Chapter 5

Role of MicroRNAs in Cardiovascular Calcification

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Additional information is available at the end of the chapter

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1. Introduction

With a growing older population, cardiovascular diseases are becoming an increasing economic and social burden in Western societies. Cardiovascular calcification is a major characteristic of chronic inflammatory disorders — such as chronic renal disease (CRD), type 2 diabetes (T2D), atherosclerosis and calcific aortic valve disease (CAVD) — that associate with significant morbidity and mortality. Cardiovascular calcification also associates with osteoporosis in humans and animal models [1, 2] — the so-called "calcification paradox" [3]. The concept that similar pathways control both bone remodeling and vascular calcification is currently widely accepted, but the precise mechanisms of calcification remain largely unknown. Osteogenic transition of vascular smooth muscle cells (SMCs), valvular interstitial cells (VIC) 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 [4]. Loss of mineralization inhibitors, such as matrix γ-carboxyglutamic acid Gla protein (MGP) and fetuin-A also contribute to cardiovascular calcification. The physiological balance between induction and inhibition of calcification becomes dysregulated in CRD, T2D, atherosclerosis, and CAVD. Consequently, calcification may occur at several sites in the cardiovascular system, including the intima and media of vessels and cardiac valves [3].

The central role of miRNAs as fine-tune regulators in the cardiovascular system and bone biology has gained acceptance and has raised the possibility for novel therapeutic targets. 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.

2. Micro-RNA biology: Biosynthesis and function

Micro-RNAs (miRNAs) are a large class of evolutionarily conserved, small, endogenous, non-coding RNAs serving as essential post-transcriptional modulators of gene expression [5]. miRNAs regulate biological processes by binding to mRNA 3′-untranslated region (UTR) sequences to attenuate protein synthesis or messenger RNA (mRNA) stability [6]. 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. miRNA dysregulation often results in impaired cellular function and disease progression. It has been estimated that the whole human genome encodes for about 1000 miRNAs which may be located within introns of coding or non-coding genes, within host exons or within intergenic regions [7].

miRNA biogenesis is shown in Figure 1. The transcription process is mediated by the RNA-polymerase II that produces long precursor RNAs known as "primary miRNA" (pri-miRNA) with a typical hairpin morphology [8]. A nuclear endonuclease, called DROSHA, then crops the distal stem portion of pri-RNA obtaining shorter chains (pre-miRNA) [9]. Pre-miRNA is transported to the cytoplasm by the nuclear receptor Exportin-5 [10] and processed by DICER, an RNase III, to short double-stranded RNA sequence containing the miRNA and the 'star strand' (miRNA*). miRNA* is degraded after stripping the miRNA strand to obtain mature miRNA [11]. Mature miRNA interact with proteins like Argonaute endonuclease (Arg 2), in order to form the RNA-induced silencing complex (RICS), which directs mature miRNA towards the targeted mRNA and bind on their 3′ untranslated region (UTR) [6].

A single miRNA may modulate hundreds of miRNAs, and one mRNA has multiple predicted binding sites for miRNAs in their 3′UTR. Furthermore, after cleavage of a target mRNA, miRNAs are not Destroyed; so they may recognise and modulate other mRNAs [5, 12].

3. miRNAs and cardiovascular disease

Cardiovascular calcification is an independent risk factor for cardiovascular morbidity and mortality. Several risk factors can accelerate atherosclerosis and cardiovascular calcification, including age, hypercholesterolemia, metabolic syndrome, CRD, and T2D. Cardiovascular calcification can be distinguished by location — as intimal (atherosclerotic) , medial (CRD, T2D), or valvular [3]. Atherosclerotic calcification occurs as a part of atherogenic progress in the vessel intima. Small hydroxyapatite mineral crystals (microcalcification) can be visualized in early lesions [13]. Medial calcification occurs primarily in association with CRD and T2D, independently of hypercholesterolemia. Aortic valve calcification leads to impaired movement of aortic valve leaflets, and causes valve dysfunction [2]. All three processes shared risk factors and etiological factors, including inflammation and oxidative stress.

