattern specific to aortic stenosis (AS), which is typically caused by calcific aortic valve disease, using whole bicuspid valves.[43] miR-30b was decreased in the aortic valves of patients requiring replacement due to AS, compared to those requiring replacement due to aortic insufficiency.[43] Another group compared bicuspid and tricuspid aortic valve leaflets by miRNA microarray, and found a number of modulated miRNAs.[44] Particularly, miR-141 had the most dramatic change, showing a 14.5-fold decrease in the bicuspid versus tricuspid valves; however the levels of calcification were comparable between the two groups. *In vitro*, miR-141 represses the valvular interstitial cell, the resident cell within cardiac valve leaflets, response to osteogenic stimuli, in part through blocking BMP2-dependent calcification.[44] Likewise, Itoh *et al.* identified miR-141 as a pre-osteoblast differentiation-related miRNA, which modulated the BMP2-induced pre-osteoblast differentiation by direct

translational repression of Dlx5, a transcription factor for osterix.[45] miR-26a is another miRNA, which is repressed in aortic valve leaflets of patients with AS.[43] miR-26a, was previously identified as a Smad-regulating miRNA related to osteoblastogenesis; it functionally represses osteoblast differentiation by targeting Smad1 and Smad5 expression.[46] Human aortic valvular interstitial cells showed decreased mRNA levels of both BMP-2 and Smad1 when treated with a miR-26a mimic.

While BMP-2 is a known inducer of osteogenic differentiation, activation of Wnt signaling is crucial for controlling osteoblast function [47] and for the programming of vascular cells during cardiovascular calcification.[39] Activation of the Wnt/β-catenin signaling pathway occurs in human calcified aortic valve stenosis,[48] in *low density lipoprotein*-deficient mice, [49, 50] and in osteogenic SMCs *in vitro*.[51] miR-29a potentiates osteoblastogenesis by modulating Wnt signaling. Interestingly, miR-29a/b was repressed in high-phosphate induced calcifying rat SMC, calcified abdominal aortas from rats with CKD induced by 5/6 nephrectomy and human radial arteries with chronic kidney failure.[52] miR-29a/b directly targeted a disintegrin and metalloproteinase with thrombospondin motifs-7 (ADAMTS-7) as shown by luciferase assay. A miR-29a/b mimic inhibited, and a miR-29a/b inhibitor enhanced, high-phosphate-induced SMC calcification though alteration of ADAMTS-7, BMP-2, p-Smad 1/5/8 and Runx2 protein expression. Because miR-29 also associated with reduced extracellular matrix components, such as collagen and elastin,[53] low cellular miR-29a/b levels may also cause vascular thickening, fibrosis and elastolysis, which accelerate arterial and aortic valve calcification.[54]

Likewise, the canonical Wnt signaling induces miR-29a expression, which negatively targets regulators of Wnt signaling, including Dickkopf (Dkk) 1, sFRP2, Kremen, and osteonectin.[55, 56] Dkk1 is an extracellular antagonist of the canonical Wnt signaling that plays a crucial role in bone remodeling.[57, 58] Dkk1 was also shown to prevent vascular calcification by preventing warfarin-induced activation of β-catenin, and osteogenic transdifferentiation of SMCs[59] and tumor necrosis factor-α-induced induction of ALP activity.[49] Dkk1 serum levels in CKD patients correlates negatively with arterial stiffness,[60] and matrix metalloproteinase-2, another target of miR-29,[61] promotes arterial calcification in CKD.[62]

Studies about miRNA expression in human calcified tissue are rare (Table 2). Li *et al.* analyzed the expression of miRNAs in patients with peripheral artery disease (arteriosclerosis obliterans), characterized by fibrosis of the tunica intima and calcification of the tunica media.[63] miR-21, miR-130a, miR-27b, let-7f, and miR-210 were significantly increased, while miR-221 and miR-222 were decreased in the sclerotic intima samples, compared to normal vessels.[63] Higher levels of miR-21 and miR-210 were confirmed in a study that compared atherosclerotic lesions with non-atherosclerotic left internal thoracic arteries.[64] miR-210 is known to promote osteoblast differentiation though the inhibition of the BMP co-receptor activin type IB receptor (ALK4).[65] In line with this evidence, activin — a ligand for ALK4[66] — inhibits SMC mineralization.[67] Another study found a different miRNA pattern using atherosclerotic plaque material from the carotid artery, compared with a specimen from the arteria mammaria interna as control tissue.[20] The healthy vessel expressed higher levels of miR-520b and miR-105, whereas miR-10b, miR-218, miR-30e, miR-26b, and miR-125a were predominantly expressed in atherosclerotic plaque.[20] The investigators in these studies, however, did not examine calcification levels to determine a potential correlation with the observed changes in miRNA.

