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Non-coding RNAs in the Vasculature

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Preface

The major parts of mammalian genomes encode for transcripts that are not further translated into proteins. Indeed, RNA-sequencing techniques have led to the discovery of thousands of noncoding transcripts with unknown function. There are several types of noncoding linear RNAs such as microRNAs (miRNA) and long noncoding RNAs (lncRNA). In addition, circular RNAs (circRNA) consisting of a closed continuous loop by back splicing are another class of noncoding RNAs which appear rather stable. Noncoding RNAs are dynamically expressed in different cell types, diseases, or developmental stages to execute a wide variety of regulatory roles at virtually every step of gene expression and translation. Importantly, noncoding RNAs are currently explored as therapeutic targets and diagnostic molecules in a wide range of diseases including cardiovascular disease. In this specific book, we give an overview about the current role of noncoding RNAs specifically in the vasculature. Of note, many authors are members of the Leducq Foundation Network MIRVAD that focuses specifically on the research of miRNAs and other noncoding RNAs in vascular cell types. The first chapter gives an overview about the role of miRNAs in diabetes and related vascular complications. This includes a wide range of topics including the role of miRNAs in microvascular-related complications of diabetic patients such as nephropathy, retinopathy, or neuropathy but also macrovascular complications such as cardiac diseases and peripheral arterial disease.

Next, Chap. 2 discusses how miRNAs can be used to improve stem cell-based regenerative therapies of cardiovascular diseases, whereas Chap. 3 reports on miRNAs, which regulate lipoprotein metabolism in the liver and reverse cholesterol transport. This is of interest as liver metabolic diseases are tightly linked to the development of vascular disease. Indeed, metabolic disease can lead to severe atherosclerosis, a topic that is next discussed in Chap. 4. Finally, Chap. 5 discusses the use of circulating miRNAs as disease biomarkers and shows interesting correlations with the amount of detectable miRNAs in the blood and future cardiovascular risks.

In conclusion, this book gives an update about exciting novel developments in vascular noncoding RNA research and the implications for future novel clinical diagnostic and therapeutic strategies of cardiovascular disease.

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Regulation of Atherosclerosis by microRNAs

1

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Abstract

Our knowledge in the role of small non-coding RNA molecules in the regulation of tissue homeostasis and disease in the cardiovascular system is steadily growing. Among this group of RNA molecules, microRNAs (miRNAs) fulfill important functions in cellular behavior of endothelial cells, vascular smooth muscle cells, and macrophages by influencing the protein output levels of a high variety of genes with crucial outcomes in the atherosclerotic setting. For example, miR-155 can intensify early stages of atherosclerosis by increasing inflammatory activation and inefficient lipid handling in macrophages. However, miRNAs display also important atheroprotective roles as demonstrated for the complementary strands of miR-126, which form a dual system sustaining the endothelial proliferative reserve and promoting endothelial regeneration to counteract atherogenic effects of disturbed flow and hyperlipidemia.

Excitingly, miRNA functions are not restricted to the producing cells but can be transferred to other cells by secretion and transport within extracellular vesicles including exosomes and microvesicles. Moreover, circulating miRNAs are found in the blood stream stabilized by complex formation with lipoproteins and ribonucleoprotein complexes. Once incorporated by the recipient cells, these extracellular miRNAs regulate target mRNAs thereby acting as a new gadget in cell-cell communication also in atherosclerosis. This chapter provides novel concepts on regulatory mechanisms of miRNAs including the function of RNA sponges, miRNA tandems, and the complementary role of miRNA strand pairs and discusses their diagnostic and therapeutic potential in atherosclerosis.

1.1 Introduction

Atherosclerosis is propitiated by a lipid-induced chronic inflammation of the vessel wall. This process is orchestrated by a complex interplay of various cell types, such as endothelial cells (ECs), smooth muscle cells (SMCs), and macrophages. Atherosclerotic lesions preferentially develop at branching points of large arterial tree and the inner curvature of the aortic arch. At these so-called predilection sites, the ECs are constantly exposed to a disturbed blood flow that results in cell dysfunction by upregulating inflammatory genes such as adhesion molecules and chemokines (Nam et al. 2009; Weber and Noels 2011). Conversely, a stable or laminar flow exerts anti-inflammatory effects and protects from artery wall thickening by promoting the expression of atheroprotective genes such as *Kruppel-like factor 2 (Klf2)*, *Klf4*, and *eNOS* (Dekker et al. 2006). Under the influence of these hemodynamic forces, miRNAs serve as direct mediators of blood flow sensorial mechanisms by interfering with the inflammatory cascade through regulation of signaling pathways or directly modulating the expression of genes implicated in atherosclerosis.

Since 1993 where miRNAs emerged as key regulators of gene expression typically by repressing the target mRNA, the number of publications addressing the

interplay of these tiny molecules in regulation has been steadily growing (Lee et al. 1993). Here we outline the most recent concepts on mechanism of action of miRNAs.

1.2 miRNA-Mediated Regulatory Mechanisms

miRNAs have been demonstrated to regulate a variety of cellular mechanisms as inflammation, cell regeneration, or lipid metabolism, thereby contributing in different manner to the progression of atherosclerosis (Rayner et al. 2012). The canonical miRNA biogenesis starts with nuclear processing of primary miRNA transcripts by the Drosha complex into precursor miRNAs (Ha and Kim 2014). These precursors are then transported to the cytoplasm which are further processed by the RNase III enzyme Dicer yielding miRNA-miRNA* heteroduplexes (Ha and Kim 2014). The miRNA duplex is loaded into the pre-RISC and undergoes strand selection. In the mature RISC, the selected miRNA strand binds to mRNA targets triggering their translation inhibition or degradation (Fig. 1.1) (Ha and Kim 2014). In addition, miRNAs can be generated by noncanonical pathways independent of Drosha or Dicer activities, like miR-320 or miR-451 (Ha and Kim 2014). miRNAs target mRNAs primarily by pairing of miRNA seed sequences (i.e., the nucleotides 2–8 at the 5′ end) and the miRNA response elements (MRE) within RNAs. Although MREs are frequently present in the 3′ untranslated region (UTR) of target RNA, several miRNAs can bind to their targets by pairing to their coding regions or 5'UTRs (Helwak et al. 2013). These short seed match and incomplete base pairing between miRNA and mRNA enables a single miRNA to target up to hundreds of different RNA molecules (Friedman et al. 2009). Although miRNAs typically target mRNAs, a wide range of RNA molecules including pseudogenes, long-intergenic non-coding RNAs, ribosomal RNAs, transfer RNAs, small nuclear RNAs, and also other miRNAs has been demonstrated to be targeted by miRNAs (Helwak et al. 2013). These miRNA-mediated effects can be regulated by various factors such as abundance of miRNAs or of their targets, differential expression of miRNAs, and relative abundance of target sites (i.e., the relative number of sites within the transcriptome for an individual miRNA) (Arvey et al. 2010; Garcia et al. 2011). Recent studies have confirmed the relevance of novel miRNA mechanisms in atherosclerosis as, for instance, the role of coexisting miRNAs strands of a single miRNA precursor, the modulation by miRNAs sponges, and the paracrine effects of miRNAs in circulation (Fig. 1.1).

