We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,300

Open access books available

172,000

International authors and editors

190M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

Role of MicroRNAs in Cardiovascular Calcification

Claudia Goettsch and Elena Aikawa

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/55326>

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 mRNAs, 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

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 synthetic 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

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 decreased 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 acts 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

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 through 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].

Additionally, miR-2861 contributes to osteoporosis in mice and humans by targeting histone deacetylase 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.

Author details

Claudia Goettsch¹ and Elena Aikawa^{1,2}

*Address all correspondence to: eaikawa@partners.org

1 Center for Interdisciplinary Cardiovascular Sciences, Cardiovascular Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

2 Center for Excellence in Vascular Biology, Cardiovascular Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

References

- [1] Hjortnaes J, Bouten CV, Van Herwerden LA, Grundeman PF, Kluin J. Translating autologous heart valve tissue engineering from bench to bed. *Tissue engineering Part B, Reviews* 2009;15(3):307-17.
- [2] Rajamannan NM, Evans FJ, Aikawa E, Grande-Allen KJ, Demer LL, Heistad DD, et al. Calcific aortic valve disease: not simply a degenerative process: A review and agenda for research from the National Heart and Lung and Blood Institute Aortic Stenosis Working Group. Executive summary: Calcific aortic valve disease-2011 update. *Circulation* 2011;124(16):1783-91.
- [3] Sage AP, Tintut Y, Demer LL. Regulatory mechanisms in vascular calcification. *Nature reviews Cardiology* 2010;7(9):528-36.
- [4] Johnson RC, Leopold JA, Loscalzo J. Vascular calcification: pathobiological mechanisms and clinical implications. *Circulation research* 2006;99(10):1044-59.
- [5] Ambros V. The functions of animal microRNAs. *Nature* 2004;431(7006):350-5.
- [6] Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009;136(2):215-33.
- [7] Santovito D, Mezzetti A, Cipollone F. MicroRNAs and atherosclerosis: New actors for an old movie. *Nutrition, metabolism, and cardiovascular diseases : NMCD* 2012.
- [8] Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, et al. MicroRNA genes are transcribed by RNA polymerase II. *The EMBO journal* 2004;23(20):4051-60.
- [9] Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, et al. The nuclear RNase III Drosha initiates microRNA processing. *Nature* 2003;425(6956):415-9.
- [10] Yi R, Qin Y, Macara IG, Cullen BR. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes & development* 2003;17(24):3011-6.
- [11] Chendrimada TP, Gregory RI, Kumaraswamy E, Norman J, Cooch N, Nishikura K, et al. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* 2005;436(7051):740-4.
- [12] Hutvagner G, Zamore PD. A microRNA in a multiple-turnover RNAi enzyme complex. *Science* 2002;297(5589):2056-60.
- [13] Aikawa E, Nahrendorf M, Figueiredo JL, Swirski FK, Shtatland T, Kohler RH, et al. Osteogenesis associates with inflammation in early-stage atherosclerosis evaluated by molecular imaging in vivo. *Circulation* 2007;116(24):2841-50.
- [14] Fichtlscherer S, De Rosa S, Fox H, Schwietz T, Fischer A, Liebetrau C, et al. Circulating microRNAs in patients with coronary artery disease. *Circulation research* 2010;107(5):677-84.