In addition to the osteoblastogenesis of SMCs, the contribution of osteoclasts, bone resorbing cells that play an active role in normal bone physiology, to vascular calcification is controversial and poorly understood.[12] The observation of osteoclast-like cells in calcified

atherosclerotic lesions suggested this bone-related cell is active in the vessel wall. The evidence was strengthened recently by Sun *et al.*, who demonstrated the functional role of SMC-derived Runx2 promoting infiltration of macrophages into the calcified lesion to form osteoclast-like cells —suggesting that the development of vascular calcification is coupled with the formation of osteoclast-like cells, paralleling the bone remodeling process.[16] The receptor activator of the nuclear factor-kappa B (NF-kappa B) ligand (RANKL)/ osteoprotegerin system controls proper osteoclastogenesis, and act as a biomarker for CAD.[68, 69] Five miRNAs are linked to osteoclastogenesis as well as to cardiovascular disorders. (5) RANKL is a proposed target of miR-126, [70] which is decreased in the plasma of CAD [19] and T2D [71] patients. (2) miR-155, which is decreased in plasma of CKD[72] and CAD[19] patients, was shown to inhibit osteoclast function in Dicer-deficient osteoclasts.[73] (3) miR-146a, highly expressed in atherosclerotic arteries,[64] inhibits osteoclastogenesis by inhibition of the number of tartrate-resistant acid phosphatase-positive multinucleated cells.[74] (4) miR-223, a key factor in osteoclastogenesis,[75] was found to be increased by Pi treatment of human SMC and in the calcified aorta of *Apoe*-deficient mice.[32] Overexpression of miR-223 enhanced SMC migration, decreased actin cytoskeleton and modulated target genes, like Mef-2 and RhoB. (5) Wang *et al.* demonstrated an association of miR-133a levels in circulating monocytes - osteoclast precursors - with postmenopausal osteoporosis.[76] Women with low bone mineral density showed higher circulating miR-133a levels[76], but the number of patients per group was small (n=10). Circulating miR-133a levels were also higher in patients with CAD.[19] However, direct links between miR-133a and/or miR-223 and vascular calcification processes have not been reported.

More careful research needs to be conducted to establish direct links between miRNAs and known signaling pathways associated with osteogenic differentiation in the context of cardiovascular calcification. For example, despite the links from different studies noted above, the direct Pi – miR-143/145 – KLF4 axis in vascular calcification has not been directly examined. Additionally, future studies may also lead to a better understanding of the role of miRNA networks in controlling cellular differentiation and ultimate tissue level changes. For instance, though miR-204 is a known inhibitor of osteoblastogenesis, introduction by a mimic agomiR-204 does not alter bone formation in a mouse model of vitamin D3-induced vascular calcification.[28] The observation that miRNAs function in clusters to control cellular and tissue homeostasis may help explain some of the current inconsistencies observed between normal bone formation and vascular calcification (e.g., the correlation between osteoporosis and calcification—"the calcification paradox"). Future miRNA studies may also better connect known signaling pathways associated with osteogenesis to the ultimate pathological outcomes, thereby leading to a deeper understanding of the cellular processes that lead to calcification.

Circulating miRNAs in Diseases Associated with Vascular Calcification – A Potential miRNA Signature

Our detailed investigation using currently published literature revealed common circulating miRNAs in diseases associated with vascular calcification. We compared miRNA signatures identified in 10 CAD, 4 T2D, and 1 CKD studies (Table 2). From this analysis, seven miRNAs (miR-21, miR-27, miR-34a, miR-126, miR-146a, miR-155, and miR210) were found to be useful biomarkers shared between at least two of the compared diseases, but only miR-21 was common among all three diseases.[19,71,72] (Figure 2) miR-21 relates to key processes in the progression of atherosclerosis [77–79] and predicted targets of miR-21 include BMPR2, which has been linked to atherosclerotic calcification.[80] Atherosclerotic arteries [64] and sclerotic intima from lower-extremity vessels [63] expressed higher miR-21 levels than did healthy vessels, whereas circulating levels of miR-21 in atherosclerosis (serum), T2D (plasma), and/or CKD (plasma) were reduced.[19,71,72] The reason for this opposing

observation is not fully understood, and requires further investigation. A recent report showed that miRNAs increased in atherosclerotic abdominal aortic aneurysm tissue, while reduced levels were found in the circulation.[81] miRNAs packaged inside exsomes or apoptotic bodies may be specifically taken up by diseased tissue, which decreases circulating miRNAs.[82, 83]