1.2.1 Regulatory Mechanisms of miRNA Strands Derived from a Single Precursor

One of the doctrines in the biogenesis of miRNAs claims that for each miRNA, only one strand, the guide strand (miRNA), is assembled into the active RISC; the other strand, called passenger or antisense in terms of relative polarity (miRNA*), is

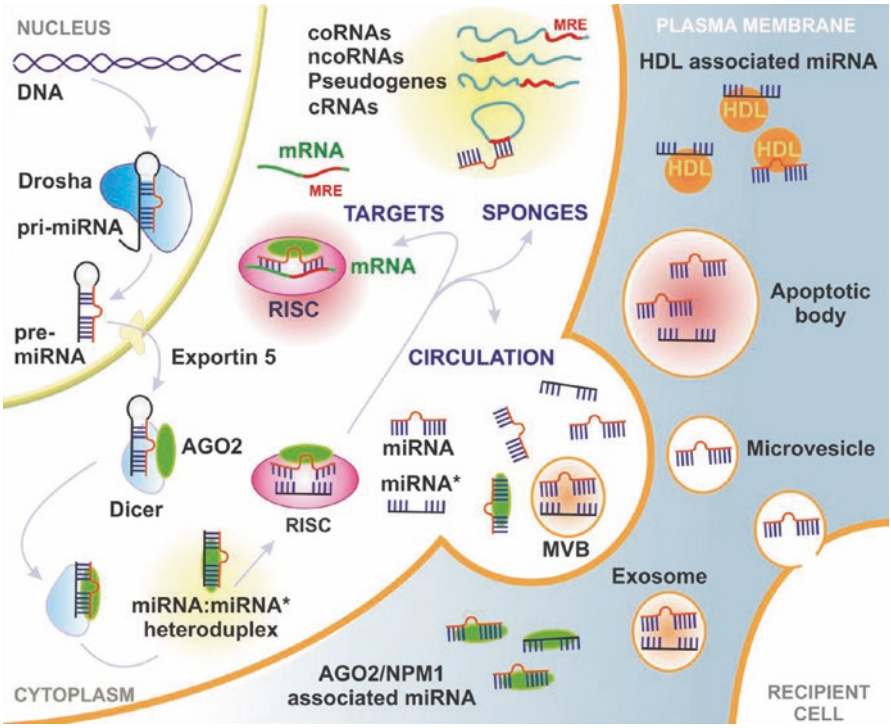

Fig. 1.1 Model of biogenesis and regulatory mechanisms of mature miRNAs. In the nucleus, miRNAs are transcribed from DNA and further processed by Drosha to a precursor hairpin miRNA (pre-miRNA). After being transported into the cytoplasm by a Exportin 5-dependent mechanism, the precursor miRNA is further cleaved by Dicer into mature miRNA heteroduplexes (miRNA-miRNA*). The processed miRNA strands can guide RISC with help of AGO2 to mRNA targets for translation inhibition, be inhibited in function by binding to the MREs of miRNA sponges or be extracellular transported into circulation. Cytoplasmic miRNAs can exit the cell enclosed within membranous vesicles as exosomes (generated from the fusion of MVB with the plasma membrane), microvesicles, or apoptotic bodies. miRNAs are released also vesicle-free associated with lipoproteins, such as HDL or RNA-binding proteins, such as AGO2 or NPM1. Once in circulation miRNAs are remarkably stable and regulate functions of the recipient cells. *RISC* RNA-induced silencing complex, *AGO2* argonaute protein 2, *MVB* multivesicular body, *NPM1* nucleophosmin1, *coRNAs* coding RNAs, *ncoRNAs* non-coding RNAs, *cRNAs* circular RNAs, *HDL* high-density lipoprotein

destroyed (Matranga et al. 2005). However, in contrast to previous beliefs, passenger strands may not always be cleaved but remain functional in translational processes thereby triggering synergistic or opposite cellular mechanisms as compared to that of the guide strand (Fig. 1.1) (Czech et al. 2009; Marco et al. 2012; Sakurai et al. 2011). Interestingly, coexisting miRNA-miRNA* pairs can either target the same mRNA molecule as described for miR-126, miR-582, and miR-17 or bind to different mRNAs as described for miR-28 (Zhang et al. 2013; Uchino et al. 2013; Yang et al. 2013; Almeida et al. 2012). Remarkably, miRNA-miRNA* pairs may

play different roles in specific cell types within a tissue but ultimately may mediate the similar effects under disease condition (Thum et al. 2008; Bang et al. 2014). For instance, miR-21-5p is expressed and upregulated in cardiac fibroblasts of the failing heart and activates ERK-MAP kinase signaling, thus contributing to fibrosis, hypertrophy, and cardiac dysfunction (Thum et al. 2008). On the other hand, the guide strand miR-21-3p is increased in pericardial fluid of mice with left ventricular pressure overload-induced hypertrophy after aortic constriction (Bang et al. 2014); it is packed in fibroblast-derived exosomes and delivered to cardiomyocytes, where it mediates their hypertrophy (Bang et al. 2014). Thus, the combined regulatory roles of miRNA pairs should be taken into account in miRNA functional studies.

1.2.2 RNA Sponges in the Regulation of miRNA Activity

The hypothesis of competing endogenous RNA (ceRNA) molecules, better known as RNA sponges, represents a novel regulatory dimension in translational biology. ceRNAs function as sponges that sequester miRNAs to influence the expression level of other transcripts sharing common miRNA response elements (MREs). RNA molecules of different fates such as non-coding RNAs, circular RNAs, and pseudogenes can compete for miRNA binding due to the presence of similar MREs (Fig. 1.1) (Salmena et al. 2011; Tay et al. 2014). Under particular conditions, RNA molecules with a large number of MREs, such as circular RNA (CDR1as) containing several miR-7 seed matches, can act as a miRNA sponge by efficiently binding and thereby eliminating specific miRNAs from their defined targets (Hansen et al. 2013). In addition, non-coding pseudogenes, such as PTENP1, which contain similar MREs as their ancestral gene (PTEN) is capable to bind and compete for the same miRNAs (Poliseno et al. 2010). Basically, the ceRNA mechanism may only be relevant for miRNAs that target two or more RNA molecules because miRNAs that bind to a single target mRNA are not regulated by ceRNAs (Helwak et al. 2013).

The sponge mechanism of ceRNAs is modulated by various parameters including stability of miRNA expression, large changes in ceRNA expression, accessibility of ceRNA influenced by cellular localization or interaction with RNA-binding proteins, and the efficiency of miRNAs binding to each MRE (Salmena et al. 2011). However, the ceRNA model may not be relevant for all miRNAs and differ between cell types. For instance, the manipulation of miR-122 target abundance in the liver in a physiological and disease setting did not significantly affect gene expression and metabolism in the tissue (Denzler et al. 2014).

