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microRNAs in the onset and development of cardiovascular disease

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Abstract

Physiological and pathological roles for small non-encoding miRNAs (microRNAs) in the cardiovascular system have recently emerged and are now widely studied. The discovery of widespread functions of miRNAs has increased the complexity of gene-regulatory processes and networks in both the cardiovascular system and cardiovascular diseases. Indeed, it has recently been shown that miRNAs are implicated in the regulation of many of the steps leading to the development of cardiovascular disease. These findings represent novel aspects in miRNA biology and, therefore, our understanding of the role of these miRNAs during the pathogenesis of cardiovascular disease is critical for the development of novel therapies and diagnostic interventions. The present review will focus on understanding how miRNAs are involved in the onset and development of cardiovascular diseases.

Keywords

cardiovascular disease; diabetes; dyslipidaemia; hypertension; microRNA (miRNA)

INTRODUCTION

miRNAs (microRNAs) are short (~19–25 nucleotides in length) single-stranded non-encoding RNAs that are well conserved in eukaryotic organisms [1,2]. By base pairing with complementary sites within target mRNA, they act as negative regulators of gene expression by inhibiting translation and/or inducing specific mRNA degradation [1,3,4]. The first miRNAs were discovered in the early 1990s; however, they were not recognized as a distinct class of post-transcriptional biological regulators until the early 2000s [5–7]. miRNAs have been identified as being encoded by the human genome, with newly identified miRNAs being reported on a regular basis. miRNA genes can be transcribed from their own promoters (intergenic) as independent transcription units or within the host protein-encoding genes (intronic) [1,8,9].

The biogenesis of miRNAs starts with RNA polymerase II-mediated transcription from the genome, leading to the formation of a pri-miRNA (primary miRNA transcript) of variable length depending on the locus [9,10] (Figure 1). The double-stranded pri-miRNA is cleaved by a large nuclear ribonuclease complex, DROSHA/DGCR8, generating a pre-miRNA (precursor miRNA) that is ~70 nucleotides in length and has a hairpin-like secondary structure [10,11]. Following nuclear processing by DROSHA/DGCR8, the pre-miRNA is then exported to the cytoplasm via the nuclear transport receptor, exportin-5, dependent

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pathway [12,13]. Once in the cytoplasm, the pre-miRNA is subject to further enzymatic processing by Dicer (another ribonuclease III enzyme) and its co-factors, generating the final short double-stranded miRNA strand (~22 nucleotides in length) [14]. The double-stranded miRNA (miRNA duplex) is composed of a mature miRNA guide strand (functional) and a passenger strand, called miRNA*. After unwinding of the duplex, the guide strand is loaded into the RISC (RNA-induced silencing complex), a multiprotein complex containing AGO2 (Argonaute 2) and other associated proteins, whereas the passenger strand is normally degraded (Figure 1) [15]. Once in the RISC, the mature miRNA seed region (nucleotides at position 2–8) participates in gene silencing by interacting with the 3′-UTR of the target mRNA [16,17]. This interaction leads predominantly to the down-regulation of the targeted mRNA (Figure 1).

Pioneering studies have also uncovered the presence of circulating extracellular miRNAs, which are stable in the plasma and not associated with cells [18]. The levels of these miRNAs can differ with disease states [18–20]. Previous findings showed that extracellular miRNAs can associate with lipid-based carriers including HDLs (high-density lipoproteins) [21,22]. HDLs were found to contain distinct miRNA signatures and HDL miRNA profiles were altered with cardiovascular disease in humans and mice [22]. Furthermore, the miRNAs present on HDLs can be delivered to cells, including human hepatocellular carcinoma cells (Huh-7) and baby hamster kidney cells overexpressing SR-BI (scavenger receptor class B, type I) [22]. As plasma HDL-C (HDL-cholesterol) levels inversely correlate with cardiovascular risk [23], the discovery of HDL-associated miRNAs and the role of HDLs in miRNA transport represents an important finding linking lipoproteins and miRNAs to cardiovascular diseases.

The aim of the present review is to (i) summarize what is known about the involvement of miRNAs in the onset of cardiovascular disease, and (ii) illustrate how miRNAs can regulate the development of cardiovascular diseases. We will discuss the involvement of miRNAs in dyslipidaemia, hypertension, insulin resistance and diabetes, CAD (coronary artery disease), vascular inflammation, MI (myocardial infarction), and heart failure.

miRNAs AND THE ONSET OF CARDIOVASCULAR DISEASE

miRNAs and dyslipidaemia

Elevated levels of HDLs protect against coronary heart disease [23], whereas elevated levels of serum cholesterol (hypercholesterolaemia), lipids (hyperlipidaemia) and TAG [triacylglycerols (triglycerides)] (hypertriglyceridaemia) promote CAD [24,25]. Cholesterol biosynthesis in the liver is fundamentally important for lipid and lipoprotein synthesis and metabolism [26,27] and miRNAs have recently been found to modulate these processes [28–30].