The identification of circulating miRNA as a novel biomarker in various diseases is a growing area of research investigation. Many pioneering studies describe specific miRNA patterns in

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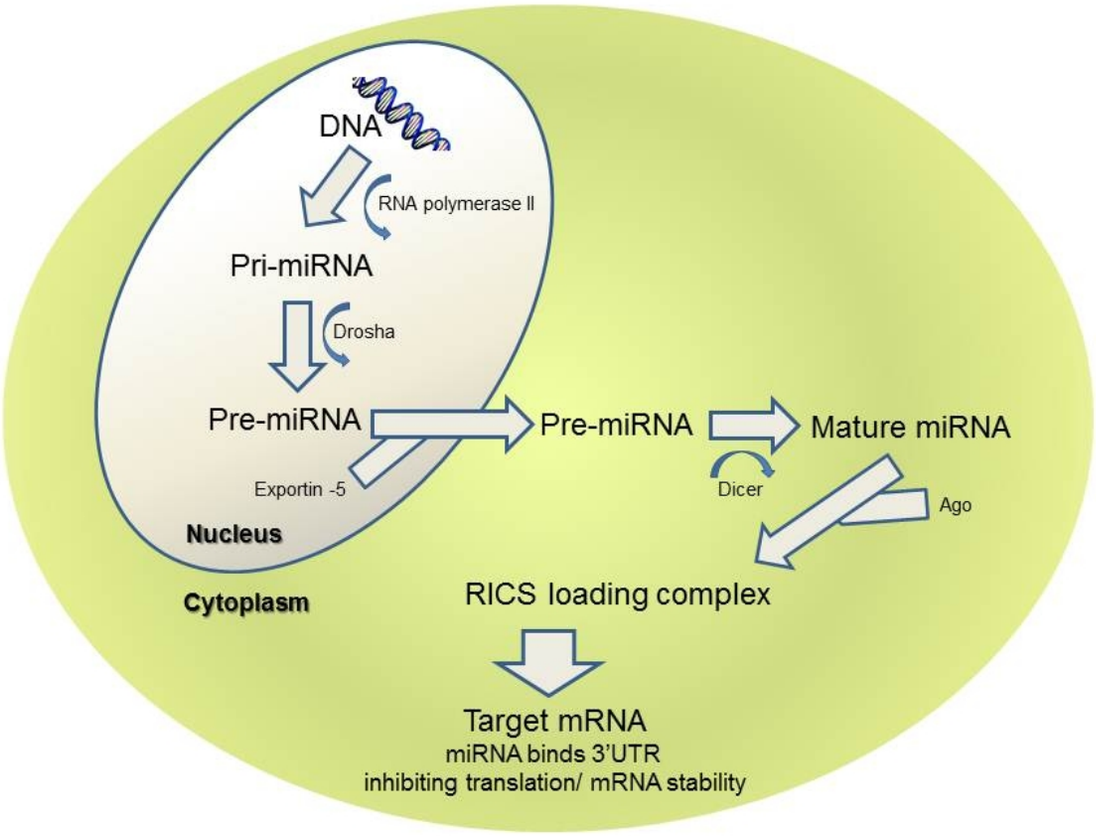

Figure 1. Schematic overview of miRNA biogenesis.

cardiovascular diseases. The first study reporting circulating miRNAs in patients with atherosclerosis was published in 2010, demonstrating a reduction of circulating vascular- and inflammation-associated miRNAs (miR-126, miR-17, miR-92a, miR-155) in patients with coronary artery disease (CAD) [14]. In addition, tissue levels of miRNAs were investigated.

Here we summarize and discuss the current knowledge on circulating and tissue miRNAs in diseases associated with cardiovascular calcification (Tables 1 and 2).

3.1. miRNAs in coronary artery disease

Studies about miRNA expression in calcified vessels are rare. 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 [15]. 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, compared to normal vessels [15]. Higher levels of miR-21 and miR-210 were confirmed in a study that compared atherosclerotic with non-atherosclerotic left internal thoracic arteries [16]. In addition, the expression of miR-34a, miR-146a, miR146b-5p, and miR-210 increased more than 4-fold in atherosclerotic arteries. Several predicted targets were downregulated [16]. Another study found a different miRNA pattern using plaque material from the carotid artery, com-

pared with the arteria mammaria interna as control tissue [17]. 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 [17]. The investigators in both studies, however, did not examine miRNAs in calcified lesions. Microcalcification is thought to cause plaque rupture [18, 19]. Destabilized human plaques are characterized by a specific miRNA expression profile (high levels of miR-100, miR-127, miR-145, miR-133a, miR-133b). Target genes of these miRNAs (Nox1, MMP9, CD40) may play a role in vascular calcification [7]. Thus, miRNAs could participate in the formation of hydroxyapatite crystals, and thereby have an important role in regulating atherosclerotic plaque toward unstable phenotypes and rupture [20].