- [15] Li T, Cao H, Zhuang J, Wan J, Guan M, Yu B, et al. Identification of miR-130a, miR-27b and miR-210 as serum biomarkers for atherosclerosis obliterans. *Clinica chimica acta; international journal of clinical chemistry* 2011;412(1-2):66-70.
- [16] Raitoharju E, Lyytikäinen LP, Levula M, Oksala N, Mennander A, Tarkka M, et al. miR-21, miR-210, miR-34a, and miR-146a/b are up-regulated in human atherosclerotic plaques in the Tampere Vascular Study. *Atherosclerosis* 2011;219(1):211-7.
- [17] Bidzhekov K, Gan L, Denecke B, Rostalsky A, Hristov M, Koeppel TA, et al. microRNA expression signatures and parallels between monocyte subsets and atherosclerotic plaque in humans. *Thrombosis and haemostasis* 2012;107(4):619-25.
- [18] Vengrenyuk Y, Carlier S, Xanthos S, Cardoso L, Ganatos P, Virmani R, et al. A hypothesis for vulnerable plaque rupture due to stress-induced debonding around cellular microcalcifications in thin fibrous caps. *Proceedings of the National Academy of Sciences of the United States of America* 2006;103(40):14678-83.
- [19] Hoshino T, Chow LA, Hsu JJ, Perlowski AA, Abedin M, Tobis J, et al. Mechanical stress analysis of a rigid inclusion in distensible material: a model of atherosclerotic calcification and plaque vulnerability. *American journal of physiology Heart and circulatory physiology* 2009;297(2):H802-10.
- [20] Cipollone F, Felicioni L, Sarzani R, Uchino S, Spigonardo F, Mandolini C, et al. A unique microRNA signature associated with plaque instability in humans. *Stroke; a journal of cerebral circulation* 2011;42(9):2556-63.
- [21] Gao W, He HW, Wang ZM, Zhao H, Lian XQ, Wang YS, et al. Plasma levels of lipometabolism-related miR-122 and miR-370 are increased in patients with hyperlipidemia and associated with coronary artery disease. *Lipids in health and disease* 2012;11(1):55.
- [22] Kirschner MB, Kao SC, Edelman JJ, Armstrong NJ, Vallely MP, van Zandwijk N, et al. Haemolysis during sample preparation alters microRNA content of plasma. *PloS one* 2011;6(9):e24145.
- [23] Wu C, Gong Y, Sun A, Zhang Y, Zhang C, Zhang W, et al. The human MTHFR rs4846049 polymorphism increases coronary heart disease risk through modifying miRNA binding. *Nutrition, metabolism, and cardiovascular diseases : NMCD* 2012.
- [24] Zhi H, Wang L, Ma G, Ye X, Yu X, Zhu Y, et al. Polymorphisms of miRNAs genes are associated with the risk and prognosis of coronary artery disease. *Clinical research in cardiology : official journal of the German Cardiac Society* 2012;101(4):289-96.
- [25] Takahashi Y, Satoh M, Minami Y, Tabuchi T, Itoh T, Nakamura M. Expression of miR-146a/b is associated with the Toll-like receptor 4 signal in coronary artery disease: effect of renin-angiotensin system blockade and statins on miRNA-146a/b and Toll-like receptor 4 levels. *Clin Sci (Lond)* 2010;119(9):395-405.

- [26] Tabuchi T, Satoh M, Itoh T, Nakamura M. MicroRNA-34a regulates the longevity-associated protein SIRT1 in coronary artery disease: effect of statins on SIRT1 and microRNA-34a expression. *Clin Sci (Lond)* 2012;123(3):161-71.
- [27] Minami Y, Satoh M, Maesawa C, Takahashi Y, Tabuchi T, Itoh T, et al. Effect of atorvastatin on microRNA 221 / 222 expression in endothelial progenitor cells obtained from patients with coronary artery disease. *European journal of clinical investigation* 2009;39(5):359-67.
- [28] Hoekstra M, van der Lans CA, Halvorsen B, Gullestad L, Kuiper J, Aukrust P, et al. The peripheral blood mononuclear cell microRNA signature of coronary artery disease. *Biochemical and biophysical research communications* 2010;394(3):792-7.
- [29] Taurino C, Miller WH, McBride MW, McClure JD, Khanin R, Moreno MU, et al. Gene expression profiling in whole blood of patients with coronary artery disease. *Clin Sci (Lond)* 2010;119(8):335-43.
- [30] Hulsmans M, Sinnaeve P, Van der Schueren B, Mathieu C, Janssens S, Holvoet P. Decreased miR-181a Expression in Monocytes of Obese Patients Is Associated with the Occurrence of Metabolic Syndrome and Coronary Artery Disease. *The Journal of clinical endocrinology and metabolism* 2012;97(7):E1213-8.
- [31] Satoh M, Tabuchi T, Minami Y, Takahashi Y, Itoh T, Nakamura M. Expression of let-7i is associated with Toll-like receptor 4 signal in coronary artery disease: effect of statins on let-7i and Toll-like receptor 4 signal. *Immunobiology* 2012;217(5):533-9.
- [32] Sondermeijer BM, Bakker A, Halliani A, de Ronde MW, Marquart AA, Tijssen AJ, et al. Platelets in patients with premature coronary artery disease exhibit upregulation of miRNA340* and miRNA624*. *PloS one* 2011;6(10):e25946.
- [33] Zampetaki A, Kiechl S, Drozdov I, Willeit P, Mayr U, Prokopi M, et al. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. *Circulation research* 2010;107(6):810-7.
- [34] Balasubramanyam M, Aravind S, Gokulakrishnan K, Prabu P, Sathishkumar C, Ranjani H, et al. Impaired miR-146a expression links subclinical inflammation and insulin resistance in Type 2 diabetes. *Molecular and cellular biochemistry* 2011;351(1-2):197-205.
- [35] Meng S, Cao JT, Zhang B, Zhou Q, Shen CX, Wang CQ. Downregulation of microRNA-126 in endothelial progenitor cells from diabetes patients, impairs their functional properties, via target gene Spred-1. *Journal of molecular and cellular cardiology* 2012;53(1):64-72.
- [36] Kong L, Zhu J, Han W, Jiang X, Xu M, Zhao Y, et al. Significance of serum microRNAs in pre-diabetes and newly diagnosed type 2 diabetes: a clinical study. *Acta diabetologica* 2011;48(1):61-9.
- [37] Neal CS, Michael MZ, Pimlott LK, Yong TY, Li JY, Gleadle JM. Circulating microRNA expression is reduced in chronic kidney disease. *Nephrology, dialysis, trans-*