We have recently identified *miR-27b* as a candidate post-transcriptional hub of lipid metabolism genes [30]. *miR-27b* was experimentally determined to target 27 of 151 lipid-associated genes in human hepatoma cell lines, including *NDST1* (heparan sulfate N-deacetylase/N-sulfotransferase 1), *ANGPTL3* (angiopoietin-like 3), *PPARG* (peroxisome proliferator-activated receptor γ) and *GPAM* (glycerol-3-phosphate acyltransferase 1) [30]. Using small RNA sequencing and real-time PCR, we found *miR-27b* and its target genes to be significantly modulated in mouse livers in response to diet-induced hyperlipidaemia [30]. *miR-27b* was found to be significantly increased in response to elevated plasma TAGs and hepatosteatosis, whereas ANGPTL3 and GPAM, both targets of *miR-27b*, were significantly repressed in the livers of mice fed on a high-fat diet [30]. ANGPTL3 is secreted from the liver and inhibits the hydrolysis of TAGs by lipoprotein lipase [31]. GPAM is the first committed enzymatic step in *de novo* TAG biosynthesis in the liver [32,33]. Therefore,

during hypertriglyceridaemia and hepatosteatosis, hepatic *miR-27b* increases, which leads to inhibition of *de novo* TAG biosynthesis. These results support a role for miRNAs in the hepatic response to diet-induced hypertriglyceridaemia.

It has been demonstrated that *miR-21* and *miR-27b* negatively regulate PPARα, which is mainly expressed in the liver and regulates the expression of multiple genes involved in fatty acid transport, catabolism and energy homoeostasis [31]. PPARα protein levels in human liver-derived cell lines were found to be decreased by the overexpression of *miR-21* and *miR-27b*, but not by that of *miR-22*, *miR-24*, *miR-181a* and *let-7a* [31]. In addition to *miR-21* and *miR-27b*, it has been reported in another study that PPARα is also regulated by *miR-10b* in the human hepatocyte cell line L02 [34]. This later study was performed using L02 cells cultured with high concentrations of non-esterified (free) fatty acids (as a non-alcoholic fatty acid liver disease model). Contrary to what has been reported for *miR-21* levels, which are highly expressed in the human liver, *miR-10b* levels are up-regulated in a human hepatocyte cell line by treatment with high concentrations of non-esterified fatty acids [34]. *PPARA* (PPARα) is the direct target of *miRNA-10b* and PPARα protein levels were significantly decreased after overexpressing *miR-10b* in L02 cells incubated with high concentrations of non-esterified fatty acids [34]. It has therefore been speculated that the role of *miR-10b* in PPARα regulation might be specific to certain pathological conditions such as non-alcoholic fatty acid liver disease.

miRNAs and cholesterol metabolism

Cellular cholesterol homoeostasis is tightly regulated and achieved through a delicate balance of cholesterol biosynthesis, cholesterol efflux from cells to acceptors in the extracellular space and cellular cholesterol uptake through scavenger receptors, such as the LDLR [LDL (low-density lipoprotein) receptor] and LRPs (LDLR-related proteins). Cellular cholesterol homoeostasis is essential for functional signal transduction, membrane integrity, cell proliferation, lipid metabolism and many other key processes. SREBP2 (sterol-regulatory-element-binding protein 2) transcriptionally regulates most of the enzymes in the cholesterol biosynthetic pathway, as well as the LDLR, the major route of cholesterol entry into cells [35]. Although multiple ATP-binding cassette transporters and other transmembrane proteins mediate cholesterol efflux from cells into the extracellular space, ABCA1 (ATP-binding cassette transporter A1) is the major route of hepatic cholesterol and phospholipid efflux to apoA-I (apolipoprotein A-I), the main HDL apolipoprotein. This results in the formation of discoidal HDLs that are the precursors of the mature spherical HDLs that predominate in the plasma [36]. Likewise, ABCA1-mediated cholesterol efflux from cholesterol-loaded macrophages is a key mechanism for the prevention of foam cell formation and ultimately the development of atherosclerosis [37].

The most widely studied miRNA feedback network in the area of cholesterol metabolism is *miR-33a/b* [38–43]. *miR-33a* is harboured within intron 16 of *SREBP2*, and is thus co-transcribed with this master lipid transcription factor [40–42]. Previous evidence supports *miR-33a* as a key mediator in the cellular response to depleted cholesterol stores. In low cholesterol states, an intricate sterol-sensing network is activated that cleaves SREBP2 from the endoplasmic reticulum, allowing it to enter the nucleus where it transcriptionally activates the cholesterol biosynthetic pathway and up-regulates LDLR expression, thus increasing cellular cholesterol synthesis and LDL-cholesterol uptake [44]. These events are accompanied by the co-transcriptional activation of SREBP2 and *miR-33a*, the latter of which directly targets and decreases *ABCA1* mRNA levels, which represses cholesterol efflux from the cells. Interestingly, *miR-33a* and *miR-33b* are localized within the introns of *SREBP* genes, therefore they collaborate with their protein encoding host transcripts to control cholesterol and lipid metabolism. In addition to *ABCA1*, *miR-33a/b* targets multiple

other genes involved in cholesterol metabolism, including *ABCG1* (ATP-binding cassette transporter G1; cholesterol efflux), *NPC1* (Niemann–Pick C1; cholesterol storage), *ABCB11* (ATP-binding cassette transporter B11; bile secretion) and *ATP8B1* (phospholipid-transporting ATPase IC; bile acid secretion) [40–42,45]. *miR-33a/b* was also found to play a key role in fatty acid *β*-oxidation by directly targeting *CROT* (carnitine *O*-octanyl transferase), *CPT1A* (carnitine palmitoyltransferase 1A) and *HADHB* (hydroxyacyl-CoA dehydrogenase-3-ketoacyl-CoA thiolase-enoyl-CoA hydratase *β*-subunit) [38,46]. Similar to the regulation of cellular cholesterol levels by SREBP2/*miR-33b*, SREBP1/*miR-33a* promotes fatty acid synthesis and antagonizes fatty acid oxidation. In non-human primates, inhibition of *miR-33a/b* resulted in reduced VLDL (very-low-density lipoprotein) secretion and a reduction in plasma TAG levels [47]. Likewise, inhibition of *miR-33* in mice was found to increase ABCA1 expression and plasma HDL-C levels. Plasma HDL-C levels are also increased in non-human primates in which *miR-33a/b* is inhibited [43,47].