Fichtlscherer *et al.* authored the first study investigating circulating miRNA in CAD [14]. Plasma levels of miR-17, miR-92a, miR-126, miR-145, and miR-155 were reduced in CAD compared to healthy controls, whereas miR-133a and miR-208a were increased [14]. Another study demonstrated a positive correlation of plasma miR-122 and miR-370 levels with the presence and severity of CAD [21]. Both miRNAs were significantly increased in hyperlipidemia patients, compared to controls [21]. Increased levels of miR-27b, miR-130a, and miR-210 were observed in the serum of arteriosclerosis obliterans patients [15].

Comparison of published studies is challenging mainly because of the different sources of circulating miRNAs, which include serum, whole blood, PBMCs, EPCs, and platelets (Table 1). The miRNA profiles obtained from the different studies, therefore, are often not the same. 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 controls [22].

Polymorphisms in the 3'UTR may alter miRNA binding, leading to post-transcriptional dysregulation of the target gene and aberrant protein level. Functional single-nucleotide polymorphisms (SNPs) of miRNA-binding sites associate with the risk of cardiovascular disease. Wu *et al.* discovered a SNP in the miR-149 binding site of the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene that associated with increased risk for CAD [23]. Furthermore, a larger study in a Chinese population of 956 CAD patients and 620 controls revealed that a SNP in the binding sites for miR-196a2 and miR-499 associated with the occurrence and prognosis of CAD [24].

3.2. miRNAs in diabetes and chronic renal disease (CRD)

T2D is a major risk factor for cardiovascular disease. Zampetaki *et al*. identified a plasma miRNA signature for T2D that includes reduced levels of miR-223, miR-15, miR-20b, miR-21, miR-24, miR-29b, miR-126, miR-150, miR-191, miR-197, miR-320, and miR-486, and elevated levels of miR-28-3p [33]. Reduced miR-126 levels antedated diabetes manifestation, and might explain the impaired peripheral angiogenic signaling in patients with T2D. Reduction of circulating miR-21 and miR-126 was confirmed by Meng *et al.*, who also found a decrease of miR-27a,b and miR-130a in T2D patients [35]. Another study demonstrated mostly elevated miRNA levels (miR-9, miR-29a, miR-30d, miR-34a, miR-124a, miR-146a, and miR-375) in serum from T2D patients, compared with pre-diabetic and/or normal glucose tolerance

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	[14]
miR-146a/b	CAD	PBMC	Increased	[25]
miR-34a	CAD	EPC	Increased	[26]
miR-221, -222	CAD	EPC	Increased	[27]
miR-135a, -147	CAD	PBMC	Decreased	[28]
miR-140, -182	CAD	Whole blood	Decreased	[29]
miR-122, -370	CAD	Plasma	Increased	[21]
miR-181a	CAD	Monocytes	Decreased	[30]
Let-7i	CAD	Monocytes	Decreased	[31]
miR-340, -624	CAD	Platelets	Increased	[32]
miR-20b, -21, -24, -29b, -15a, -126, -150, -191, -197, -223, -320, -486 miR-28-3p	T2D	Plasma	Decreased Increased	[33]
miR-146a	T2D	PBMC	Decreased	[34]
miR-21, -27a, b, -126, -130a	T2D	EPC	Decreased	[35]
miR-9, -29a, -30d, -34a, -124a, -146a, -375	T2D	Serum	Increased	[36]
miR-16, -21, -155, -210, -638	CRD	Plasma	Decreased	[37]
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	[38]
miR-21, -27b, -130a, -210	AO	Serum	Increased	[15]

CRD, chronic renal 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.

Table 1. Circulating miRNA in diseases associated with vascular calcification.

conditions [36]. In contrast, reduced miR-146a levels in PMBCs from Asian Indian T2D patients associated with insulin resistance, poor glycemic control, and several proinflammatory cytokine genes [34]. miR-146a participates in the transcriptional circuitry regulating fibronectin in T2D animals.[39].