1.2.3 Circulating miRNAs: Messengers on the Move

miRNAs are not intracellularly confined; indeed, they are remarkably stable in the extracellular space being detected in various physiological fluids of the human body, such as plasma and breast milk (Mitchell et al. 2008; Kosaka et al. 2010a; Weber et al. 2010). Variation on the miRNA profile in circulation has been described

in numerous diseases, including cardiovascular diseases and cancer (Etheridge et al. 2011; Fichtlscherer et al. 2011; Creemers et al. 2012). miRNAs even in an unprocessed form do exist in the cell enclosed in vesicles such as microvesicles, exosomes, or apoptotic bodies or are released building a complex with other molecules such as Argonaute proteins or nucleophosmin1 (NPM1) or associated with high-density lipoproteins (HDL) (Fig. 1.1) (Kosaka et al. 2010b; Vickers and Remaley 2012; Arroyo et al. 2011a). Arroyo et al. have demonstrated that vesicle-associated miRNAs represent a minority in the circulation, whereas the majority of miRNAs are present in a non-membrane-bound form (Arroyo et al. 2011b). Some miRNAs can become enriched in secretory vesicles, such as exosomes and apoptotic bodies, through sequence-specific interactions with RNA-binding proteins that regulate the loading into exosomes (Villarroya-Beltri et al. 2013; Zernecke et al. 2009). Similar to secreted proteins, miRNAs released from cells can mediate communication with remote cells. For instance, the atheroprotective laminar flow increases Klf2 levels triggering the release of miR-143 and miR-145 from ECs in microvesicles, which are taken up by neighboring smooth muscle cells (SMC) mediated thereby a beneficial contractile SMC phenotype (Hergenreider et al. 2012).

1.3 Regulation of Inflammatory Macrophage Activation by a miRNA Tandem

Inflammatory monocytes are recruited to the inflamed endothelium of the artery wall where they differentiate into macrophages. By the ingestion of modified low-density lipoproteins (LDLs), macrophages produce free cholesterol that is further exported to the liver in HDL form (Cuchel and Rader 2006). However, excessive lipid accumulation leads to a chronic, unresolved inflammatory response of macrophages that exacerbate atherosclerosis.

Macrophages undergo specific differentiation depending on the local tissue environment. Two distinct states of polarized activation of macrophages have been defined: the classically activated (M1) macrophage phenotype and the alternatively (M2) macrophage phenotype. Both phenotypes display different roles, and whereas activated M1 macrophages act as effector cells during the immune response, M2-activated macrophages are rather involved in immunosuppression. Several M1-M2 intermediates have been described and identified in atherosclerosis (Wolfs et al. 2011; Murray et al. 2014; Locati et al. 2013). Inflammatory activation of macrophages induces signaling cascades such as TLR/Myd88 and NF-κβ, transcription factors, inflammatory mediators, and miRNAs (Tedgui and Mallat 2006). During this M1-type activation, numerous miRNAs are downregulated, whereas a small set of miRNAs are upregulated such as miR-147 and miR-155 (Curtale et al. 2013; Ma et al. 2011; O'Connell et al. 2010; Nazari-Jahantigh et al. 2012a; Graff et al. 2012).

miR-155 is encoded by exon 3 of B-cell receptor inducible (BIC) gene and controls several aspects of macrophage function such as inflammatory response, lipid uptake, and apoptosis (Nazari-Jahantigh et al. 2012a; Koch et al. 2012). miR-155 expression is reduced during macrophage differentiation while it is strongly induced

upon M1 polarization through MyD88- and TRIF-dependent signaling cascades (Nazari-Jahantigh et al. 2012a; O'Connell et al. 2007). In addition, LDL and mildly oxidized LDL can induce the expression of miR-155 in macrophages (Nazari-Jahantigh et al. 2012a). miR-155 expression is negatively regulated in macrophages by the transcription factors Ets2 and YY1 (Quinn et al. 2014; Tian et al. 2014) and by the protein kinase AKT1, which is activated by TLR signaling (Androulidaki et al. 2009). PI3K-AKT signaling limits the expression of inflammatory mediators in macrophages, and the suppression of AKT1 promotes the inflammatory M1-type phenotype by upregulation of miR-155 (Guha and Mackman 2002; Arranz et al. 2012; Androulidaki et al. 2009).

Beyond that, miR-155 can also be indirectly regulated by other miRNA, and so miR-342-5p can increase the expression of miR-155 in macrophages by targeting AKT1 thereby triggering the expression of pro-inflammatory mediators such as NO, TNFα, and IL6 (Wei et al. 2013a). Although the expression of miR-342-5p is not regulated during TLR4-mediated macrophage activation, its effect on AKT1 expression is only detectable in inflammatory macrophages (Wei et al. 2013a). The effects of miR-342-5p on AKT1 levels are based on the relative abundance of two of its targets, Bmpr2 and AKT1 (Wei et al. 2013a). In unstimulated macrophages, miR-342-5p preferentially targets Bmpr2 that is highly expressed and which appears to have a more efficient binding site for miR-342-5p than AKT1 (Wei et al. 2013a). During inflammatory activation, however, the levels of Bmpr2 are downregulated and due to the lack of its preferential target miR-342-5p switch to target also AKT1 expression leading finally to an increase of miR-155 and subsequently high expression of inflammatory mediators (Fig. 1.2) (Wei et al. 2013a). The expression of Bmpr2 mRNA in unstimulated macrophages inhibits their inflammatory activation by preventing the targeting of AKT1 by miR-342-5p. This represents a beautiful example of ceRNA-based regulatory system controlling macrophage polarization and also illustrates how two miRNAs in tandem, miR-342-5p and miR-155, orchestrate the inflammatory macrophage activation.

The process by which miR-155 upregulates the expression of the pro-inflammatory mediators, like the chemokine Ccl2, in macrophages is by targeting the transcriptional repressor Bcl6. Bcl6 is upregulated during macrophage differentiation by the activation of NF-κB signaling, and counter-regulates the NF-κβ-mediated transcriptional upregulation of many inflammatory genes. In addition to Bcl6, miR-155 can target other transcripts, such as Socs1 and Sfpi1, which can contribute to the regulation of macrophage inflammatory responses (Nazari-Jahantigh et al. 2012b). However, in contrast to Bcl6, Socs1 and Sfpil1 are not involved in the inflammatory activation of macrophages mediated by miR-155, and their functional role is not fully understood (Nazari-Jahantigh et al. 2012a). Whether the different miR-155 targets act as ceRNAs and thus regulate the targeting of Bcl6 by miR-155 is currently unclear.