Of note within the miRNAs shared between two of the diseases, miR-146a is an inflammation-related miRNA, implicated in atherosclerosis and osteoclastogenesis.[74] While circulating miR-146a obtained from peripheral blood mononuclear cells (PBMC) is increased in CAD patients,[84] T2D studies showed controversial results; miR-146a serum levels were increased,[85] and levels in monocytes were decreased.[86] In addition, miR-146a was more highly expressed in atherosclerotic arteries in an animal model[64] and is associated with CKD *in vivo*.[87] Additionally, miR-155, another inflammation-associated miRNA, was decreased in serum of CAD [19] and plasma of CKD patients.[72] Deficiency of miR155 enhanced atherosclerotic plaque development and decreased plaque stability, [88] suggesting that it acts as an anti-inflammatory and atheroprotective miRNA. miR-155 is also highly expressed in endothelial cells (ECs) and SMCs, where it targets angiotensin-II receptor.[89] The renin–angiotensin system participates in vascular calcification,[90] and angiotensin-receptor blockade can inhibit arterial calcification by disrupting vascular osteogenesis *in vivo*.[91] Furthermore, miR-155 repressed osteoblastogenesis by targeting Smad proteins.[46] Thus, high expression of miR-155 may prevent vascular calcification by inhibiting the BMP signaling pathway or the renin–angiotensin system. Comparison of circulating miRNAs in published studies is challenging mainly because of the different sources of circulating miRNAs, which include serum, whole blood, PBMCs, endothelial progenitor cells, and platelets (Table 2) and employ different protocols. The miRNA profiles obtained from the different studies, therefore, are often not the same. Future studies need to take standardized approach to identify circulating miRNAs. In this context, a recent report suggested the necessity of careful selection for reference miRNAs by showing that hemolysis may significantly affect the levels of plasma miRNAs previously used as endogenous controls.[92] Therefore, hemolysis should be determined, to avoid the phenomenon based on red blood cells.[93] Alternatively, the determination of miRNAs known to be enriched in red blood cells, like miR-451 and miR-16 could be performed.[92] A novel LNA™-based qPCR method can identify samples affected by sources of pre-analytical variation such as cellular contamination.[93]

Allowing for these limitations, a set of circulating miRNAs (consisting of miR-21, miR-27, miR-34a, miR-126, miR-146a, miR-155, and miR-210) is dysregulated in various pro-inflammatory diseases and may represent a miRNA signature for vascular calcification. Of note, systemic and local inflammation paradoxically affects cardiovascular calcification and bone loss, which supports the concept of inflammation-dependent cardiovascular calcification previously proposed by our group and others.[9, 54, 94–96] Given the observation that miRNAs often function in clusters and networks,[71] the subset of circulating miRNAs identified in our analysis may turn out to be a portion of a larger network that regulates cardiovascular calcification, wherein the up- or down- regulation of a single miRNA is less important than the function of the network as a whole. Using a network analysis, key miRNA clusters may then be identified as the core regulators of the calcification process. Future studies may be able to utilize this subset as a starting point when trying to compile the larger networks for calcification.

Extracellular Vesicles in miRNA Transport and Calcification

miRNAs are present in blood (plasma, platelets, erythrocytes, nucleated blood cells) with high stability that is conferred by encapsulation in extracellular vesicles,[97, 98] association

with a protein complex with the RNA-binding protein Argonaute (Ago) 2,[124] or in lipoprotein complexes (HDL).[99] These associations prevent the degradation of the miRNAs while in the circulation. However, the mechanism by which the majority of miRNAs are extracellular transported remains controversial. Exported miRNAs are found both within and outside of 16,500 and 120,000 × g centrifugation pellets, which contains most of the cell-derived vesicles.[97] Other studies showed that the majority of miRNAs are independent of vesicles and co-purify with the Ago2 complex, which is known as a key intracellular effector protein of miRNA-mediated RNA silencing.[100, 101] Arroy *et al.* quantified 88 plasma miRNAs (isolated by 120,000 × g centrifugation, 70 min) from healthy donors and found that 90% of them are present in a non-membrane-bound form.[100] Another study compared microparticles isolated from a 16,000 × g (90 min) centrifugation of plasma from patients with stable CAD and patients with acute coronary syndrome as a control. In CAD patients, most plasma miRNAs associate with extracellular vesicles, and only a small amount are found in extracellular vesicle-free plasma.[102] However, the number of cases is very small (n=5 per group) and a healthy control group is missing. To determine if the differing patterns of vesicle-associated miRNAs may be used to characterize different diseases, larger study collectives with standardized protocols are needed.