The high incidence of cardiovascular complications in patients with CRD is partly explained by more aggressive development of atherosclerotic lesions and accelerated calcification [40]. To our knowledge, only one study reports circulating miRNA in patients with CRD. Neal *et*

al. found that plasma levels of total and specific miRNAs (miR-16, miR-21, miR-155, miR-210, and miR-638) are reduced in CRD patients, compared to patients with normal renal function [37]. A strong correlation exists between detected circulating miRNAs and estimated glomerular filtration rate [37]. Interestingly, miR-638 was the only miRNA that showed a differential urine excretion in CRD patients [37]. Transforming growth factor beta (TGF-β), a pro-fibrotic key mediator of CRD, reduces levels of miR-192 [41] and miR-29a [42] and increases miR-377 levels [43] *in vitro* and *in vivo*, thereby promoting the expression of extracellular matrix components.

3.3. miRNAs and aortic valve disease

Aortic stenosis (AS) is typically caused by calcific aortic valve disease. To our knowledge, no study to date describes a specific miRNA signature in the circulation of patients with AS. Nigam *et al*. identified a miRNA pattern specific to AS using tissue from whole bicuspid valves and linking them to calcification-related genes, such as Smad1/3, Runx2, and BMP2 [44]. miR-26a, miR-30b, and miR-195 were decreased in the aortic valves of patients requiring replacement due to AS, compared to those requiring replacement due to aortic insufficiency [44]. Another group compared bicuspid with tricuspid aortic valve leaflets by miRNA microarray, and found a number of modulated miRNAs [45]. Particularly, miR-141 had the most dramatic change, showing a 14.5-fold decrease in the bicuspid versus tricuspid valve tissue, while the levels of calcification were comparable between the two groups.

3.4. Similar miRNA profiles may represent common miRNAs in diseases associated with cardiovascular calcification

Our detailed investigation using currently published literature revealed common circulating miRNAs in diseases associated with vascular calcification. Seven miRNAs (miR-21, miR-27, miR-34a, miR-126, miR-146a, miR-155, and miR210) were useful biomarkers in atherosclerosis, T2D, and/or CRD, and only miR-21 was common among all three diseases [14, 33, 37] (Table 3).

Atherosclerotic arteries [16] and sclerotic intima from lower-extremity vessels [15] expressed higher miR-21 levels than did healthy vessels. Circulating levels of miR-21 in atherosclerosis, T2D, and/or CRD were reduced [14, 33, 37]. The reason for this discrepancy is unknown, and requires further investigation.

miR-146a is an inflammation-related miRNA, implicated in atherosclerosis and osteoclastogenesis [46]. Circulating miR-146a is increased in CAD patients [25] and T2D [36]. In addition, miR-146a was more highly expressed in atherosclerotic arteries in an animal model [16], and associated with CRD *in vivo* [47]. miR-155, another inflammation-associated miRNA, is decreased in CAD [14] and CRD [37]. Deficiency of miR155 enhanced atherosclerotic plaque development and decreased plaque stability [48], 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 [49]. The renin–angiotensin system participates in cardiovascular calcification [50, 51]. Angiotensin-receptor blockade can inhibit

miRNA	Disease	Tissue type	Finding	Reference number
miR-21, -34a, -146a, -146b-5p, -210	CAD	Atherosclerotic arteries	Increased	[16]
miR-105, -520b	CAD	Atherosclerotic carotid artery	Decreased	[17]
miR-10b, -26b, -30e, -125a, -218,			Increased	
miR-100, -127, -133a,b -145	CAD	Destabilized plaque	Increased	[20]
miR-221, -222	AO	Sclerotic intima from lower extremities vessels	Decreased	[15]
miR-21, -27b, -210, -130a, let-7f			Increased	
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	AS	Bicuspid aortic valve	Decreased	[45]
miR-30e, -32, -145, -151, -152, -190, -373, -768			Increased	
miR-26a, -30b, -195	AS	Whole bicuspid valves	Decreased	[44]

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

Table 2. miRNAs expressed in human calcified tissue.