plantation : official publication of the European Dialysis and Transplant Association - European Renal Association 2011;26(11):3794-802.

- [38] Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nature cell biology* 2011;13(4):423-33.
- [39] Feng B, Chen S, McArthur K, Wu Y, Sen S, Ding Q, et al. miR-146a-Mediated extracellular matrix protein production in chronic diabetes complications. *Diabetes* 2011;60(11):2975-84.
- [40] Aikawa E, Aikawa M, Libby P, Figueiredo JL, Rusanescu G, Iwamoto Y, et al. Arterial and aortic valve calcification abolished by elastolytic cathepsin S deficiency in chronic renal disease. *Circulation* 2009;119(13):1785-94.
- [41] Krupa A, Jenkins R, Luo DD, Lewis A, Phillips A, Fraser D. Loss of MicroRNA-192 promotes fibrogenesis in diabetic nephropathy. *Journal of the American Society of Nephrology : JASN* 2010;21(3):438-47.
- [42] Wang B, Komers R, Carew R, Winbanks CE, Xu B, Herman-Edelstein M, et al. Suppression of microRNA-29 expression by TGF-beta1 promotes collagen expression and renal fibrosis. *Journal of the American Society of Nephrology : JASN* 2012;23(2):252-65.
- [43] Wang Q, Wang Y, Minto AW, Wang J, Shi Q, Li X, et al. MicroRNA-377 is up-regulated and can lead to increased fibronectin production in diabetic nephropathy. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2008;22(12):4126-35.
- [44] Nigam V, Sievers HH, Jensen BC, Sier HA, Simpson PC, Srivastava D, et al. Altered microRNAs in bicuspid aortic valve: a comparison between stenotic and insufficient valves. *The Journal of heart valve disease* 2010;19(4):459-65.
- [45] Yanagawa B, Lovren F, Pan Y, Garg V, Quan A, Tang G, et al. miRNA-141 is a novel regulator of BMP-2-mediated calcification in aortic stenosis. *The Journal of thoracic and cardiovascular surgery* 2012.
- [46] Nakasa T, Shibuya H, Nagata Y, Niimoto T, Ochi M. The inhibitory effect of microRNA-146a expression on bone destruction in collagen-induced arthritis. *Arthritis and rheumatism* 2011;63(6):1582-90.
- [47] Ichii O, Otsuka S, Sasaki N, Namiki Y, Hashimoto Y, Kon Y. Altered expression of microRNA miR-146a correlates with the development of chronic renal inflammation. *Kidney international* 2012;81(3):280-92.
- [48] Donners MM, Wolfs IM, Stoger LJ, van der Vorst EP, Pottgens CC, Heymans S, et al. Hematopoietic miR155 deficiency enhances atherosclerosis and decreases plaque stability in hyperlipidemic mice. *PloS one* 2012;7(4):e35877.