The miRNA pattern found within released vesicles is different from that associated with Ago2 complexes,[100] implicating a cell-type-specific miRNA release utilizing different export systems. Depending on the size and type, extracellular vesicles are broadly classified as ectosomes (also called shedding microvesicles), exosomes, matrix vesicles, and apoptotic bodies. Ectosomes are large extracellular vesicles 50–100 nm in diameter. Exosomes are small membranous vesicles of endocytic origin with diameter of 40–100 nm. Matrix vesicles, which have been shown to calcify, are 30–300 nm in diameter and produced by blebbing of the plasma membrane. Apoptotic bodies, 500–1000 nm in diameter, are released from fragmented apoptotic cells. Cells may select miRNA and pre-miRNA for cellular release as cargo within these vesicles in a context dependent manner.[103, 104] miRNA profiles of extracellular vesicles are different from their maternal cell profiles, indicating an active mechanism of selective miRNA packing from cells into vesicles,[102] whereas Ago2–miRNA complexes may be passively produced by dead cells, released by live cells, or actively transported though cell-membrane–associated channels or receptors.[105] Additionally, blockade of sphingomyelinase inhibits exosome generation, miRNA secretion, and subsequent intercellular miRNA transfer implicating a ceramide-dependent mechanism in miRNA packaging and release within extracellular vesicles.[106, 107]

Cells utilize extracellular vesicles to transport miRNA and mediate intercellular communication over long distances or on a local tissue level.[98] Endothelial apoptotic bodies can convey miR-126 to atherosclerotic lesions, demonstrating unique paracrine-signaling function for miRNA during atherosclerosis.[71, 83] A recent report provided evidence that miRNA-containing vesicles regulate intercellular communication between ECs and SMCs by selective packaging of the previously discussed regulators of SMC phenotype, miR-143/145, in EC-derived vesicles that are then transported to SMCs in the vessel wall.[107]

The mechanism by which miRNAs are received by target cells in a biologically active state is still unknown. In physiological conditions, extracellular vesicles may bind to the membrane proteins of the surface of target cells through receptor–ligand interaction, resulting in intracellular stimulation of genetic pathways. They can also fuse with cell–target membranes and release genetic content in a nonselective manner. Furthermore, vesicles can bind to surface receptors on target cells with endocytotic internalization by recipient cells, followed by fusion with the membranes, leading to a release of their content into the cytosol

of target cells and allowing the vesicle contents to directly associate with intracellular components.[108] (Figure 3A, 3B)

In addition to their known role as regulators of intercellular communication, extracellular vesicles have also been observed to participate in matrix mineralization. In non-pathological biomineralization in cartilage and bone, chrondrocytes and osteoblasts release extracellular vesicles, which serve as nucleation site for hydroxyapatite and initiate mineralization.[109] These structures are also observed in ectopic calcification. The release of vesicles from SMC is an established key event in the initiation and promotion of SMC calcification.[110] Treatment of SMCs with elevated calcium levels promotes the production of calcifying matrix vesicles, and the loss of fetuin-A, an inhibitor of mineral nucleation.[111] These vesicles act as early nucleation sites for calcification. The phosphatidylserine-membrane complex from SMC-derived and macrophage-derived matrix vesicles redistributes and nucleates hydroxyapatite.[112–114] In addition, hydroxyapatite nanocrystals shed from vesicles may further promote mineralization via direct effects on the SMC phenotype.[115] Using a proteomic approach it was also shown that calcified extracellular vesicles-derived from SMC contain a specific protein profile compared with non-calcifying vesicles,[110] which might be caused by a specific packaging mechanism.

In a pro-osteogenic environment, the specific, physiologic vesicle-mediated transport of miRNA in the vasculature may be disturbed through differential miRNA packaging into the vesicles, decreased miRNA stability by increased enzymatic activity (methyl transferase, 3′-5′ exonuclease) within the vesicles, increased matrix vesicle degradation, or blocked or non-specific uptake of vesicle into the target cell due to mineral nucleation on the outer membrane (Figure 3C). In fact, SMC-derived matrix vesicles have been observed to calcify in a cell-independent manner when exposed to mineralizing conditions on a collagen I substrate.[112] These results may explain the observations of an increased number of matrix vesicles [116] and numerous microcalcifications throughout the collagen I-rich fibrous caps of atherosclerotic plaques. [117] These microcalcifications have been hypothesized to contribute directly to plaque rupture that leads to thrombosis.[118] Given these observations, miRNAs packed in extracellular vesicles may contribute to vascular calcification in two ways. First, vesicles packaged with miRNA for paracrine signaling within the plaque may become entrapped and form microcalcifications. Second, in turn, this may prevent the miRNAs within the vesicles from reaching the intended target cell, leading to phenotypic changes that promote further calcification. Therefore, insight into the underlying mechanism of selective packing of miRNAs into extracellular vesicles and selective uptake into the target cell will help increase understanding of the role of miRNA-containing vesicles in physiological intercellular communication as well as unintended disruption of this communication, which may prevent calcification in the vascular system.