The current in vitro findings on the miR-155-mediated regulatory mechanisms in macrophages have been validated also in models of atherosclerosis in vivo. miR-155 expression is upregulated in mouse and human atherosclerotic lesions and is increased in $CD14^+$ monocytes from patient with coronary heart disease

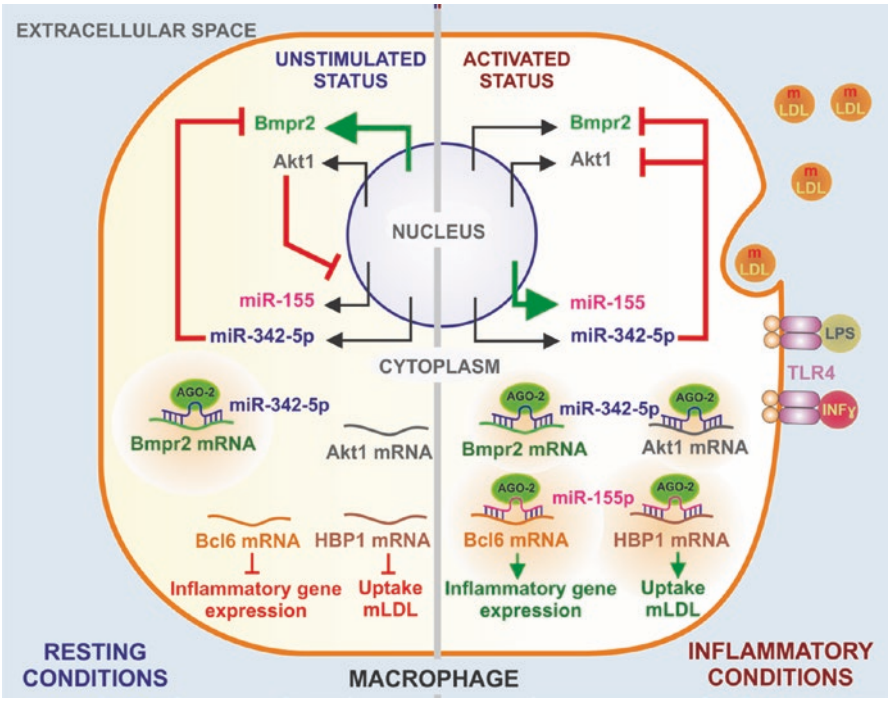

Fig. 1.2 Tandem mechanism of miR-342-5p and miR-155 in inflammatory macrophage activation. Under resting conditions (left side of the illustration), AKT1 and Bmpr2 compete for the binding to miR-342-5p in macrophages. The preferential binding of miR-342-5p to Bmpr2 facilitates basal translation of AKT1, which inhibits the expression of miR-155. The lack of miR-155 enables the expression of Bcl6 and HBP1 thereby impairing the production of inflammatory factors and the uptake of modified LDL (mLDL). Under inflammatory conditions (right side of the illustration), the transcription level of Bmpr2 is downregulated increasing the availability of miR-342-5p for the binding to AKT1 mRNA. This suppression of AKT1 results in upregulation of miR-155, which targets Bcl6 and HBP1 unleashing inflammatory gene expression and mLDL uptake

(Nazari-Jahantigh et al. 2012a; Du et al. 2014). The increased expression of the prototypical M1 macrophage miRNA, miR-155, indicates that primarily M1-type macrophages contribute to the coronary heart diseases and atherosclerosis (Nazari-Jahantigh et al. 2012a). In contrast to vascular cells, miR-155 deficiency in macrophages attenuates atherosclerosis due to the increased Bcl6 expression, which impairs Ccl2 production and thereby limits the lesional macrophage accumulation (Nazari-Jahantigh et al. 2012a; Aiello et al. 1999; Wei et al. 2013c). In line with this data, miR-155 deficiency in hematopoietic cells and whole body attenuates atherosclerosis by an increment of Bcl6 and SOCS1 levels in lesional macrophages, subsequently leading to a reduction of inflammatory genes including Ccl2, TNFα, IL6, and IL1b (Du et al. 2014). Therefore, overexpression of miR-155 in inflammatory macrophages within atherosclerotic lesions amplifies the vascular inflammation exacerbating the progress of atherosclerosis (Nazari-Jahantigh et al. 2012a; Du

et al. 2014). Notably, miR-342-5p levels are also increased in mouse atherosclerotic lesions after 3 and 10 months of high-fat diet feeding (Wei et al. 2013a). miR-342-5p suppression attenuates atherosclerosis in Apoe$^{-/-}$ mice (Wei et al. 2013a). Inhibition of miR-342-5p enhances the AKT1 expression in lesional macrophages, which presumably results in reduced atherosclerosis by downregulating miR-155 expression (Wei et al. 2013a). This indicates that miR-342-5p and miR-155 regulatory tandem in inflammatory macrophages indeed exacerbate atherosclerosis.

Conversely, in hematopoietic cells, miR-155 deficiency increases atherosclerosis in LDLR$^{-/-}$ mice without significant change in lesional macrophage content (Donners et al. 2012). This discrepancy could be due to distinct expression levels of miR-155 targets at the different stages of atherosclerosis and possible switching of the targets in macrophages during the development of lesions. Therefore, miR-155 probably mediates opposing effects at different stages of atherosclerosis by switching the target transcript.

Besides its role in inflammatory activation, miR-155 regulates additional macrophage functions as, for instance, the lipid uptake. By the targeting of HMG box-transcription protein1 (HBP1), a negative regulator of MIF, miR-155, increases the uptake of oxLDL by macrophages (Tian et al. 2014). A systemic delivery of antagomirs of miR-155 in Apoe$^{-/-}$ mice thereby reduces the expression of HBP1 and decreases atherosclerosis presumably by limiting not only the expression of IL6 and TNFα but also the lesional macrophage lipid accumulation (Tian et al. 2014). Similarly, miR-155 deficiency in macrophages reduced the formation of macrophage-derived foam cells in atherosclerotic lesions (Nazari-Jahantigh et al. 2012a). These data illustrates the crucial role of miR-155 at the interface between inflammatory activation and lipid handling in macrophages.

Taken together, the regulatory tandem of miR-342-5p and miR-155 enhances the inflammatory activation of macrophages in atherosclerosis. The functional studies clearly demonstrate that Bcl6 is targeted by miR-155 mediating an enhanced inflammatory response and thereby increase in lesional macrophage accumulation exacerbating the progress of atherosclerosis. However, the functional role of other miR-155 targets such as Socs1 in atherosclerosis remains to be further determined. Finally, the ceRNA-based regulation of miR-342-5p activity thereby modulating the miR-155-mediated macrophage inflammatory response beautifully illustrates a safeguard mechanism to avoid inappropriate activation of macrophages.

1.4 Combined Roles of miR-126-3p and miR-126-5p in Endothelial Regeneration and Their Use in Therapeutic Approaches

miR-126, the highest miRNA expressed in ECs, is encoded by exon 7 of the *Egfl7* gene (Fish et al. 2008; Wang et al. 2008). Out of the miR-126 precursor, two mature miRNA strands are generated, miR-126-3p (guide strand) and miR-126-5p (passenger strand). The key role of the miR-126-3p in the vasculature was highlighted in studies with genetic deletion or inhibition of this strand. miR-126-3p regulates

vascular integrity and angiogenesis by targeting Spred-1 and PIK3R2 as determined in mouse and zebrafish, respectively (Wang et al. 2008; Fish et al. 2008). In addition, miR-126-3p regulates vascular inflammation by targeting VCAM1 in ECs (Harris et al. 2008). Notably, the expression of miR-126-5p is upregulated in vascular precursor cells and ECs during embryogenesis, and its expression can be even higher than the expression level of miR-126-3p (Fish et al. 2008; Neth et al. 2013). Moreover, miR-126-5p expression levels are comparable to that of miR-126-3p in various adult mouse tissues (Schober et al. 2014). This data limelights that both mature miRNA strands generated from miR-126 are functional in endothelial cells.