miR-122 was the first miRNA to be identified as having an important role in the regulation of lipid metabolism [48,49]. Anti-*miR-122* therapy in mice and non-human primates resulted in a significant reduction in cholesterol levels [29,48]. These results have recently been confirmed in *miR-122*-deficient mouse models [50,51]. The mechanisms by which *miR-122* induces its effects on lipid metabolism are still poorly understood, but previous studies have demonstrated that genes involved in cholesterol biosynthesis, such as *HMGCR* (3-hydroxy-3-methylglutaryl-CoA reductase) and *HMGCS1* (3-hydroxy-3-methylglutaryl-CoA synthase 1) are down-regulated by this miRNA [50–52]. In addition to its effects on cholesterol biosynthesis, *miR-122* seems to be involved in TAG metabolism, with the TAG secretion rate being significantly reduced in *miR-122*-deficient mice [50].

In the liver, *miR-122* accounts for 70–80% of the miRNA, depending on the technique used [53]. As such, *miR-122* has been widely studied in the liver and has been found to play a role in hepatitis C, inflammation and hepatocellular carcinoma as well as iron, glucose, lipid and cholesterol metabolism [29,50,51,54–58]. Many cholesterol-regulating genes, including genes in the HMGCR, SQLE (squalene expoxidase) and lipoprotein synthesis/microsomal transfer protein pathways are inversely associated with *miR-122* [29,50,52]. Although, *miR-122* indirectly regulates cholesterol and lipid homoeostasis, its direct intermediary targets are currently unknown. Interestingly, *miR-370* was found to regulate lipid metabolism both though direct targeting of lipid genes and modulation of *miR-122* [59].

Recently, another miRNA, *miR-144*, has been reported to regulate the expression of *ABCA1* in macrophages and hepatocytes [60]. Indeed, *miR-144* expression was up-regulated in LXR (liver X receptor) agonist (T090)-treated mouse peritoneal macrophages. Human monocytes (THP-1) and human hepatic (Huh-7) cells treated with T090 also showed increased expression of *miR-144* [60]. *miR-144* is an intergenic miRNA located in the same locus as *miR-451*, which is also induced by stimulating mouse primary macrophages, THP-1 and Huh-7 cells, with T090 [60]. As expected, overexpression of *miR-144* in mouse peritoneal macrophages inhibited ABCA1 expression. Although *ABCG1* is not a direct target of *miR-144*, *ABCG1* mRNA levels were also down-regulated in macrophages transfected with *miR-144*. This may have been an indirect effect involving the retinoid X receptor *β*, which is a predicted target for *miR-144* [60]. Further evidence of the involvement of *miR-144* in the regulation of ABCA1 expression was obtained by transfecting J774 murine macrophages with *miR-144*, which inhibited the efflux of cholesterol to apoA-I. On the other hand, antagonism of endogenous *miR-144* in J774 murine macrophages increased ABCA1 expression as well as the efflux of cellular cholesterol to apoA-I [60]. *miR-144* is widely expressed in mouse tissues and is particularly abundant in the liver, spleen and aorta. A recent study has reported a role for *miR-144* in regulating lipoprotein metabolism and showed that the overexpression or inhibition of *miR-144* reduces and increases circulating

HDL levels respectively [60]. The injection of *miR-144* mimic nanoparticles into mice significantly reduced *ABCA1* and *ABCG1* mRNA and protein levels in the liver. The inhibition of ABCA1 expression after 6 days of treatment with *miR-144* particles also decreased total cholesterol and HDL-C levels without changing the TAG or cholesterol distribution in other lipoproteins. Finally, *in vivo* inhibition of *miR-144* with *miR-144* inhibitor-conjugated nanoparticles showed increased liver ABCA1 protein expression and plasma HDL-C levels [60].

Other miRNAs that have been reported to play key roles in lipid metabolism include *miR-106*, *miR-26* and *miR-758*, which target and repress ABCA1 in multiple cell types including macrophages and hepatocytes [61–63]. Similar to *miR-33a/b*, *miR-758* was also found to be sensitive to cellular sterol levels and was down-regulated in response to cholesterol loading [61]. *miR-1*, *miR-206* and *miR-613* have all recently been reported to directly target LXRα and, thus, repress lipogenesis [64,65]. *miR-613* suppresses lipogenesis by inhibiting expression of LXRα and its target genes, including *SREBP1c*, *FAS* (fatty acid synthase), *ChREBP* (carbohydrate responsive element-binding protein) and *ACC* (acetyl-CoA carboxylase) [65,66]. Although *NR1H3* (encoding LXRα) is a target of miRNAs, recent evidence suggests that LXRα also controls the expression of specific miRNAs, with *miR-26* being repressed by LXRα in macrophages [63]. Most interestingly, *miR-26* was also found to directly target the LXRα genes, *ABCA1* and *ARL7* (ADP-ribosylation factor-like 7). ARL7 has been shown to be induced by cholesterol loading and participates in apoA-I-dependent cholesterol export [63].