- [49] Zhu N, Zhang D, Chen S, Liu X, Lin L, Huang X, et al. Endothelial enriched micro-RNAs regulate angiotensin II-induced endothelial inflammation and migration. *Atherosclerosis* 2011;215(2):286-93.
- [50] Jia G, Stormont RM, Gangahar DM, Agrawal DK. Role of Matrix Gla Protein in Angiotensin II-Induced Exacerbation of Vascular Stiffness. *American journal of physiology Heart and circulatory physiology* 2012.
- [51] O'Brien KD, Shavelle DM, Caulfield MT, McDonald TO, Olin-Lewis K, Otto CM, et al. Association of angiotensin-converting enzyme with low-density lipoprotein in aortic valvular lesions and in human plasma. *Circulation* 2002;106(17):2224-30.
- [52] Armstrong ZB, Boughner DR, Drangova M, Rogers KA. Angiotensin II type 1 receptor blocker inhibits arterial calcification in a pre-clinical model. *Cardiovascular research* 2011;90(1):165-70.
- [53] Shavelle DM, Takasu J, Budoff MJ, Mao S, Zhao XQ, O'Brien KD. HMG CoA reductase inhibitor (statin) and aortic valve calcium. *Lancet* 2002;359(9312):1125-6.
- [54] Taipaleenmaki H, Bjerre Hokland L, Chen L, Kauppinen S, Kassem M. Mechanisms in endocrinology: micro-RNAs: targets for enhancing osteoblast differentiation and bone formation. *European journal of endocrinology / European Federation of Endocrine Societies* 2012;166(3):359-71.
- [55] New SE, Aikawa E. Molecular imaging insights into early inflammatory stages of arterial and aortic valve calcification. *Circulation research* 2011;108(11):1381-91.
- [56] Hjortnaes J, Butcher J, Figueiredo JL, Riccio M, Kohler RH, Kozloff KM, et al. Arterial and aortic valve calcification inversely correlates with osteoporotic bone remodeling: a role for inflammation. *European heart journal* 2010;31(16):1975-84.
- [57] Geng Y, Hsu JJ, Lu J, Ting TC, Miyazaki M, Demer LL, et al. Role of cellular cholesterol metabolism in vascular cell calcification. *The Journal of biological chemistry* 2011;286(38):33701-6.
- [58] Rajamannan NM, Subramaniam M, Rickard D, Stock SR, Donovan J, Springett M, et al. Human aortic valve calcification is associated with an osteoblast phenotype. *Circulation* 2003;107(17):2181-4.
- [59] Fadini GP, Rattazzi M, Matsumoto T, Asahara T, Khosla S. Emerging Role of Circulating Calcifying Cells in the Bone-Vascular Axis. *Circulation* 2012;125(22):2772-81.
- [60] Doebling LC, Heeger C, Aherrahrou Z, Kaczmarek PM, Erdmann J, Schunkert H, et al. Myeloid CD34+CD13+ precursor cells transdifferentiate into chondrocyte-like cells in atherosclerotic intimal calcification. *The American journal of pathology* 2010;177(1):473-80.