Conclusion and emerging concepts

In this review, we have discussed an emerging role of miRNAs in cardiovascular calcification, and we have also analyzed the literature of diseases that are known to correlate with calcification in an attempt to establish a potential signature for cardiovascular calcification that should be investigated further. *In vitro* and *in vivo* studies have established miRNAs as biomarkers, thereby providing circulating miRNA signatures for different diseases. But these circulating miRNAs may not have biological functions while circulating. Instead, they may act as intercellular communicators, and this communication might be disturbed in calcification disorders, where calcifying matrix vesicles fail to properly delivery miRNAs to the target cell. Studies to fully exploit this potential novel mechanism of cardiovascular calcification are needed.

Moreover, miRNA biology is very complex. Multiple miRNAs can target the same gene (e.g., Runx2–miRNA cluster), and one miRNA might have several targets. Only a small amount of these fine-tuned targets may alter biological responses and phenotypes. Understanding the role of miRNA in vascular calcification may be helpful in considering the paradoxical clinical observations of the concurrence of cardiovascular calcification and osteoporosis. Despite its global clinical burden, no medical therapies are available to treat cardiovascular calcification. Targeting of miRNA represents a novel therapeutic opportunity for treating cardiovascular calcification. As cardiovascular calcification and bone remodeling share common mechanisms, we need an in-depth understanding of miRNA function and their association with the molecular pathogenesis of osteoporosis and vascular calcification. This knowledge will be critical for the developing of a more specific therapy for cardiovascular calcification that does not adversely affect physiological bone homoeostasis.

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Nonstandard Abbreviations

Ago	Argonaute
AS	aortic stenosis
ALP	tissue non-specific alkaline phosphatase
BMP	bone morphogenetic protein
CAD	coronary artery disease
CKD	chronic kidney disease
Dkk-1	Dickkopf 1
EC	endothelial cell
KLF	krüppel-like factor
miRNA	microRNA
PBMC	peripheral blood mononuclear cells
Pi	inorganic phosphate
RANKL	receptor activator of the nuclear factor-kappa B (NF-kappa B) ligand
SMC	smooth muscle cells
T2D	type 2 diabetes
UTR	untranslated region
Wnt	wingless-type MMTV integration site family member

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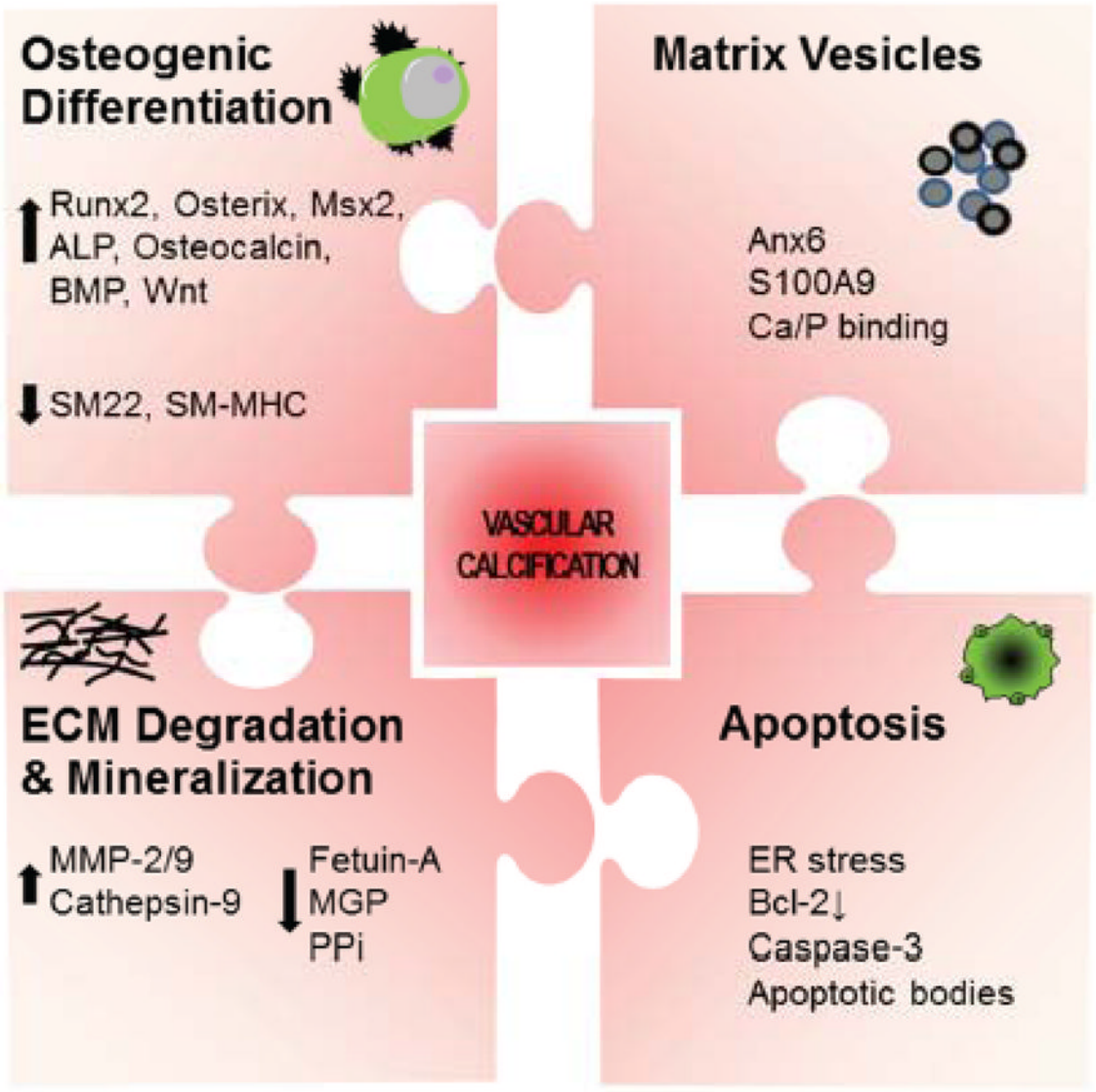