CAD	T2D	CRD
miR-21 ↓	miR-21 ↓	miR-21 ↓
miR-27 ↓	miR-27 ↓	
miR-34a ↑	miR-34a ↑	
miR-126 ↓	miR-126 ↓	
miR-155 ↓		miR-155 ↓

CRD, chronic renal disease; T2D, type 2 diabetes; CAD, coronary artery disease

Table 3. Common circulating miRNA in diseases associated with vascular calcification.

arterial calcification by disrupting vascular osteogenesis *in vivo* [52]. In addition, an observational study showed reduced progression of AV disease in patients taking angiotensin-converting enzyme inhibitors [53]. Furthermore, miR-155 represses osteoblastogenesis by targeting Smad proteins [54]. Thus, high expression of miR-155 may prevent cardiovascular

calcification by inhibiting the BMP signalling pathway or the renin–angiotensin system, making it a promising anti-calcification therapeutic target.

In summary, 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 cardiovascular 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 [13, 40, 55-57].

4. miRNA and osteogenesis in the vascular wall

Cardiovascular calcification is an active, cell-regulated process. Various studies provide evidence of phenotypic transition or transition/dedifferentiation of mature SMCs or VICs into an osteogenic phenotype — a key feature in cardiovascular calcification. In medial calcification, SMCs undergo dedifferentiation from a contractile to a pro-atherogenic synthestic phenotype, lose the expression of their marker genes, acquire osteogenic markers, and deposit a mineralized bone-like matrix. In valvular calcification, VICs can undergo the transition to osteoblast-like bone-forming cells [58]. Recently, a novel concept emerged of circulating cells harboring osteogenic potential that can home to atherosclerotic lesions and contribute to intimal calcification [59, 60]. Comparing the sources of cells that contribute to atherosclerotic intimal calcification revealed that SMCs are the major contributors that reprogram its lineage towards osteochondrogenesis/blastogenesis; circulating bone marrow-derived cells, however, also contribute to early osteochondrogenic differentiation in atherosclerotic vessels [61]. The master transcription factors, including Runx2/Cbfa1, Msx2, and Osterix, designate cells for osteoblast lineages through the induction of downstream genes such as alkaline phosphatase, osteopontin, and osteocalcin. Here we summarize miRNAs involved in SMC differentiation, as well as in osteogenesis, with targets involved in cardiovascular calcification.

The SMC phenotype is dependent on the miR-143/145 cluster [62-64]. Circulating miR-145 levels are reduced in CAD patients [14]. miR-145 is one of the most recognized arterial miRNAs [65]. Inhibition of miR-143/145 promotes a phenotypic switch to the synthetic, pro-atherogenic SMC state [62], including the inhibition of SMC marker-like alpha-smooth muscle actin and smooth muscle myosin heavy chain [66] — both diminished in osteogenic SMCs [67]. miR-145 modulates SMC differentiation by targeting Krüppel-like factor 4 (KLF4) [63]. KLF4 mediates high phosphate-induced conversion of SMCs into osteogenic cells [68]. Conversely, miR-145-deficient mice [69] and overexpression of miR-145 [66] both reduce neointima formation in vascular injury.

Similar to miR-145, miR-133 has a potent inhibitory role on the vascular SMC phenotypic switch [70]. Runx2, a cell-fate determining osteoblastic transcription factor, is a target of miR-133 [71]. Runx2 acts as a critical regulator of osteogenic lineage and a modulator of bone-related genes [72]. Runx2 is essential and sufficient for regulating osteogenesis in SMC and VIC [73, 74, 75, 76]. Discovered in the bone biology field, a program of miRNAs controls Runx2

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expression to prevent skeletal disorders [77]. Three of these miRNAs (miR-133a, miR-135a, and miR-218) are altered in cardiovascular diseases associated with vascular calcification [14, 17, 20, 28]. Klotho mutant mice, which display vascular calcification due to hyperphosphatemia and through a Runx2-dependent mechanism [78], show overexpression of miR-135a (together with miR-762, miR-714, and miR-712) in the aortic media, which causes SMC calcification by disruption of Ca^{2+} transporters and increasing intracellular Ca^{2+} concentrations [79]. More recently, miR-204, another candidate of the Runx2-cluster, was found to contribute to SMC calcification *in vitro* and *in vivo* [80]. Downstream targets of Runx2 are bone-specific genes like osteopontin, osterix and osteocalcin, all present in the cardiovascular osteogenic cell phenotype [2, 81]. We recently demonstrated that miR-125b, which inhibits osteoblast differentiation [82] regulates the transition of SMCs into osteoblast-like cells partially by targeting the transcription factor osterix, providing the first miR-dependent mechanism in the progression of vascular calcification [83]. Additionally, miRNA-processing enzymes — essential for SMC function [84] — were reduced in calcified SMCs [83].