- [61] Naik V, Leaf EM, Hu JH, Yang HY, Nguyen NB, Giachelli CM, et al. Sources of cells that contribute to atherosclerotic intimal calcification: an in vivo genetic fate mapping study. *Cardiovascular research* 2012;94(3):545-54.
- [62] Elia L, Quintavalle M, Zhang J, Contu R, Cossu L, Latronico MV, et al. The knockout of miR-143 and -145 alters smooth muscle cell maintenance and vascular homeostasis in mice: correlates with human disease. *Cell death and differentiation* 2009;16(12):1590-8.
- [63] Cordes KR, Sheehy NT, White MP, Berry EC, Morton SU, Muth AN, et al. miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. *Nature* 2009;460(7256):705-10.
- [64] Boettger T, Beetz N, Kostin S, Schneider J, Kruger M, Hein L, et al. Acquisition of the contractile phenotype by murine arterial smooth muscle cells depends on the Mir143/145 gene cluster. *The Journal of clinical investigation* 2009;119(9):2634-47.
- [65] Ji R, Cheng Y, Yue J, Yang J, Liu X, Chen H, et al. MicroRNA expression signature and antisense-mediated depletion reveal an essential role of MicroRNA in vascular neointimal lesion formation. *Circulation research* 2007;100(11):1579-88.
- [66] Cheng Y, Liu X, Yang J, Lin Y, Xu DZ, Lu Q, et al. MicroRNA-145, a novel smooth muscle cell phenotypic marker and modulator, controls vascular neointimal lesion formation. *Circulation research* 2009;105(2):158-66.
- [67] Steitz SA, Speer MY, Curinga G, Yang HY, Haynes P, Aebersold R, et al. Smooth muscle cell phenotypic transition associated with calcification: upregulation of Cbfa1 and downregulation of smooth muscle lineage markers. *Circulation research* 2001;89(12):1147-54.
- [68] Yoshida T, Yamashita M, Hayashi M. Kruppel-like factor 4 contributes to high phosphate-induced phenotypic switching of vascular smooth muscle cells into osteogenic cells. *The Journal of biological chemistry* 2012.
- [69] Xin M, Small EM, Sutherland LB, Qi X, McAnally J, Plato CF, et al. MicroRNAs miR-143 and miR-145 modulate cytoskeletal dynamics and responsiveness of smooth muscle cells to injury. *Genes & development* 2009;23(18):2166-78.
- [70] Torella D, Iaconetti C, Catalucci D, Ellison GM, Leone A, Waring CD, et al. MicroRNA-133 controls vascular smooth muscle cell phenotypic switch in vitro and vascular remodeling in vivo. *Circulation research* 2011;109(8):880-93.
- [71] Li Z, Hassan MQ, Volinia S, van Wijnen AJ, Stein JL, Croce CM, et al. A microRNA signature for a BMP2-induced osteoblast lineage commitment program. *Proceedings of the National Academy of Sciences of the United States of America* 2008;105(37):13906-11.
- [72] Komori T. Regulation of bone development and extracellular matrix protein genes by RUNX2. *Cell and tissue research* 2010;339(1):189-95.

- [73] Byon CH, Javed A, Dai Q, Kappes JC, Clemens TL, Darley-USmar VM, et al. Oxidative stress induces vascular calcification through modulation of the osteogenic transcription factor Runx2 by AKT signaling. *The Journal of biological chemistry* 2008;283(22):15319-27.
- [74] Sun Y, Byon C, Yuan K, Chen J, Mao X, Heath JM, et al. Smooth Muscle Cell-Specific Runx2 Deficiency Inhibits Vascular Calcification. *Circulation research* 2012.
- [75] Speer MY, Li X, Hiremath PG, Giachelli CM. Runx2/Cbfa1, but not loss of myocardin, is required for smooth muscle cell lineage reprogramming toward osteochondrogenesis. *Journal of cellular biochemistry* 2010;110(4):935-47.
- [76] Miller JD, Weiss RM, Serrano KM, Castaneda LE, Brooks RM, Zimmerman K, et al. Evidence for active regulation of pro-osteogenic signaling in advanced aortic valve disease. *Arteriosclerosis, thrombosis, and vascular biology* 2010;30(12):2482-6.
- [77] Zhang Y, Xie RL, Croce CM, Stein JL, Lian JB, van Wijnen AJ, et al. A program of microRNAs controls osteogenic lineage progression by targeting transcription factor Runx2. *Proceedings of the National Academy of Sciences of the United States of America* 2011;108(24):9863-8.
- [78] Lim K, Lu TS, Molostvov G, Lee C, Lam FT, Zehnder D, et al. Vascular Klotho deficiency potentiates the development of human artery calcification and mediates resistance to fibroblast growth factor 23. *Circulation* 2012;125(18):2243-55.
- [79] Gui T, Zhou G, Sun Y, Shimokado A, Itoh S, Oikawa K, et al. MicroRNAs that target Ca(2+) transporters are involved in vascular smooth muscle cell calcification. *Laboratory investigation; a journal of technical methods and pathology* 2012.
- [80] Cui RR, Li SJ, Liu LJ, Yi L, Liang QH, Zhu X, et al. MicroRNA-204 Regulates Vascular Smooth Muscle Cell Calcification in vitro and in vivo. *Cardiovascular research* 2012.
- [81] Shanahan CM, Crouthamel MH, Kapustin A, Giachelli CM. Arterial calcification in chronic kidney disease: key roles for calcium and phosphate. *Circulation research* 2011;109(6):697-711.
- [82] Mizuno Y, Yagi K, Tokuzawa Y, Kanesaki-Yatsuka Y, Suda T, Katagiri T, et al. miR-125b inhibits osteoblastic differentiation by down-regulation of cell proliferation. *Biochemical and biophysical research communications* 2008;368(2):267-72.
- [83] Goettsch C, Rauner M, Pacyna N, Hempel U, Bornstein SR, Hofbauer LC. miR-125b regulates calcification of vascular smooth muscle cells. *The American journal of pathology* 2011;179(4):1594-600.
- [84] Albinsson S, Suarez Y, Skoura A, Offermanns S, Miano JM, Sessa WC. MicroRNAs are necessary for vascular smooth muscle growth, differentiation, and function. *Arteriosclerosis, thrombosis, and vascular biology* 2010;30(6):1118-26.