Multiple miRNAs have been reported to target receptors associated with lipoprotein or cholesterol uptake. *miR-146a* represses TLR4 (Toll-like receptor 4) signalling, and inhibits oxLDL (oxidized LDL) cholesterol uptake in macrophages [67]. Most interestingly, oxLDL stimulation was found to decrease *miR-146a* expression [67]. *miR-155*, a miRNA associated with inflammation, was also found to repress lipid uptake in oxLDL-stimulated dendritic cells through targeting and down-regulation of the scavenger receptor, CD36, and the lectin-type LOX-1 (oxidized LDL receptor 1). *miR-155* also regulates LOX-1 and SR-A (scavenger receptor A) pathways in macrophages [68]. Most interestingly, oxLDL stimulation was previously found to up-regulate *miR-155*, along with *miR-9*, *miR-146a*, *miR-125a-5p* and *miR-146b-5p*, in monocytes [69]. In that study, *miR-125-5p* repressed lipid uptake through targeting of ORP6 (oxysterol-binding protein-like 9) in monocytes [69]. In a separate study, inhibition of *miR-155* was found to increase both lipid uptake and inflammation in oxLDL-stimulated THP-1 macrophages [70]. *miR-125*, *miR-455-5p*, *miR-185*, *miR-96* and *miR-223* have all been reported to target SR-BI and reduce HDL-C uptake [22,71,72]. Recently, *miR-217* was found to mediate ethanol-induced SIRT1 (sirtuin 1) repression and contribute to ethanol-induced lipid accumulation and fatty acid synthesis [73]. Chronic ethanol feeding in mice also caused a significant increase in hepatic *miR-217* levels [73]. Similar to *miR-33*, *miR-217* is a key modulator of fatty acid oxidation and fatty acid synthesis [73].

Recently, there has been significant interest in identifying single nucleotide polymorphisms and functional variants within miRNAs themselves, and in mRNA target sites [74–78]. A recent study has found a gain-of-function variant in the *LPL* (lipoprotein lipase) gene that appears to abrogate a *miR-410* target site [79]. The LPL variant rs13702 minor allele was found through meta-analyses to be significantly associated with increased HDL-C and decreased TAG levels [79]. *miR-467b* has also been reported to directly target LPL and inhibit lipid accumulation in macrophages [80]. Although over 1400 miRNAs have been identified in humans, each cell type typically contains 100–300 unique miRNAs. Most are transcribed in the cell; however, some are likely transferred to the cells from the extracellular compartment via lipoproteins or microvesicles [81].

miRNAs in insulin resistance and diabetes

Insulin resistance and T2D (Type 2 diabetes mellitus) are major risk factors of cardiovascular disease, together with associated endothelial dysfunction and micro- and macro-vascular complications [82]. Insulin resistance, a condition where tissues such as muscle and adipose tissue fail to adequately respond to the physiological actions of insulin, often progresses to T2D and coronary heart disease [83]. This is usually seen when pancreatic β-cells are unable to produce sufficient insulin to maintain normal blood sugar levels when insulin resistance is present, leading to hyperglycaemia. The inability of the β-cells to produce sufficient insulin under hyperglycaemic conditions is what characterizes the transition from insulin resistance to T2D [83]. It has been demonstrated that plasma miRNA levels are changed in patients with T2D. Previous studies have shown that plasma levels of *miR-24*, *miR-21*, *miR-20b*, *miR-15a*, *miR-126*, *miR-191*, *miR-197*, *miR-223*, *miR-320*, *miR-486*, *miR-150* and *miR-29b* are lower in patients with T2D, whereas *miR-28-3p* and *miR-375* tend to be elevated [84,85].

miR-126 is an miRNA that is consistently associated with T2D. In a population study, reduced levels of *miR-126* in plasma were systematically associated with T2D [84]. One of the mechanisms by which *miR-126* is involved in the development of insulin resistance is through the inhibition of IRS1 (insulin receptor substrate 1) [86]. This miRNA plays an important role in maintaining vascular integrity, angiogenesis and wound repair [87,88]. It also facilitates VEGF (vascular endothelial growth factor) signalling by repressing two negative regulators of the VEGF pathway: SPRED1 (Sprouty-related protein) and PIK3R2 (phosphoinositol-3 kinase regulatory subunit 2)/p85-β [87]. *miR-126* is the most important miRNA in endothelial apoptotic bodies, where it is significantly reduced by a high glucose concentration [84,89]. *miR-126* in apoptotic bodies is responsible for their cardioprotective properties. Indeed, *miR-126*-carrying, but not *miR-126*-deficient, apoptotic bodies confer a protection against diet-induced atherosclerosis in the carotid arteries of apoE$^{-/-}$ mice, which is associated with a reduced infiltration of macrophages into the artery wall and an increase in the number of smooth muscle cells [89].

miRNAs play key roles in the regulation of insulin secretion through both pancreatic development and insulin exocytosis. Enrichment of pancreatic β-cells with *miR-375*, was found to negatively regulate insulin exocytosis and secretion [90]. The mechanisms by which *miR-375* modulates insulin secretion are independent of changes in glucose metabolism and intracellular Ca^{2+} signalling, but are related to a direct effect on insulin exocytosis through the repression of *Mtpn* (myotrophin), a gene implicated in actin depolymerization [90]. *Mtpn* is a predicted and validated target of *miR-375* and the inhibition of *miR-375* induces the repression of Mtpn which could contribute to the defect in exocytosis [90]. On the other hand, inhibition of *miR-375* expression enhances insulin secretion [90]. Furthermore, homozygous deletion of *miR-375* in mice is associated with hyperglycaemia due to decreased total pancreatic β-cell mass and plasma insulin levels [90]. *miR-375* levels are elevated in people with T2D [85].

miR-9 is an islet-specific miRNA that is an important regulator of insulin secretion [91]. *miR-9* may increase insulin secretion from β-cells by regulating expression of the nuclear protein Sirt1 *in vivo* during glucose-dependent insulin secretion [92]. Sirt1 is a nuclear NAD-dependent protein deacetylase, the expression of which is known to fluctuate in tissues such as the liver, white adipose tissue, brown adipose tissue and muscle under different metabolic conditions such as calorie restriction and starvation [93]. *miR-9* targets and regulates Sirt1 expression in insulin-secreting β-cells. Interestingly, Sirt1 levels are down-regulated during glucose-stimulated insulin secretion *in vivo* in pancreatic β-islets, consistent with *miR-9* levels being up-regulated. This targeting is relevant in diabetes as it highlights the functional interplay between insulin secretion, miRNAs and Sirt1 expression [92].