Another potent regulator of vascular and valvular calcification is the BMP signaling pathway (reviewed in detail elsewhere [85]). BMP2 and BMP4 are potent osteogenic differentiation factors detected in calcified valve and atherosclerotic lesions [86-88]. BMPs elicit their effects through activation of 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 [85]. MiR-26a, miR-135, and miR-155 were previously reported as Smad-regulating miRNAs related to osteoblastogenesis; they functionally repress osteoblast differentiation by targeting Smad1 and Smad5, respectively [54]. miR-155 is one of the circulating miRNAs that is decreased in CAD [14] and CRD [37] (discussed earlier). miR-26a was repressed in aortic valve leaflets of patients with aortic stenosis, and human aortic valvular interstitial cells showed decreased mRNA levels of BMP2 and Smad1 when treated with miR-26a mimic [44]. The same group found lower expression of miR-30b, which targets Smad1 and Smad3. Another group reported deceased miR-141 levels together with increased BMP2 levels in bicuspid versus tricuspid aortic valve leaflets, and showed *in vitro* that miR-141 represses the VIC response to calcification, in part through BMP2-dependent calcification [45]. 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 [89].

Activation of canonical wingless-type (WNT) signaling is crucial for osteoblast function [90] and for the programming of valvular and vascular cells during cardiovascular calcification [85]. Activation of the Wnt/β-catenin signaling pathway occurs in human calcified aortic valve stenosis [91], in LDL receptor (LDLR)-deficient mice [92, 93], and in osteogenic SMCs *in vitro* [94]. Dickkopf (Dkk)1 is an extracellular antagonist of the canonical Wnt signaling that plays a crucial role in bone remodeling by binding to and inactivating signaling from LDLR-related protein 5/6 [95, 96]. Dkk-1 may also play a role in vascular calcification. In CRD patients, Dkk1 serum levels correlated negatively with arterial stiffness [97]. Dkk-1 prevents warfarin-induced activation of β-catenin, and osteogenic transdifferentiation of SMCs [98] and TNF α-induced induction of alkaline phosphatase activity [92]. Remarkably, two miRNAs targeting

bone Dkk-1 (miR-335-5p, miR-29a) increase with age [99, 100] — a risk for cardiovascular calcification. miR-335-5p directly targets and represses Wnt inhibitor Dkk-1, thereby enhancing Wnt signaling and promoting osteoblast differentiation [101]. To date, no publications exist regarding the role of miR-335-5p in the cardiovascular system. Yet, the age-dependent increase of miR-335 in rat renal tissue inhibited the expression and function of the enzymes implicated in oxidative stress defense [99]. Likewise, miR-29a potentiates osteoblastogenesis by modulating Wnt signaling. Canonical Wnt signaling induces miR-29a expression, which negatively targets regulators of Wnt signaling, including Dkk-1, sFRP2, Kremen, and osteonectin [102, 103]. miR-29 increased age-dependently in mouse aortic tissue and associated with reduced extracellular matrix components, such as collagen and elastin [100]. Elastolysis accelerates arterial and aortic valve calcification [40]. Furthermore, MMP-2, another target of miR-29 [104], was shown to promote arterial calcification in CRD [105] and valvular calcification [106].

The contribution of osteoclasts to cardiovascular calcification is still controversial [59]. 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 [74]. The receptor activator of the nuclear factor-kappa B (NF-kappa B) ligand (RANKL)/osteoprotegerin (OPG) system controls proper osteoclastogenesis, and actes as a biomarker for CAD [107, 108]. *In silico* analysis revealed RANKL as a target of miR-126 [109], which is decreased in the plasma of CAD [14] and T2D [33] patients. miR-146a, highly expressed in atherosclerotic arteries [16], inhibits osteoclastogenesis [46]. The number of tartrate-resistant acid phosphatase-positive multinucleated cells was significantly reduced by miR-146a in a dose-dependent manner [46]. Furthermore, miR-155, which is decreased in plasma of CRD [37] and CAD [14] patients, was shown to inhibit osteoclast function [110].