Figure 1.
Major pathways/molecules involved in cardiovascular calcification.

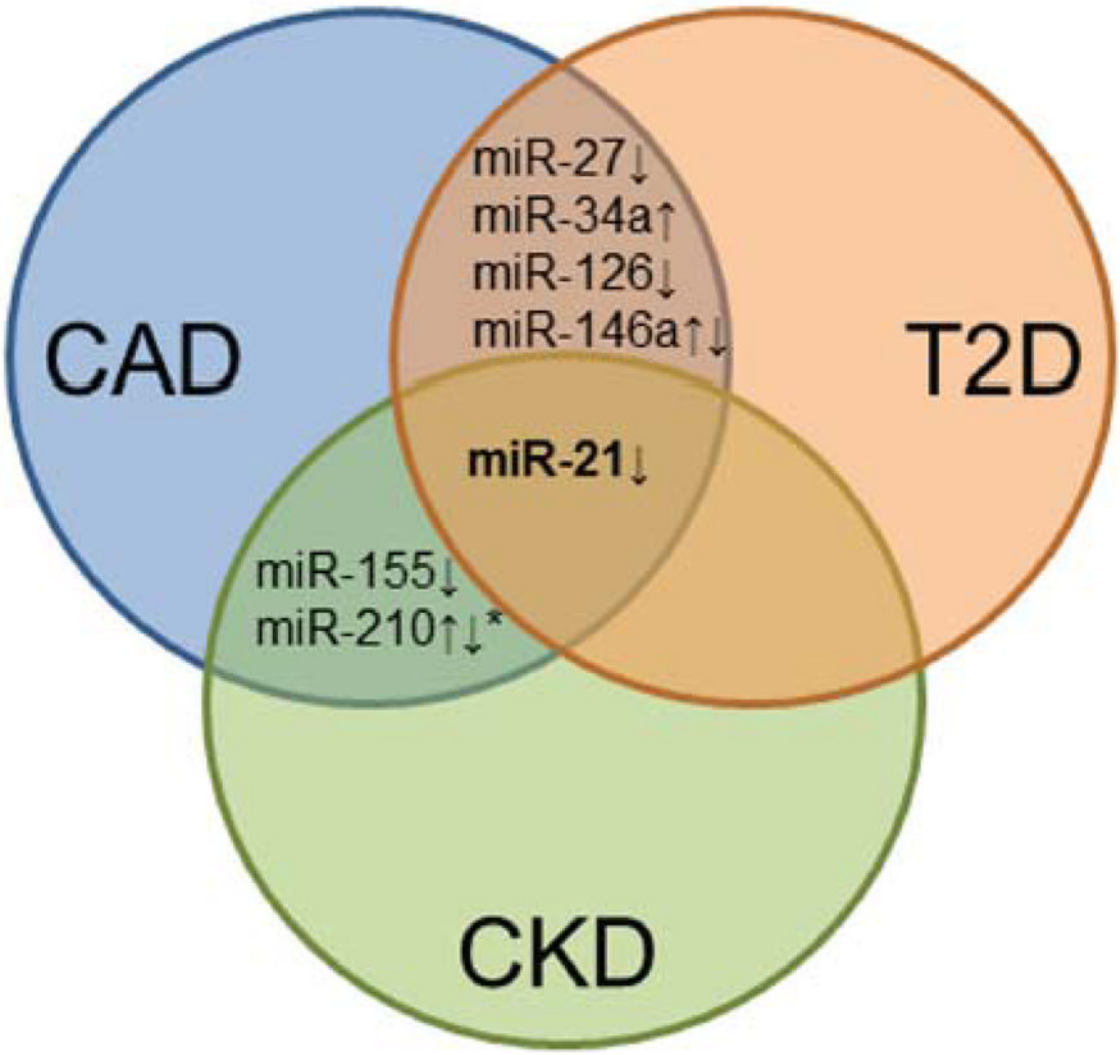

Figure 2. Common circulating miRNA in diseases associated with vascular calcification

Venn diagram shows overlapping miRNAs identified in at least two of the three cardiovascular diseases (CRD, chronic renal disease; T2D, type 2 diabetes; CAD, coronary artery disease).*Also found in arteriosclerosis obliterans.

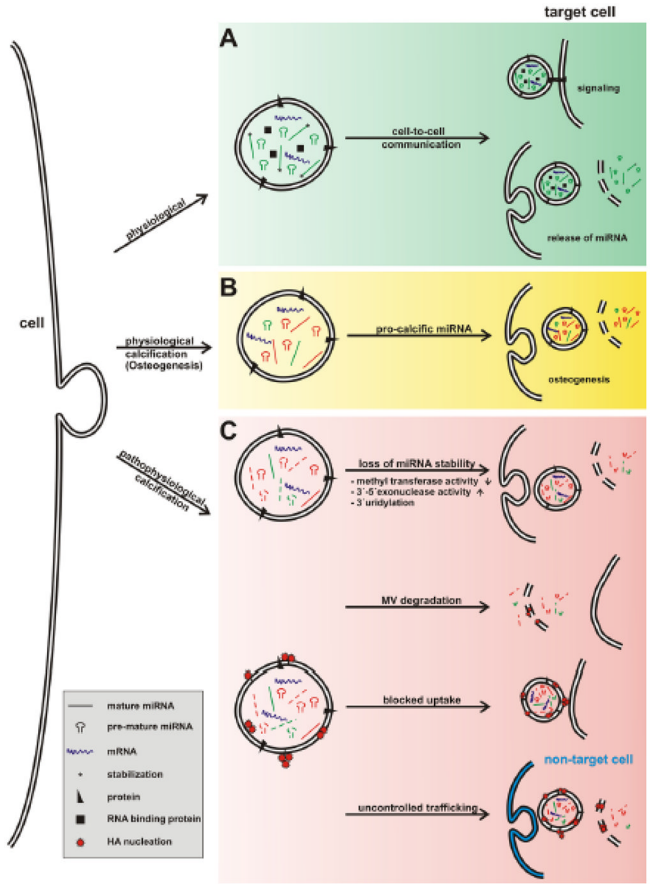

Figure 3. Alteration of matrix vesicle transport of circulating miRNAs in cardiovascular calcification

(A) In physiological conditions or during **(B)** bone calcification (osteogenesis), extracellular vesicles bind to the membrane proteins of the surface of target cells through receptor–ligand interaction, causing signaling processes. They can also fuse with cell–target membrane or bind to surface receptors on target cells with endocytotic internalization by recipient cell, leading to a release of their content into the cytosol of target cells. **(C)** Mechanisms associated with extracellular vesicle-miRNA–mediated pathological calcification. Potential mechanisms associated with extracellular vesicle-miRNA–mediated pathological calcification include (1) a different miRNA packaging into vesicles due to the osteogenic environment; (2) increased degradation due to increased enzymatic activity within the vesicle; (3) blocked vesicle uptake into the target cell; and (4) uptake in non-target cells due to mineral nucleation on the outer membrane.