Increased *miR-29a* and *miR-29b* levels in the muscle, fat and liver of diabetic Goto–Kakizaki rats are associated with insulin resistance [94]. *miR-29a* and *miR-29b* are also highly expressed in the pancreatic islets of diabetic mice and are involved in insulin release through the modulation of Mct1 (monocarboxylate transporter 1) in the plasma membrane [95]. In pancreatic β-cells, elevated glucose concentrations stimulate mitochondrial oxidative metabolism to raise intracellular ATP/ADP levels, prompting insulin secretion. Mct1 enables circulating pyruvate/lactate to enter β-cells, where it acts as a substrate for mitochondrial oxidation, leading to an increase in the cytosolic ATP/ADP ratio. The inhibition of *miR-29a* in primary mouse islets with LNAs (locked nucleic acids) increases *Mct1* mRNA levels, demonstrating that *miR-29a* contributes to the β-cell-specific silencing of the Mct1 transporter and may thus affect insulin release [95].

miR-223 is up-regulated in insulin-resistant human hearts and is involved in glucose uptake in cardiomyocytes [96]. *miR-223* also increases cellular glucose uptake through the up-regulation of Glut4 (glucose transporter 4) protein expression. This effect is independent of PI3K (phosphoinositide 3-kinase) signalling and AMP kinase activity [96]. Overexpression of *miR-223 in vitro* also inhibits the insulin-stimulated phosphorylation of Akt and GSK3β (glycogen synthase kinase 3 β-subunit) in cardiomyocytes [96].

miR-124a is also abundant in pancreatic β-cells and is implicated in islet development, through the regulation of FOXA2 (forkhead box A2), a transcription factor important for pancreatic development and β-cell differentiation, and RAB27A, a GTPase involved in insulin secretion [97,98]. FOXA2 regulates genes involved in glucose metabolism and insulin secretion, including the ATP-sensitive K^+ (K_{ATP}) channel subunits Kir6.2 and Sur-1. Correspondingly, *miR-124a* overexpression decreases, and anti-*miR-124a* increases, *Kir6.2* and *Sur-1* mRNA levels [98]. Furthermore, *miR-124a* modified basal and glucose- or KCl-stimulated changes in intracellular free Ca^{2+} concentrations in MIN6 and INS-1 β-cells without affecting the secretion of insulin, indicating an altered sensitivity of the β-cell exocytotic machinery to Ca^{2+} [98].

miRNAs also regulate insulin resistance by modulating insulin-signalling pathways in target tissues. As the predominant action of insulin in the liver is to prevent gluconeogenesis through a cascade of phosphorylation events that terminate with diminished *PEPCK* (phosphoenolpyruvate carboxykinase) promoter activation [99], it has been demonstrated that *miR-29a* inhibits insulin actions in the liver by preventing the insulin-mediated inhibition of *PEPCK* gene expression. This effect is mediated by a direct targeting of the regulatory p85α subunit of PI3K [99]. Furthermore, *miR-29* was shown to subsequently inhibit insulin-mediated glucose import by 3T3-L1 adipocytes through mechanisms that involve indirect down-regulation of Akt activation [94].

miRNAs AND CARDIOVASCULAR DISEASES

CAD (coronary artery disease)

A recent study has shown that circulating levels of vascular- and inflammation-associated miRNAs are significantly down-regulated in patients with CAD [100]. Interestingly, most of the highly expressed miRNAs and significantly down-regulated miRNAs in the circulation of patients with CAD are expressed in ECs (endothelial cells). These include *miR-126*, members of the *miR-17~92* cluster (*miR-17*, *miR-20a* and *miR-92a*), *miR-130a*, *miR-221*, members of the *let-7* family (*let-7d*), *miR-21* and *miR-145*. *miR-155* is also significantly decreased in the plasma of patients with CAD [100]. On the other hand, cardiac muscle-enriched miRNAs, such as *miR-133a* and *miR-208a*, are increased in these patients [100]. This indicates that these miRNAs could potentially be used as biomarkers in patients with CAD.

Proliferation of VSMCs (vascular smooth muscle cells) makes a significant contribution to vascular neointimal formation. The role of miRNAs in VSMC proliferation has been extensively studied and miRNAs are key determinants of VSMC differentiation and phenotypic switching [101]. *miR-143/145* have been shown to up-regulate the VSMC proliferative response to balloon injury in rat carotid arteries through alterations in cytoskeletal dynamics and organization [102]. Therefore restoration of *miR-145* in balloon-injured arteries via an adenovirus expressing *miR-145* inhibits neointimal growth [101]. It has also been shown that *miR-143/145*-enriched vesicles from endothelial cells decrease atherosclerotic fatty lesion formation in the aortas of apoE$^{-/-}$ mice [103].

miR-21 is also associated with VSMC proliferation and vascular neointimal lesion formation and is significantly up-regulated in atherosclerotic plaques [104,105]. It has been shown that anti-sense oligonucleotide inhibition of *miR-21* reduces neointima formation following vascular balloon injury through a mechanism that involves increased levels of the pro-apoptotic proteins Bcl-2 and Pten (phosphatase and tensin homologue) [104].