Indeed, miR-126-5p plays a key function in EC proliferation improving reendothelialization after vascular injury by targeting Delta-like 1 homolog (DLK1) (Schober et al. 2014). DLK1 is a noncanonical inhibitor of NOTCH1 highly expressed in various tissues during development and is downregulated in most tissues after birth (Rodriguez et al. 2012; Baladron et al. 2005; Falix et al. 2012). Interestingly, DLK1 is upregulated following vascular injury and counter-regulates increased EC proliferation during endothelial repair probably by targeting the NOTCH1 receptor (Rodriguez et al. 2012; Schober et al. 2014). These effects in the modulation of reendothelialization in injured arteries are exclusive of miR-126-5p indicating functional specialization of the individual miR-126 strands (Schober et al. 2014). The guide strand miR-126-3p supports endothelial recovery promoting the angiogenic growth factor signaling through targeting of Spred1 (Jansen et al. 2013).

Endothelial proliferation is rare in the adult vasculature; however, increased EC turnover occurs at predilection sites of atherosclerosis in response to increased levels of stress-induced EC death and detachment (Bartling et al. 2000; Zeng et al. 2009; Foteinos et al. 2008). Notably, miR-126-5p, but not miR-126-3p, is downregulated at predilection sites characterized by a disturbed flow, probably due to differences in the stability and processing of the individual miR-126 strands (Schober et al. 2014). The reduced miR-126-5p levels at predilection sites upregulate DLK1 expression, thereby controlling and limiting EC proliferation (Schober et al. 2014). Laminar blood flow not only protects EC functions and limits EC turnover but also provides a higher proliferative capacity by inducing miR-126-5p expression at non-predilection sites (Schober et al. 2014).

The prevalence of endothelial ER stress and enhanced EC apoptosis are characteristics of predilection sites (Zeng et al. 2009; Civelek et al. 2009). During atherosclerosis, modified LDL induces EC apoptosis by activating ER stress via LOX-1 receptor (Hong et al. 2014). Under this stress conditions, resident ECs surrounding the damaged regions proliferate in order to heal the damaged part (Itoh et al. 2010). Modified LDL has been shown to inhibit EC replication in vitro and hyperlipidemia significantly reduces EC proliferation at predilection sites in vivo (Schober et al. 2014; Chen et al. 2000). Under these hyperlipidemic conditions, the DLK1-mediated antiproliferative response becomes detrimental at the predilection sites and results in impaired EC regeneration. At the non-predilection sites, however, the high levels of miR-126-5p suppress DLK1 expression thereby enabling EC regeneration in

response to hyperlipidemia (Schober et al. 2014). Consequently, a replenishment of the miR-126-5p pool by an exogenous therapy with mimics under hyperlipidemic condition improves EC proliferation at predilection sites and limits atherogenesis (Schober et al. 2014). Hence, the miR-126-5p strand plays an atheroprotective role in response to hyperlipidemic stress by maintaining the endothelial proliferative reserve through targeting DLK1 (Schober et al. 2014). Similarly, the restoration of other miRNAs have been shown to also be an adequate strategy in atherosclerosis. Hyperlipidemia downregulates also miR-181b crucial for the inhibition of the NF-κβ signaling in endothelial cells; thus, using exogenous miR-181b helps to reduce atherosclerosis (Loyer et al. 2014; Sun et al. 2014). Conversely, sometimes the inhibition of miRNAs is beneficial. During the atheroprotective laminar flow, the levels of miR-92a are decreased, which in turn increases KLF2 expression to maintain endothelial homeostasis (Loyer et al. 2014; Sun et al. 2014). Indeed, large-scale miRNA profiling in endothelial cells identified miR-92a as a candidate, which is preferentially upregulated by the combination of disturbed flow and oxidized LDL through STAT3, as confirmed in atheroprone regions and mouse models (Loyer et al. 2014; Sun et al. 2014). Specific in vivo blockade of miR-92a expression targeting Socs5 reduced endothelial inflammation, decreased atherosclerotic plaque size, and promoted a more stable lesion phenotype. Hence, miR-92a antagomirs may serve as another atheroprotective therapeutic strategy.

Besides its functions in EC proliferation, miR-126-5p promotes also leukocyte adhesion by targeting SetD5 and represses transendothelial migration by degrading ALCAM in retinal and pulmonary ECs, respectively (Poissonnier et al. 2014). However, it seems that this is not involved in atherosclerosis as miR-126 mature strands inhibit the recruitment of inflammatory monocytes to the tumor microenvironment (Zhang et al. 2013). Thus, the miR-126-5p strand can regulate crucial cell functions such as proliferation and inflammation depending on the transcripts targeted and the tissue where it is present.

Both miR-126 mature strands, miR-126-5p and miR126-3p, act synergistically for the preservation of vasculature. Whereas miR-126-5p protective activities are principally intracelullarly mediated, miR-126-3p needs to be release for its atheroprotective activities. miR-126-3p is delivered to vascular cells via endothelial-derived apoptotic bodies, reducing atherosclerosis through the repression of RGS16 that leads to an upregulation of CXCL12 expression (Zernecke et al. 2009). The chemokine CXCL12 not only triggers the recruitment of angiogenic progenitor cells to the atherosclerotic sites but also increases its own mRNA in an autoregulatory loop thereby also supporting endothelial function (Akhtar et al. 2013; Zernecke et al. 2009; Kuhlmann et al. 2005; Wei et al. 2013b). This system of atheroprotection appears to be disrupted upon disease, and so circulating levels of miR-126-3p has been detected to be substantially reduced in patients with coronary artery disease or insulin resistance/diabetes (Fichtlscherer et al. 2010; Zampetaki et al. 2010).

In addition to the vesicle-mediated transfer, miR-126-3p is transmitted by Ago2 protein from ECs to SMCs, where it targets Foxo3, Bcl2, and Irs1 thereby

increasing SMC turnover (Zhou et al. 2013). These data indicate that miR-126-3p is mainly transferred to the vascular cells and improves cellular regeneration while miR-126-5p stays within ECs and controls endothelial regeneration. Understanding the functional roles of miR-126 mature strands in regulation of endothelial cell function provides new insights on complimentary regulatory mechanisms mediated by miRNA pairs in atherosclerosis (Fig. 1.3).

However, the mechanisms determining differential stability and trafficking of sister strands are not fully understood. It needs to be further studied how one strand of a miRNA duplex can be tagged as export good preferentially leaving the cell, whereas the antisense strand is rather maintained in the cell.