- [85] Bostrom KI, Rajamannan NM, Towler DA. The regulation of valvular and vascular sclerosis by osteogenic morphogens. *Circulation research* 2011;109(5):564-77.
- [86] Bostrom K, Watson KE, Horn S, Wortham C, Herman IM, Demer LL. Bone morphogenetic protein expression in human atherosclerotic lesions. *The Journal of clinical investigation* 1993;91(4):1800-9.
- [87] Dhore CR, Cleutjens JP, Lutgens E, Cleutjens KB, Geusens PP, Kitslaar PJ, et al. Differential expression of bone matrix regulatory proteins in human atherosclerotic plaques. *Arteriosclerosis, thrombosis, and vascular biology* 2001;21(12):1998-2003.
- [88] Seya K, Yu Z, Kanemaru K, Daitoku K, Akemoto Y, Shibuya H, et al. Contribution of bone morphogenetic protein-2 to aortic valve calcification in aged rat. *Journal of pharmacological sciences* 2011;115(1):8-14.
- [89] Itoh T, Nozawa Y, Akao Y. MicroRNA-141 and -200a are involved in bone morphogenetic protein-2-induced mouse pre-osteoblast differentiation by targeting distal-less homeobox 5. *The Journal of biological chemistry* 2009;284(29):19272-9.
- [90] Monroe DG, McGee-Lawrence ME, Oursler MJ, Westendorf JJ. Update on Wnt signaling in bone cell biology and bone disease. *Gene* 2012;492(1):1-18.
- [91] Miller JD, Chu Y, Brooks RM, Richenbacher WE, Pena-Silva R, Heistad DD. Dysregulation of antioxidant mechanisms contributes to increased oxidative stress in calcific aortic valvular stenosis in humans. *Journal of the American College of Cardiology* 2008;52(10):843-50.
- [92] Al-Aly Z, Shao JS, Lai CF, Huang E, Cai J, Behrmann A, et al. Aortic Msx2-Wnt calcification cascade is regulated by TNF-alpha-dependent signals in diabetic Ldlr-/- mice. *Arteriosclerosis, thrombosis, and vascular biology* 2007;27(12):2589-96.
- [93] Cheng SL, Shao JS, Halstead LR, Distelhorst K, Sierra O, Towler DA. Activation of vascular smooth muscle parathyroid hormone receptor inhibits Wnt/beta-catenin signaling and aortic fibrosis in diabetic arteriosclerosis. *Circulation research* 2010;107(2):271-82.
- [94] Faverman L, Mikhaylova L, Malmquist J, Nurminskaya M. Extracellular transglutaminase 2 activates beta-catenin signaling in calcifying vascular smooth muscle cells. *FEBS letters* 2008;582(10):1552-7.
- [95] Glinka A, Wu W, Delius H, Monaghan AP, Blumenstock C, Niehrs C. Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* 1998;391(6665):357-62.
- [96] Mukhopadhyay M, Shtrom S, Rodriguez-Esteban C, Chen L, Tsukui T, Gomer L, et al. Dickkopf1 is required for embryonic head induction and limb morphogenesis in the mouse. *Developmental cell* 2001;1(3):423-34.
- [97] Thambiah S, Roplekar R, Manghat P, Fogelman I, Fraser WD, Goldsmith D, et al. Circulating sclerostin and Dickkopf-1 (DKK1) in predialysis chronic kidney disease