miR-221/222 are highly expressed, but have opposing effects in VSMCs and ECs. Although *miR-221/222* are involved in VSMC proliferation and migration, they have anti-migratory effects in ECs [106,107]. *CDKN1B* (encoding p27^{Kip1}; cyclin-dependent kinase inhibitor 1B), *CDKN1C* (encoding p57^{Kip2}; cyclin-dependent kinase inhibitor 1C) and the proto encogene *c-Kit* are target genes of *miR-221/222* in both VSMCs and ECs; however, p27^{Kip1} and p57^{Kip2} are highly expressed in VSMCs, but not in ECs. In contrast, c-Kit is highly expressed in ECs, but not in VSMCs. The cellular effects of these target genes are also distinct. p27^{Kip1} and p57^{Kip2} have been shown to induce anti-proliferative effects on VSMCs and ECs. In contrast, c-Kit has pro-proliferative properties in these vascular cells. Therefore in VSMCs *miR-221/222* were found to have pro-proliferative and pro-migratory effects by targeting *CDKN1B* and *CDKN1C* [108]. In ECs, in contrast, *miR-221/222* were found to have anti-migratory effects by targeting c-Kit and transcription 5A, as well as endothelial and nitric oxide synthase [107]. These opposing effects of *miR-221/222* have also been observed *in vivo*, where they increase neointimal growth, but decrease re-endothelialization in a balloon injury rat carotid artery model [106].

miR-126 is another miRNA that may also exert cardioprotective effects. *miR-126*, which is enriched in apoptotic bodies, has been shown to mediate the atheroprotective effects of endothelial apoptotic bodies. *miR-126* in endothelial apoptotic bodies mediates these effects by targeting *RGS16* (regulator of G-protein 16) to induce the expression of the CXC chemokine CXCL12 through its CXCR4 (CXC receptor 4) [89]. In the context of arterial injury, CXCL12, through CXCR4, is implicated in the recruitment of progenitor cells [identified by the presence of Sca-1 (stem cell antigen-1) and the absence of lineage markers on their surface] from the bone marrow to the damaged tissue in order to compensate for apoptosis processes [109,110]. Finally, results from this study showed that *miR-126*-carrying, but not *miR-126*-deficient, apoptotic bodies conferred a protection against diet-induced atherosclerosis in the carotid artery [89].

miR-195 also plays an important role in VSMC proliferation and migration, as well as in neointimal formation [111]. *miR-195*, introduced by adenovirus, reduces neointimal formation in balloon-injured rat carotid arteries [111].

miRNAs in hypertension—The persistent elevation of systemic blood pressure, or systemic hypertension, is classified as ‘essential’ in 90–95% of cases, when there are no obvious medical causes. The remaining causes of hypertension involve various identifiable medical conditions affecting the kidneys, the heart or the endocrine system. Patients with systemic hypertension have an elevated incidence of cardiovascular disease and heart failure

[112]. Several mechanisms are involved in the pathogenesis of hypertension, including increased vascular tone, the hyperactivation of the RAAS (renin–angiotensin–aldosterone system), vascular endothelial dysfunction, VSMC and cardiac hypertrophy, and increased activity of the sympathetic nervous system; all of which are risk factors for cardiovascular diseases [113–115]. miRNAs have been shown to be involved in all these processes [116].

Expression of *miR-143* and *miR-145* is decreased in acute and chronic vascular stress [117]. Mice lacking both *miR-143* and *miR-145* have decreased blood pressure due to reduced vasoconstriction [102], impaired vasodilation and decreased medial thickness [118]. Neointima formation in response to vascular injury is profoundly impaired in mice lacking these miRNAs due to disarray of actin stress fibres and diminished migratory activity of VSMCs [102]. Indeed, the *miR-143* and *miR-145* cluster plays an important role in vascular differentiation and vascular function. Furthermore, it has been demonstrated that the *miR-143/145* cluster is required for the acquisition of the contractile phenotype for VSMCs in mice [119]. Because the *miR-143/miR-145* cluster is expressed mostly in the VSMC compartment, both during development and postnatally, the loss of *miR-143* and *miR-145* expression induces incomplete differentiation of VSMCs, leading to structural modifications of the aorta [117]. Overexpression of *miR-143* and *miR-145* on the other hand decreased neointimal formation in a rat model of acute vascular injury [117].

Although miRNAs are highly expressed in the heart, *miR-21* was shown to be the most significantly up-regulated miRNA in mouse hypertrophic hearts and is aberrantly increased in acute MI and vascular neointimal lesions [104,117,120–122]. *miR-21* is involved in cardiac hypertrophy, remodelling and fibrosis in response to pressure overload [123]. In a mouse model of pressure overload of the left ventricle, silencing of *miR-21* using a specific *miR-21* antagomir reduced cardiac ERK (extracellular-signal-regulated kinase)/MAPK (mitogen-activated protein kinase) activity, and attenuated cardiac hypertrophy, fibrosis and cardiac dysfunction [123]. However, the involvement of *miR-21* in cardiac fibrosis and hypertrophy seems to be more complex, as other studies have shown that targeted deletion of *miR-21* in mice, or inhibition of *miR-21* with 8-mer LNAs, were not sufficient to improve fibrotic lesions or hypertrophic responses to cardiac stress stimuli [124].

miR-208a is a cardiac-specific miRNA that is involved in stress-dependent cardiac growth [125]. It has been shown that the transgenic overexpression of *miR-208a* induces hypertrophic growth in mice [126]. On the other hand, therapeutic inhibition of *miR-208a* using LNA-based therapy in the Dahl hypertensive rat model improves cardiac survival and function and prevents cardiac remodelling in hypertension-induced heart failure [127].