Taken together, osteogenic processes in both bone and the cardiovascular system are tightly controlled by miRNAs (Figure 2). Further studies are needed to elucidate whether interplay of miRNAs could explain the bone-vascular axis "calcification paradox," or whether they act independently in the calcifying vessel and bone.

5. Circulating miRNAs as biomarkers and extracellular communicators

miRNAs are present in blood (plasma, platelets, erythrocytes, nucleated blood cells) with high stability. miRNAs can circulate in extracellular vesicles [111], in a protein complex (Ago2), or in a lipoprotein complex (HDL) [38], which prevents their degradation. Depending on the size and type, extracellular vesicles are broadly classified as ectosomes (also called shedding microvesicles), exosomes, matrix vesicles (MVs), and apoptotic bodies. Ectosomes are large extracellular vesicles 50-1000 nm in diameter; exosomes are small membranous vesicles of endocytic origin, 40-100 nm in diameter; MVs are 30-300 nm in diameter, are produced by

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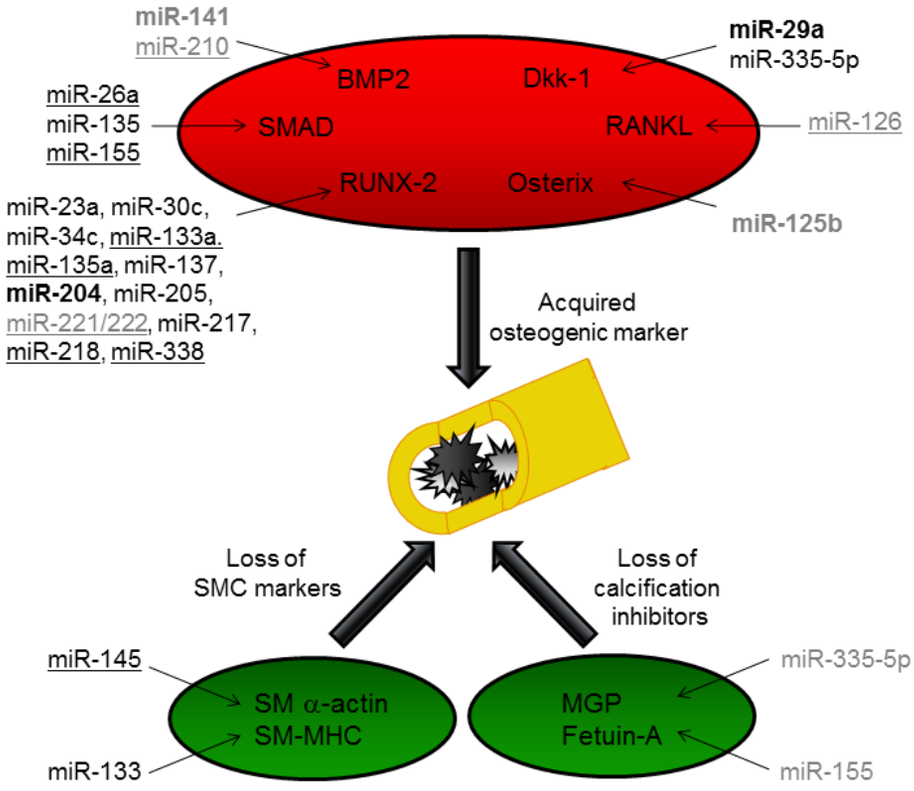

Figure 2. Potential and established miRNAs contributing to osteogenic regulation of vascular calcification. Bold, established miRNAs in vascular calcification; underlined, dysregulated in cardiovascular disease (circulating or tissue); gray, predicted miRNA binding sites.

blebbing of the plasma membrane, and can calcify; and apoptotic bodies, 50-5000 nm in diameter, are released from fragmented apoptotic cells.

The majority of miRNAs are independent of vesicles [111] and co-purify with the Ago2 complex [112, 113]. But in CAD patients, most plasma miRNAs associate with extracellular vesicles, and only a small amount are found in extracellular vesicle-free plasma [114]. A cell-type-specific miRNA release and different export systems are implicated, as the miRNA release pattern within vesicles is different from that associated with Ago2 complexes [112]. Thus, cells can select miRNA and pre-miRNA for controlled cellular release [115, 116]. miRNA profiles of extracellular vesicles are different from their maternal cell profiles, indicating an active mechanism of selective miRNA packing from cells into vesicles [114]. We have limited knowledge about miRNA secretion. Blockade of sphingomyelinase inhibits exosome generation and miRNA secretion, and intercellular miRNA transfer implicates a ceramide-dependent mechanism [117, 118]. Ago2–miRNA complexes may be passively produced by dead cells, released by live cells, or actively transported though cell-membrane–associated channels or receptors [119].