Table 1

Specific miRNAs, their targets and effects in cardiovascular calcification

miRNA/Regulation	Target	Cell source	Observation by using inhibitor/mimic	Reference number
miR-125b ↓	**SP7**	Human CASMC	Inhibit ALP activity and matrix mineralization	23
miR-141 ↓	-	Porcine VIC	Blocks TGF-β-mediated ALP activity	44
miR-223 ↑	**Mef2c RhoB SMα actin**	Human SMC	Enhance VSMC migration	32
miR-143 miR-145 ↓	**KLF4**	Human SMC	-	32
miR-30d/e ↓	Runx2	Human SMC	Inhibit ALP activity and matrix mineralization	42
miR-204 ↓	Runx2	Mouse SMC	Inhibit ALP activity and osteocalcin secretion	28
miR-29a/b ↓	ADAMTS-7	Rat SMC	Inhibit matrix mineralization	52
miR-135a*, miR-762, miR-714, or miR-712* ↑	**NCX PMCA NCKX4**	Mouse SMC	Promote matrix mineralization	25

Bold; direct binding to 3′UTR not shown in this study, Arrow indicate regulation of miRNA

ALP, alkaline phosphatase; CASMC, coronary artery SMC

Table 2

miRNAs expressed in human atherosclerotic/calcified tissue.

miRNA	Disease	Tissue type	Finding	Control tissue	Reference number
miR-***21***, -34a, *-146a*, -146b-5p, ***-210***	CAD	Atherosclerotic arteries	Increased	Non-atherosclerotic left internal thoracic arteries	64
miR-105, -520b miR-10b, -26b, **-30e**, -125a, -218,	CAD	Atherosclerotic carotid artery	Decreased Increased	Arteriamamm ariainterna	20
miR-**30b**	CAD	Calcified carotid artery	Decreased	Non-calcified carotid artery from non CAD patients	42
miR-100, -127, *-133a*,b -145	CAD	Destabilized plaque	Increased	Stabilized plaque	21
miR-221, -222 miR-***21***, *-27b*, ***-210***, *-130a*, let-7f	AO	Sclerotic intima from lower extremities vessels	Decreased Increased	Non-sclerotic intima from lower extremities vessels	63
miR-22, *-27a*, -141, -124, -125b, -185, -187, -194, -211, -330, -370, -449, -486, -551, -564, -575, -585, -622, -637, -648, -1202, -1282, -1469, -1908, -1972 miR-**30e**, -32, *-145*, -151, -152, -190, -373, -768	AS	Bicuspid aortic valve	Decreased Increased	Tricuspid aortic valve	44
miR-26a, -**30b**, -195	AS	Whole bicuspid valves	Decreased	Replacement due to aortic insufficiency	43

Bold: Found in more than one study. *Italic*: Found also in circulation (Table 1).

CAD, coronary artery disease; AS, aortic stenosis; AO, arteriosclerosis obliterans.

Table 3

Circulating miRNA in diseases associated with vascular calcification.

miRNA	Disease	Source	Finding	Reference number
miR-17**, *-21***,-20a, -22a, ***-27a***, -92a, **-126**, *-145*, **-155,*-221***, -130a, -208b, let-7d miR-*133a*, -208a	CAD	Serum	Decreased Increased	19
miR-146a/b			Increased	84
miR-34a	CAD	EPC	Increased	119
*miR-221, **-222***	CAD	EPC	Increased	120
miR-135a, -147	CAD	PBMC	Decreased	22
miR-140, -182	CAD	Whole blood	Decreased	121
miR-122, -370	CAD	Plasma	Increased	122
miR-181a	CAD	Monocytes	Decreased	123
Let-7i	CAD	Monocytes	Decreased	124
miR-340, -624	CAD	Platelets	Increased	125
miR-20b, ***-21***, -24, -29b, -15a, **-126**, -150, -191, -197, **-223**, -320, -486 miR-28-3p	T2D	Plasma	Decreased Increased	71
miR-146a	T2D	PBMC	Decreased	86
miR-21, ***-27a***, b, **-126**, **-130a**	T2D	EPC	Decreased	126
miR-9, -29a, -30d, **-34a**, -124a, ***-146a***, -375	T2D	Serum	Increased	85
miR-16, ***-21***, **-155**, ***-210***, -638	CKD	Plasma	Decreased	72
miR-188-5p, -135*, -323-3p, -509-3p, -520-3p, -572, -573, 629*, -632 miR-24, -106a, -191, -218, **-222**, **-223**, -342-3p, -412, let-7p	HC	HDL	Decreased Increased	99
miR-***21***, *-27b*, ***-130a,-210***	AO	Serum	Increased	63

Bold: Found in more than one study. *Italic*: Found also in tissue (Table 2).

CKD, chronic kidney disease; T2D, type 2 diabetes; CAD, coronary artery disease; AS, aortic stenosis; HC, familial hypercholesterolemia; AO, arteriosclerosis obliterans; PBMC, peripheral blood mononuclear cell; EPC, endothelial progenitor cell; HDL, high-density lipoprotein.