- (CKD): relationship with bone density and arterial stiffness. *Calcified tissue international* 2012;90(6):473-80.
- [98] Beazley KE, Deasey S, Lima F, Nurminskaya MV. Transglutaminase 2-mediated activation of beta-catenin signaling has a critical role in warfarin-induced vascular calcification. *Arteriosclerosis, thrombosis, and vascular biology* 2012;32(1):123-30.
 - [99] Bai XY, Ma Y, Ding R, Fu B, Shi S, Chen XM. miR-335 and miR-34a Promote renal senescence by suppressing mitochondrial antioxidative enzymes. *Journal of the American Society of Nephrology : JASN* 2011;22(7):1252-61.
 - [100] Boon RA, Seeger T, Heydt S, Fischer A, Hergenreider E, Horrevoets AJ, et al. Micro-RNA-29 in aortic dilation: implications for aneurysm formation. *Circulation research* 2011;109(10):1115-9.
 - [101] Zhang J, Tu Q, Bonewald LF, He X, Stein G, Lian J, et al. Effects of miR-335-5p in modulating osteogenic differentiation by specifically downregulating Wnt antagonist DKK1. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 2011;26(8):1953-63.
 - [102] Kapinas K, Kessler C, Ricks T, Gronowicz G, Delany AM. miR-29 modulates Wnt signaling in human osteoblasts through a positive feedback loop. *The Journal of biological chemistry* 2010;285(33):25221-31.
 - [103] Kapinas K, Kessler CB, Delany AM. miR-29 suppression of osteonectin in osteoblasts: regulation during differentiation and by canonical Wnt signaling. *Journal of cellular biochemistry* 2009;108(1):216-24.
 - [104] Jones JA, Stroud RE, O'Quinn EC, Black LE, Barth JL, Elefteriades JA, et al. Selective microRNA suppression in human thoracic aneurysms: relationship of miR-29a to aortic size and proteolytic induction. *Circulation Cardiovascular genetics* 2011;4(6):605-13.
 - [105] Chen NX, O'Neill KD, Chen X, Kiattisunthorn K, Gattone VH, Moe SM. Activation of arterial matrix metalloproteinases leads to vascular calcification in chronic kidney disease. *American journal of nephrology* 2011;34(3):211-9.
 - [106] Freeman RV, Otto CM. Spectrum of calcific aortic valve disease: pathogenesis, disease progression, and treatment strategies. *Circulation* 2005;111(24):3316-26.
 - [107] Mohammadpour AH, Shamsara J, Nazemi S, Ghadirzadeh S, Shahsavand S, Ramezani M. Evaluation of RANKL/OPG Serum Concentration Ratio as a New Biomarker for Coronary Artery Calcification: A Pilot Study. *Thrombosis* 2012;2012:306263.
 - [108] Kiechl S, Schett G, Schwaiger J, Seppi K, Eder P, Egger G, et al. Soluble receptor activator of nuclear factor-kappa B ligand and risk for cardiovascular disease. *Circulation* 2007;116(4):385-91.

- [109] Dombkowski AA, Sultana Z, Craig DB, Jamil H. In silico analysis of combinatorial microRNA activity reveals target genes and pathways associated with breast cancer metastasis. *Cancer informatics* 2011;10:13-29.
- [110] Mizoguchi F, Izu Y, Hayata T, Hemmi H, Nakashima K, Nakamura T, et al. Osteoclast-specific Dicer gene deficiency suppresses osteoclastic bone resorption. *Journal of cellular biochemistry* 2010;109(5):866-75.
- [111] Wang K, Zhang S, Weber J, Baxter D, Galas DJ. Export of microRNAs and microRNA-protective protein by mammalian cells. *Nucleic acids research* 2010;38(20):7248-59.
- [112] Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proceedings of the National Academy of Sciences of the United States of America* 2011;108(12):5003-8.
- [113] Turchinovich A, Weiz L, Langheinz A, Burwinkel B. Characterization of extracellular circulating microRNA. *Nucleic acids research* 2011;39(16):7223-33.
- [114] Diehl P, Fricke A, Sander L, Stamm J, Bassler N, Htun N, et al. Microparticles: major transport vehicles for distinct microRNAs in circulation. *Cardiovascular research* 2012;93(4):633-44.
- [115] Pigati L, Yaddanapudi SC, Iyengar R, Kim DJ, Hearn SA, Danforth D, et al. Selective release of microRNA species from normal and malignant mammary epithelial cells. *PloS one* 2010;5(10):e13515.
- [116] Chen TS, Lai RC, Lee MM, Choo AB, Lee CN, Lim SK. Mesenchymal stem cell secretes microparticles enriched in pre-microRNAs. *Nucleic acids research* 2010;38(1):215-24.
- [117] Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T. Secretory mechanisms and intercellular transfer of microRNAs in living cells. *The Journal of biological chemistry* 2010;285(23):17442-52.
- [118] Hergenreider E, Heydt S, Treguer K, Boettger T, Horrevoets AJ, Zeiher AM, et al. Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs. *Nature cell biology* 2012;14(3):249-56.
- [119] Creemers EE, Tijssen AJ, Pinto YM. Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease? *Circulation research* 2012;110(3):483-95.
- [120] Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nature cell biology* 2007;9(6):654-9.