Extracellular vesicles use miRNA to mediate intercellular communication over long distances or on a local tissue level [120]. Endothelial apoptotic bodies can convey miR-126 to atherosclerotic lesions, which demonstrate uniquely paracrine-signaling function for miRNA during

atherosclerosis [33, 121]. miRNA-containing vesicles can regulate intercellular communication between ECs and SMCs by selective packing of miR-143/145 in endothelial-derived vesicles, which are then transported to SMCs to control their phenotype [118].

How miRNAs are taken up by target cells and remain biologically active is still unknown. We know little about the mechanisms of vesicle-mediated cargo transfer. 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 [122].

A key event in the initiation and promotion of VIC and SMC calcification is the release of extracellular vesicles [81, 123]. Treatment of SMCs with elevated calcium levels promotes the production of calcifying vesicles (MVs), and the loss of fetuin-A, an inhibitor of mineral nucleation [124]. These vesicles act as early nucleation sites for calcification. The phosphatidylserine-membrane complex from SMC-derived and macrophage-derived MVs redistributes and nucleates hydroxyapatite [125-127]. In addition, hydroxyapatite nanocrystals shed from vesicles may further promote mineralization via direct effects on SMC phenotype [128].

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, which may prevent calcification in the cardiovascular system.

6. miRNAs in the "calcification paradox"

Osteoporosis frequently associates with cardiovascular calcification, and the severity of aortic calcification associates positively with bone loss [2, 129, 130]. The "calcification paradox" could be explained by the shared molecular pathways in bone remodeling and cardiovascular calcification [3]. How these two processes associate with each other and whether osteoporosis leads to cardiovascular calcification - or whether both disorders just share common risk factors - is unclear. In this section, we link cardiovascular calcification and bone loss and show commonalities in the systems' miRNA pathways/patterns.

Studies of miRNA in patients with bone disease are lacking. A recent clinical study first reported miRNA as a potential biomarker for postmenopausal osteoporosis. Wang *et al*. demonstrated an association of miR-133a levels in circulating monocytes - osteoclast precursors - with postmenopausal osteoporosis [131]. Women with low bone mineral density showed higher circulating miR-133a levels [131], but the number of patients per group was small (n=10). Circulating miR-133a levels were also higher in patients with CAD [14]. Unfortunately, the study investigating bone mineral density in patients with osteoporosis did not mention characteristics of the cardiovascular patient population. miR-133a belongs to the Runx2-targeting miRNA cluster [77].

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Additionally, miR-2861 contributes to osteoporosis in mice and humans by targeting histone deacethylase 5, and thereby increasing Runx2 [132]. No studies of miR-2861 in the cardiovascular system have been reported. Patients with rheumatoid arthritis also suffer from vascular calcification in different vessel beds, in addition to osteoporosis; the pathogenesis includes pro-inflammatory cytokines and site-specific inflammation (reviewed in detail elsewhere [133]). miR-146a, a negative regulator of inflammation and osteoclastogenesis, also associates with rheumatoid arthritis [134]. Similar to patients with CAD, in patients with rheumatoid arthritis, miR-146a is up-regulated in PBMCs [25].

7. Conclusion and perspectives

In vitro and *in vivo* studies have established miRNAs as biomarkers focusing on different aspects and providing circulating miRNA signatures for different diseases. But these circulating miRNAs may not have biological functions within the cell while circulating — instead, they act as intercellular communicators, and this communication may be disturbed by calcified vesicles. More studies are needed to fully exploit this potentially novel mechanism of cardiovascular calcification.

Moreover, miRNA biology is very complex. Multiple miRNAs can target the same gene (e.g., Runx2–miRNA cluster), and one miRNA may 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 calcification disorders. As vascular calcification and bone remodeling share common mechanisms, we have to understand in greater detail the functions of miRNAs and their association with the molecular pathogenesis of osteoporosis and vascular/valvular calcification.

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