MI and heart failure

MI induced by coronary artery occlusion is accompanied by cardiac remodelling at the site of infarction injury. This remodelling process involves fibrous tissue formation and extracellular matrix deposition. These processes are mediated by cardiac fibroblasts [128]. miRNAs were identified to play potential roles in post-MI-induced cardiac remodelling [129]. Among these miRNAs, the *miR-29* family was found to be dramatically down-regulated in the region of fibrous tissue formation in mice post-MI induced by the occlusion of the left coronary artery. The down-regulation of *miR-29* correlated with the up-regulation of *miR-29* targets which include various collagen and extracellular matrix protein genes, such as fibrillin 1, collagen type I, α1 and α2, and collagen type III α1 [129].

miR-199b is up-regulated in the hearts of animal models of cardiac hypertrophy and has been shown to play a role in heart failure and cardiac hypertrophy [130]. One major intracellular signalling pathway involved in heart failure is the activation of the pro-hypertrophic phosphatase calcineurin and its downstream transcriptional effector NFAT

(nuclear factor of activated T-cells) [131,132]. *miR-199b* promotes calcineurin/NFAT-mediated cardiac hypertrophy by active down-regulation of its direct target gene *DyrK1a* [dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1a] [130]. The *in vivo* injection of the *miR-199b* antagomir (chemically modified antisense oligonucleotide specific for *miR-199b*) prevented the development of cardiac disease in mice subjected to transverse aortic constriction pressure overload [130].

miR-499 is a cardiac abundant miRNA and is down-regulated in response to hypoxic and ischaemic stress in cardiomyocytes [133]. It has been shown that under ischaemia/reperfusion, the hearts of *miR-499* transgenic mice showed less apoptosis and reduced infarct size when compared with control mice. The cardioprotective properties of *miR-499* result from the fact that this miRNA prevents cardiomyocyte apoptosis by targeting the α and β isoforms of calcineurin [133]. Calcineurin is a serine and threonine protein phosphatase that is known to dephosphorylate pro- and anti-apoptotic factors, leading to their activation and inactivation respectively [134]. On the other hand, the knockdown of *miR-499* using a cholesterol-modified antagomir (an antisense oligonucleotide with a cholesterol moiety modification) induces myocardial apoptosis and increases the infarct size [133].

Vascular inflammation

Endothelial activation and vascular inflammation are considered to be the first steps in atherosclerotic lesion development and cardiovascular disease. It has been shown that pro-inflammatory cytokines, such as TNF-α (tumour necrosis factor-α), increase the expression of adhesion molecules in ECs, which recruit inflammatory cells such as monocytes to the site of inflammation [135,136]. Adhesion molecule expression is mainly mediated by NF-κB (nuclear factor κB) signalling pathways [137].

It has recently been shown that activation of ECs with TNF-α decreases *miR-181b* expression [138]. This study shows that the overexpression, both *in vitro* and *in vivo*, of *miR-181b* blocks the expression of adhesion molecules such as VCAM-1 (vascular adhesion molecule 1). Furthermore, the systemic administration of *miR-181b* mimetics decreases EC activation and leucocyte recruitment in LPS (lipopolysaccharide)-induced lung injury. *miR-181b* represses the nuclear translocation of NF-κB by targeting importin-α3. These results suggest that the inhibitory effects of *miR-181b* on TNF-α-induced expression of adhesion molecules are mediated by the inhibition of NF-κB nuclear translocation [138].

Previous studies have demonstrated that *miR-126* and *miR-195* are also involved in vascular inflammation. Indeed, *miR-126*, which is highly expressed in ECs, supresses VCAM-1 expression in ECs and decreases leucocyte binding to TNF-α-activated ECs [139]. *miR-195* significantly reduces the synthesis of IL (interleukin)-1β, IL-6 and IL-8 in rat VSMCs [111].

Inflammation-induced atherogenesis also involves components of the innate immune system (macrophages and dendritic cells) and of the adaptive immune system (T-lymphocytes) [140]. It has been demonstrated that miRNAs are expressed in activated B-cells, T-cells, macrophages and dendritic cells [141] and miRNA-dependent regulators of immune cells are involved in the control of vascular inflammation and atherosclerosis [142,143]. For example, *miR-155*, *miR-146a* and *miR-29a* are up-regulated in patients with CAD and *miR-125a* decreases the secretion of inflammatory cytokines such as IL-2, IL-6 and TNF-α from oxLDL-stimulated monocyte-derived macrophages [62,142].

Among the above miRNAs, previous studies have indicated that *miR-155*, a typical multi-functional miRNA, plays a crucial role in immunity, inflammation and cardiovascular disease [143]. *miR-155* is involved in the prevention of atherosclerotic lesion development

and progression, and is significantly up-regulated in both arteries and mononuclear cells in a mouse model of atherosclerosis [142]. One of the major mechanisms underlying the anti-atherogenic effects of *miR-155* is likely to be the inhibition of inflammation, which involves MAP3K10 (mitogen-activated protein kinase kinase kinase 10) [142]. *miR-155* was also shown to be up-regulated in the aorta of apoE$^{-/-}$ mice fed on a high-fat diet for 3–10 months [143,144]. *In situ* hybrizidation studies established that *miR-155* in atherosclerotic plaques was derived from macrophages and smooth muscle cells. This study also showed that the polarization of murine bone marrow derived macrophages into pro-inflammatory M1-type macrophages by stimulation with LPS and IFN-γ (interferon γ) induced *miR-155* expression [144].

miR-223 regulates progenitor proliferation as well as granulocyte differentiation and activation during inflammation [145]. A recent role for *miR-223* relates to its ability to regulate macrophage polarization. Indeed *miR-223* levels are dramatically elevated in bone marrow-derived macrophages after treatment with IL-4 to induce anti-inflammatory M2 macrophages, whereas treatment with LPS to induce M1 pro-inflammatory macrophages leads to the slight decrease in *miR-223* [146]. Although *miR-223* was initially thought to be restricted to myeloid cells regulating multiple inflammatory genes in monocytes and macrophages [145,147,148], several groups have now reported functional *miR-223* expression in non-myeloid cell types, including cardiomyocytes, hepatocytes and ECs [96,149]. Hepatic *miR-223* levels in mice are also significantly increased in post ischaemia/reperfusion injury [150]. *miR-223* was also shown to protect against diet-induced adipose tissue inflammatory response [146].