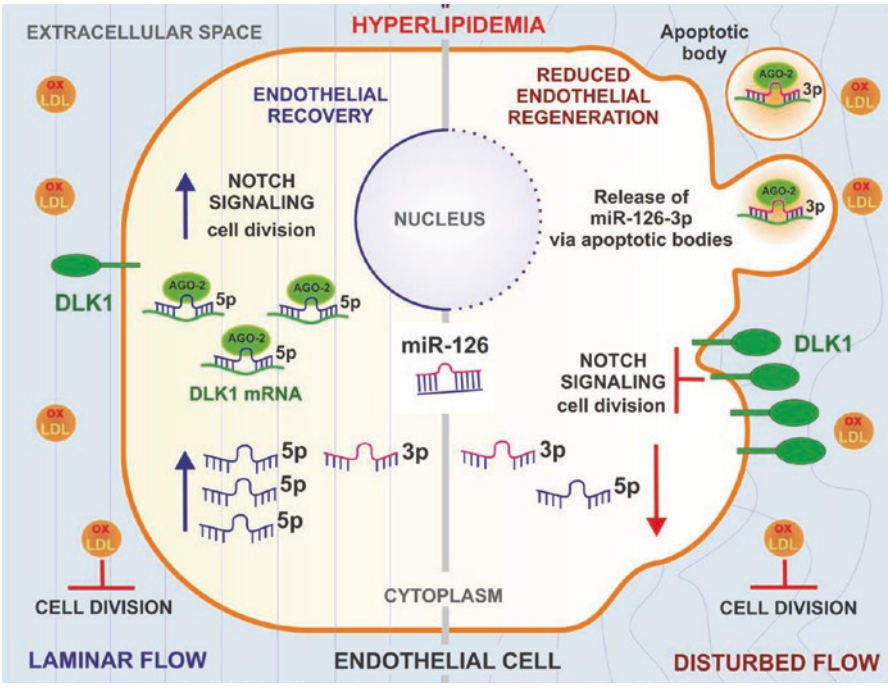

Fig. 1.3 Complementary role of miR-126-3p/5p pair on vascular EC homeostasis during atherosclerosis. miR-126-5p levels are sensitive to blood flow and regulate the proliferative capacity of endothelial cells through degradation of DLK1 a noncanonical inhibitor of NOTCH1, subsequently improving cell regeneration. This mechanism becomes detrimental under hyperlipidemic conditions where oxidized LDL strongly impairs endothelial cell (EC) proliferation. At the regions of laminar flow (left side of the illustration), the high levels of miR-126-5p are still capable to maintain EC regenerative potential, whereas the low levels of miR-126-5p at the predilection sites (right side of the illustration) are not sufficient to counteract this process. As a last atheroprotective effort, the apoptotic ECs under disturbed flow and hyperlipidemic conditions release miR-126-3p via apoptotic bodies to neighboring cells which may support EC regeneration at predilection sites

1.5 Lipoprotein-Mediated miRNA Transfer

Lipoproteins are complex aggregates of lipids and proteins that render lipids suitable for transport in circulation between tissues. Recently, it has been shown that HDL, besides acting as a cellular cholesterol carrier, is also functioning as a miRNA transporter (Fig. 1.1) (Vickers and Remaley 2012; Wagner et al. 2013). Wagner et al. demonstrated circulating miRNAs to be bound to HDL and also, but in a lower amount, to LDL fractions from plasma of healthy subjects as well as in plasma of patients with stable coronary artery disease (CAD) or acute coronary syndromes (ACS) (Wagner et al. 2013). The miRNA levels between patients and healthy subjects were not significantly different. In vitro, miRNAs bound to HDL were not taken up by ECs, SMCs, and peripheral blood mononuclear cells (PBMCs), yet patient-derived HDL transiently modulated miRNA expression in SMCs and PBMCs. Endogenous expression of miR-92a, miR-126, and miR-223 in PBMCs was increased after longer incubation with HDL derived from patients with stable CAD or ACS, in contrast to the effect of HDL from healthy subjects in which the expression of these miRNAs was reduced (Wagner et al. 2013). These results are in line with previous studies showing distinct biological functions for HDL derived of CAD patients versus healthy subjects in several cellular models (Besler et al. 2011). Interestingly, short incubation times of 1 h reduced the expression of miR-126 as a consequence of lower transcription or destabilization of the unprocessed form pri-miR-126 (Wagner et al. 2013). Thus, lipoprotein-bound miRNAs appeared to be not efficiently delivered to endothelial, smooth muscle, and peripheral blood mononuclear cells suggesting that this pool of miRNAs is not well suited to regulate the function of cells in vitro.

In earlier studies, Vickers et al. did not only describe a singular profiling for miRNA-HDL complexes but also revealed a specific signature in patients with familial hypercholesterolemia. The mechanisms by which miRNAs bind to HDL are still not clarified; what it is known is that only mature miRNAs are present and bound through divalent cation bridges (Janas and Yarus 2006; Vickers and Remaley 2012). HDL-miRNA complexes include miR-22, miR-105, and miR-106a being miR-223 the most abundant miRNA. In human familiar hypercholesterolemia, miR-223 is dramatically upregulated; however, it could not be validated as a possible biomarker in circulation for atherosclerosis (Vickers and Remaley 2012; Fichtlscherer et al. 2010). It remains unclear how this miRNAs can display biological functions, as only unprocessed forms of miRNAs are able to bind to the RNA-induced silencing complex. Nevertheless, Vickers et al. demonstrated that indeed HDL is able to transport miR-105 to hepatocytes for altering gene expression (Vickers and Remaley 2012). It was further shown that miR-223 derived from HDL can indeed be transferred to ECs, where it directly targets ICAM-1 as an example of an extracellular miRNA regulating gene expression in cells where it is not transcribed (Wagner et al. 2013). These data together suggest that anti-inflammatory properties of HDL are in part conferred through miR-223 delivery and translational repression of ICAM-1 in ECs. Even though the contribution of

HDL-miRNAs to the total miRNA-pool appears to be rather low, the finding of miRNAs in lipoprotein fractions may represent a new system of intercellular communications.

In conclusion, the identification of this high variety of miRNAs and their carriers in circulation challenges a new generation of potential biomarkers and therapeutic approaches. In this fashion, circulating miRNAs associated with their lipid-based carriers have already emerged as important mediators of novel treatment strategies. Specific miRNAs or miRNA inhibitors combined with particular lipoproteins or packed into vesicles after application into blood circulation may alter the extracellular miRNA signature and thus gain a therapeutic advantage. In atherogenesis, several studies have already demonstrated beneficial and protective effects by delivery of selected miRNAs linked to lipoproteins or engulfed by natural vesicles (Hergenreider et al. 2012; Zernecke et al. 2009).

1.6 Conclusions and Future Work

The discovery of miRNAs has significantly widened our understanding of the regulatory mechanisms governing gene networks in many biological processes, including disease. The therapeutic potential of these tiny gene regulators renders them as attractive targets for researchers, as they may modulate cell responses to atherogenic stimuli, which give rise to chronic unresolved vascular stress. Identifying atherosclerotic miRNAs (atheromiRs) involved in the regulation of vascular stress responses would be of high value for future therapeutic options. The complexity of these atheromiRs with different roles in distinct cell types or distinct effects depending on the specific stage of the disease represent a big adversity in terms of therapeutic expectations. Therefore, major efforts should be invested to better understand the nature of these atheromiRs. The regulatory system of miRNA-miRNA* strand pairs should be further clarified to understand, for example, how interfering with one miRNA strand would affect the regulatory system of the whole miRNA-miRNA* pair. The understanding on the paracrine effects of miRNAs is now being expanded, and future studies should be focused to understand how miRNAs are being tagged for extracellular release and in particular which are the mechanisms determining their vesicle or vesicle-free release. Furthermore, it would be necessary to understand, by which mechanisms the recipient cells are recruiting these miRNAs. A clarification of these processes together would strongly help to design strategies for clinical applications.