- [121] Zerneck A, Bidzhekov K, Noels H, Shagdarsuren E, Gan L, Denecke B, et al. Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. *Science signaling* 2009;2(100):ra81.
- [122] Meckes DG, Jr., Raab-Traub N. Microvesicles and viral infection. *Journal of virology* 2011;85(24):12844-54.
- [123] Wuthier RE, Lipscomb GF. Matrix vesicles: structure, composition, formation and function in calcification. *Frontiers in bioscience : a journal and virtual library* 2011;16:2812-902.
- [124] Reynolds JL, Joannides AJ, Skepper JN, McNair R, Schurgers LJ, Proudfoot D, et al. Human vascular smooth muscle cells undergo vesicle-mediated calcification in response to changes in extracellular calcium and phosphate concentrations: a potential mechanism for accelerated vascular calcification in ESRD. *Journal of the American Society of Nephrology : JASN* 2004;15(11):2857-67.
- [125] Chen NX, O'Neill KD, Chen X, Moe SM. Annexin-mediated matrix vesicle calcification in vascular smooth muscle cells. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 2008;23(11):1798-805.
- [126] Kapustin AN, Davies JD, Reynolds JL, McNair R, Jones GT, Sidibe A, et al. Calcium regulates key components of vascular smooth muscle cell-derived matrix vesicles to enhance mineralization. *Circulation research* 2011;109(1):e1-12.
- [127] New SE, Marchini JF, Aikawa M, Shanahan CM, Croce K, Aikawa E. Novel Role of Macrophage-derived Matrix Vesicles in Arterial Microcalcification *Circulation* 2011;124(21 Supplement):A10866.
- [128] Sage AP, Lu J, Tintut Y, Demer LL. Hyperphosphatemia-induced nanocrystals upregulate the expression of bone morphogenetic protein-2 and osteopontin genes in mouse smooth muscle cells in vitro. *Kidney international* 2011;79(4):414-22.
- [129] Naves M, Rodriguez-Garcia M, Diaz-Lopez JB, Gomez-Alonso C, Cannata-Andia JB. Progression of vascular calcifications is associated with greater bone loss and increased bone fractures. *Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA* 2008;19(8):1161-6.
- [130] Jensky NE, Hyder JA, Allison MA, Wong N, Aboyans V, Blumenthal RS, et al. The association of bone density and calcified atherosclerosis is stronger in women without dyslipidemia: the multi-ethnic study of atherosclerosis. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 2011;26(11):2702-9.

- [131] Wang Y, Li L, Moore BT, Peng XH, Fang X, Lappe JM, et al. MiR-133a in human circulating monocytes: a potential biomarker associated with postmenopausal osteoporosis. *PloS one* 2012;7(4):e34641.
- [132] Li H, Xie H, Liu W, Hu R, Huang B, Tan YF, et al. A novel microRNA targeting HDAC5 regulates osteoblast differentiation in mice and contributes to primary osteoporosis in humans. *The Journal of clinical investigation* 2009;119(12):3666-77.
- [133] Paccou J, Brazier M, Mentaverri R, Kamel S, Fardellone P, Massy ZA. Vascular calcification in rheumatoid arthritis: Prevalence, pathophysiological aspects and potential targets. *Atherosclerosis* 2012.
- [134] Pauley KM, Satoh M, Chan AL, Bubb MR, Reeves WH, Chan EK. Upregulated miR-146a expression in peripheral blood mononuclear cells from rheumatoid arthritis patients. *Arthritis research & therapy* 2008;10(4):R101.