CONCLUSIONS AND FUTURE DIRECTIONS

miRNAs play a central role in the onset and development of cardiovascular disease and their discovery has ushered in an entirely new set of drug targets that can be used to identify potential novel therapeutic strategies to treat dyslipidaemia, diabetes and cardiovascular disease. Although the field is developing very rapidly, we are most likely only at the beginning of our ability to understand the complexity and full repertoire of the post-transcriptional regulation of gene expression by miRNAs. The novel finding that miRNAs are present in plasma highlights their potential use as disease biomarkers.

One of the challenges for the future use of miRNA-based therapies arises from the fact that many miRNAs modulate multiple target genes (100 or more) involved in multiple cellular processes, and the manipulation of a single miRNA can lead to therapeutic benefits, but also to pathological effects. Using miRNA-targeted therapies for the prevention and treatment of cardiovascular disease clearly warrants further genetic and pharmacological investigation. The outcomes of such studies are awaited with interest.

Abbreviations

ABC	ATP-binding cassette transporter
ANGPTL3	angiopoietin-like 3
apo	apolipoprotein
ARL7	ADP-ribosylation factor-like 7
CAD	coronary artery disease
CXCR4	CXC receptor 4
EC	endothelial cell

FOXA2	forkhead box A2
GPAM	glycerol-3-phosphate acyltransferase 1
HDL	high-density lipoprotein
HDL-C	HDL-cholesterol
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
IL	interleukin
LDL	low-density lipoprotein
LDLR	LDL receptor
LNA	locked nucleic acid
LOX-1	oxidized LDL receptor 1
LPL	lipoprotein lipase
LPS	lipopolysaccharide
LXR	liver X receptor
Mct1	monocarboxylate transporter 1
MI	myocardial infarction
miRNA	microRNA
Mtpn	myotrophin
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor κB
$p27^{Kip1}$	cyclin-dependent kinase inhibitor 1B
$p57^{Kip2}$	cyclin-dependent kinase inhibitor 1C
oxLDL	oxidized LDL
PEPCK	phosphoenolpyruvate carboxykinase
PI3K	phosphoinositide 3-kinase
PPAR	peroxisome proliferator-activated receptor
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA transcript
RISC	RNA-induced silencing complex
SIRT1	sirtuin 1
SR-BI	scavenger receptor class B, type I
SREBP	sterol-regulatory-element-binding protein
T2D	Type 2 diabetes mellitus
TAG	triacylglycerol
TNF-α	tumour necrosis factor α
VCAM-1	vascular adhesion molecule 1
VEGF	vascular endothelial growth factor

VSMC vascular smooth muscle cell

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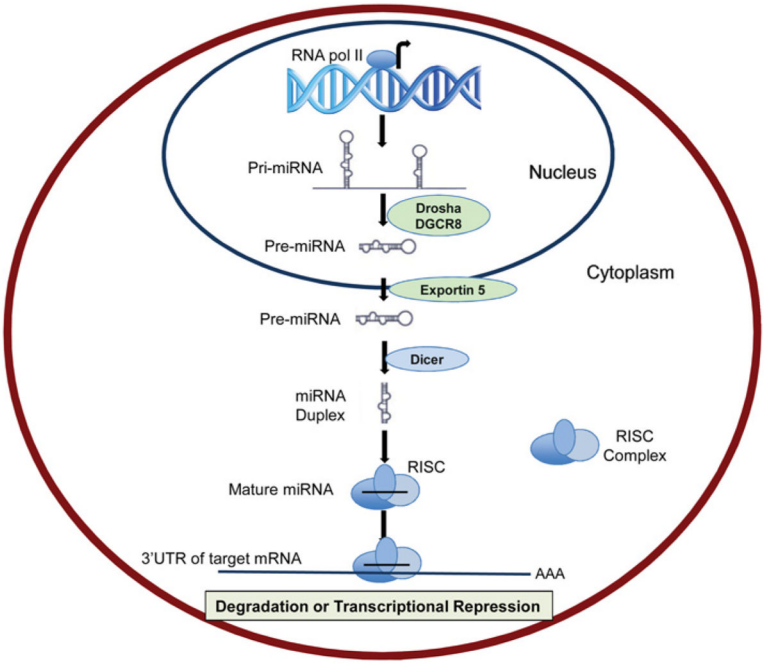

Figure 1. Schematic representation of miRNA biogenesis

miRNA biogenesis is initiated with the processing of primary miRNA transcripts in the nucleus by DROSHA/DGCR8, to generate a pre-miRNA. Pre-miRNA is exported to the cytoplasm by Exportin 5 and further processed into a miRNA duplex by Dicer. One of the single stands (mature miRNA) is incorporated into RISC and binds the 3′-UTR of the target miRNA leading to mRNA degradation or translational repression. RNA pol II, RNA polymerase II.