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References

Aiello RJ, Bourassa PA, Lindsey S, Weng W, Natoli E, Rollins BJ, Milos PM. Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol. 1999;19(6):1518–25.

Akhtar S, Gremse F, Kiessling F, Weber C, Schober A. CXCL12 promotes the stabilization of atherosclerotic lesions mediated by smooth muscle progenitor cells in Apoe-deficient mice. Arterioscler Thromb Vasc Biol. 2013;33(4):679–86. doi:10.1161/ATVBAHA.112.301162.

Almeida MI, Nicoloso MS, Zeng L, Ivan C, Spizzo R, Gafa R, Xiao L, Zhang X, Vannini I, Fanini F, Fabbri M, Lanza G, Reis RM, Zweidler-McKay PA, Calin GA. Strand-specific miR-28-5p and miR-28-3p have distinct effects in colorectal cancer cells. Gastroenterology. 2012;142(4):886–96. e889 doi:10.1053/j.gastro.2011.12.047.

Androulidaki A, Iliopoulos D, Arranz A, Doxaki C, Schworer S, Zacharioudaki V, Margioris AN, Tsichlis PN, Tsatsanis C. The kinase Akt1 controls macrophage response to lipopolysaccharide by regulating microRNAs. Immunity. 2009;31(2):220–31. doi:10.1016/j.immuni.2009.06.024.

Arranz A, Doxaki C, Vergadi E, Martinez de la Torre Y, Vaporidi K, Lagoudaki ED, Ieronymaki E, Androulidaki A, Venihaki M, Margioris AN, Stathopoulos EN, Tsichlis PN, Tsatsanis C. Akt1 and Akt2 protein kinases differentially contribute to macrophage polarization. Proc Natl Acad Sci U S A. 2012;109(24):9517–22. doi:10.1073/pnas.1119038109.

Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, Mitchell PS, Bennett CF, Pogosova-Agadjanyan EL, Stirewalt DL, Tait JF, Tewari M. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A. 2011a; doi:10.1073/pnas.1019055108.

Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, Mitchell PS, Bennett CF, Pogosova-Agadjanyan EL, Stirewalt DL, Tait JF, Tewari M. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A. 2011b;108(12):5003–8. doi:10.1073/pnas.1019055108.

Arvey A, Larsson E, Sander C, Leslie CS, Marks DS. Target mRNA abundance dilutes microRNA and siRNA activity. Mol Syst Biol. 2010;6:363. doi:10.1038/msb.2010.24.

Baladron V, Ruiz-Hidalgo MJ, Nueda ML, Diaz-Guerra MJ, Garcia-Ramirez JJ, Bonvini E, Gubina E, Laborda J. dlk acts as a negative regulator of Notch1 activation through interactions with specific EGF-like repeats. Exp Cell Res. 2005;303(2):343–59. doi:10.1016/j.yexcr.2004.10.001.

Bang C, Batkai S, Dangwal S, Gupta SK, Foinquinos A, Holzmann A, Just A, Remke J, Zimmer K, Zeug A, Ponimaskin E, Schmiedl A, Yin X, Mayr M, Halder R, Fischer A, Engelhardt S, Wei Y, Schober A, Fiedler J, Thum T. Cardiac fibroblast-derived microRNA passenger strand-enriched exosomes mediate cardiomyocyte hypertrophy. J Clin Invest. 2014;124(5):2136–46. doi:10.1172/JCI70577.

Bartling B, Tostlebe H, Darmer D, Holtz J, Silber RE, Morawietz H. Shear stress-dependent expression of apoptosis-regulating genes in endothelial cells. Biochem Biophys Res Commun. 2000;278(3):740–6. doi:10.1006/bbrc.2000.3873.

Besler C, Heinrich K, Rohrer L, Doerries C, Riwanto M, Shih DM, Chroni A, Yonekawa K, Stein S, Schaefer N, Mueller M, Akhmedov A, Daniil G, Manes C, Templin C, Wyss C, Maier W, Tanner FC, Matter CM, Corti R, Furlong C, Lusis AJ, von Eckardstein A, Fogelman AM, Luscher TF, Landmesser U. Mechanisms underlying adverse effects of HDL on eNOS-activating pathways in patients with coronary artery disease. J Clin Invest. 2011;121(7):2693–708. doi:10.1172/JCI42946.

Chen CH, Jiang W, Via DP, Luo S, Li TR, Lee YT, Henry PD. Oxidized low-density lipoproteins inhibit endothelial cell proliferation by suppressing basic fibroblast growth factor expression. Circulation. 2000;101(2):171–7.

Civelek M, Manduchi E, Riley RJ, Stoeckert Jr CJ, Davies PF. Chronic endoplasmic reticulum stress activates unfolded protein response in arterial endothelium in regions of susceptibility to atherosclerosis. Circ Res. 2009;105(5):453–61. doi:10.1161/CIRCRESAHA.109.203711.

Creemers EE, Tijsen AJ, Pinto YM. Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease? Circ Res. 2012;110(3):483–95. doi:10.1161/circresaha.111.247452.

Cuchel M, Rader DJ. Macrophage reverse cholesterol transport: key to the regression of atherosclerosis? Circulation. 2006;113(21):2548–55. doi:10.1161/CIRCULATIONAHA.104.475715.

Curtale G, Mirolo M, Renzi TA, Rossato M, Bazzoni F, Locati M. Negative regulation of Toll-like receptor 4 signaling by IL-10-dependent microRNA-146b. Proc Natl Acad Sci U S A. 2013;110(28):11499–504. doi:10.1073/pnas.1219852110.

Czech B, Zhou R, Erlich Y, Brennecke J, Binari R, Villalta C, Gordon A, Perrimon N, Hannon GJ. Hierarchical rules for Argonaute loading in Drosophila. Mol Cell. 2009;36(3):445–56. doi:10.1016/j.molcel.2009.09.028.

Dekker RJ, Boon RA, Rondaij MG, Kragt A, Volger OL, Elderkamp YW, Meijers JC, Voorberg J, Pannekoek H, Horrevoets AJ. KLF2 provokes a gene expression pattern that establishes functional quiescent differentiation of the endothelium. Blood. 2006;107(11):4354–63. doi:10.1182/blood-2005-08-3465.

Denzler R, Agarwal V, Stefano J, Bartel DP, Stoffel M. Assessing the ceRNA hypothesis with quantitative measurements of miRNA and target abundance. Mol Cell. 2014;54(5):766–76. doi:10.1016/j.molcel.2014.03.045.

Donners MM, Wolfs IM, Stoger LJ, van der Vorst EP, Pottgens CC, Heymans S, Schroen B, Gijbels MJ, de Winther MP. Hematopoietic miR155 deficiency enhances atherosclerosis and decreases plaque stability in hyperlipidemic mice. PLoS One. 2012;7(4):e35877. doi:10.1371/journal.pone.0035877.

Du F, Yu F, Wang Y, Hui Y, Carnevale K, Fu M, Lu H, Fan D. MicroRNA-155 deficiency results in decreased macrophage inflammation and attenuated atherogenesis in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol. 2014;34(4):759–67. doi:10.1161/ATVBAHA.113.302701.

Etheridge A, Lee I, Hood L, Galas D, Wang K. Extracellular microRNA: a new source of biomarkers. Mutat Res. 2011;717(1–2):85–90. doi:10.1016/j.mrfmmm.2011.03.004.

Falix FA, Aronson DC, Lamers WH, Gaemers IC. Possible roles of DLK1 in the Notch pathway during development and disease. Biochim Biophys Acta. 2012;1822(6):988–95. doi:10.1016/j.bbadis.2012.02.003.

Fichtlscherer S, De Rosa S, Fox H, Schwietz T, Fischer A, Liebetrau C, Weber M, Hamm CW, Roxe T, Muller-Ardogan M, Bonauer A, Zeiher AM, Dimmeler S. Circulating microRNAs in patients with coronary artery disease. Circ Res. 2010;107 (5):677–84. doi:10.1161/CIRCRESAHA.109.215566. CIRCRESAHA.109.215566 [pii].

Fichtlscherer S, Zeiher AM, Dimmeler S. Circulating microRNAs: biomarkers or mediators of cardiovascular diseases? Arterioscler Thromb Vasc Biol. 2011;31(11):2383–90. doi:10.1161/ATVBAHA.111.226696.

Fish JE, Santoro MM, Morton SU, Yu S, Yeh RF, Wythe JD, Ivey KN, Bruneau BG, Stainier DY, Srivastava D. miR-126 regulates angiogenic signaling and vascular integrity. Dev Cell. 2008;15(2):272–84. doi:10.1016/j.devcel.2008.07.008.

Foteinos G, Hu Y, Xiao Q, Metzler B, Xu Q. Rapid endothelial turnover in atherosclerosis-prone areas coincides with stem cell repair in apolipoprotein E-deficient mice. Circulation. 2008;117(14):1856–63. doi:10.1161/CIRCULATIONAHA.107.746008.

Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 2009;19(1):92–105. doi:10.1101/gr.082701.108.

Garcia DM, Baek D, Shin C, Bell GW, Grimson A, Bartel DP. Weak seed-pairing stability and high target-site abundance decrease the proficiency of lsy-6 and other microRNAs. Nat Struct Mol Biol. 2011;18(10):1139–46. doi:10.1038/nsmb.2115.

Graff JW, Dickson AM, Clay G, McCaffrey AP, Wilson ME. Identifying functional microRNAs in macrophages with polarized phenotypes. J Biol Chem. 2012;287(26):21816–25. doi:10.1074/jbc.M111.327031.

Guha M, Mackman N. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. J Biol Chem. 2002;277(35):32124–32. doi:10.1074/jbc.M203298200.

Ha M, Kim VN. Regulation of microRNA biogenesis. Nat Rev Mol Cell Biol. 2014;15(8):509–24. doi:10.1038/nrm3838.

Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J. Natural RNA circles function as efficient microRNA sponges. Nature. 2013;495(7441):384–8. doi:10.1038/nature11993.

Harris TA, Yamakuchi M, Ferlito M, Mendell JT, Lowenstein CJ. MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1. Proc Natl Acad Sci U S A. 2008;105(5):1516–21. doi:10.1073/pnas.0707493105.

Helwak A, Kudla G, Dudnakova T, Tollervey D. Mapping the human miRNA interactome by CLASH reveals frequent noncanonical binding. Cell. 2013;153(3):654–65. doi:10.1016/j.cell.2013.03.043.

Hergenreider E, Heydt S, Treguer K, Boettger T, Horrevoets AJ, Zeiher AM, Scheffer MP, Frangakis AS, Yin X, Mayr M, Braun T, Urbich C, Boon RA, Dimmeler S. Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs. Nat Cell Biol. 2012;14(3):249–56. doi:10.1038/ncb2441.

Hong D, Bai YP, Gao HC, Wang X, Li LF, Zhang GG, Hu CP. Ox-LDL induces endothelial cell apoptosis via the LOX-1-dependent endoplasmic reticulum stress pathway. Atherosclerosis. 2014;235(2):310–7. doi:10.1016/j.atherosclerosis.2014.04.028.

Itoh Y, Toriumi H, Yamada S, Hoshino H, Suzuki N. Resident endothelial cells surrounding damaged arterial endothelium reendothelialize the lesion. Arterioscler Thromb Vasc Biol. 2010;30(9):1725–32. doi:10.1161/ATVBAHA.110.207365.

Janas T, Yarus M. Specific RNA binding to ordered phospholipid bilayers. Nucleic Acids Res. 2006;34(7):2128–36. doi:10.1093/nar/gkl220.

Jansen F, Yang X, Hoelscher M, Cattelan A, Schmitz T, Proebsting S, Wenzel D, Vosen S, Franklin BS, Fleischmann BK, Nickenig G, Werner N. Endothelial microparticle-mediated transfer of MicroRNA-126 promotes vascular endothelial cell repair via SPRED1 and is abrogated in glucose-damaged endothelial microparticles. Circulation. 2013;128(18):2026–38. doi:10.1161/CIRCULATIONAHA.113.001720.

Koch M, Mollenkopf HJ, Klemm U, Meyer TF. Induction of microRNA-155 is TLR- and type IV secretion system-dependent in macrophages and inhibits DNA-damage induced apoptosis. Proc Natl Acad Sci U S A. 2012;109(19):E1153–62. doi:10.1073/pnas.1116125109.

Kosaka N, Iguchi H, Ochiya T. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. Cancer Sci. 2010a;101(10):2087–92. doi:10.1111/j.1349-7006.2010.01650.x.

Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T. Secretory mechanisms and intercellular transfer of microRNAs in living cells. J Biol Chem. 2010b;285(23):17442–52. doi:10.1074/jbc.M110.107821.

Kuhlmann CR, Schaefer CA, Reinhold L, Tillmanns H, Erdogan A. Signalling mechanisms of SDF-induced endothelial cell proliferation and migration. Biochem Biophys Res Commun. 2005;335(4):1107–14. doi:10.1016/j.bbrc.2005.08.006.

Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993;75(5):843–54.

Locati M, Mantovani A, Sica A. Macrophage activation and polarization as an adaptive component of innate immunity. Adv Immunol. 2013;120:163–84. doi:10.1016/B978-0-12-417028-5.00006-5.

Loyer X, Potteaux S, Vion AC, Guerin CL, Boulkroun S, Rautou PE, Ramkhelawon B, Esposito B, Dalloz M, Paul JL, Julia P, Maccario J, Boulanger CM, Mallat Z, Tedgui A. Inhibition of microRNA-92a prevents endothelial dysfunction and atherosclerosis in mice. Circ Res. 2014;114(3):434–43. doi:10.1161/CIRCRESAHA.114.302213.

Ma X, Becker Buscaglia LE, Barker JR, Li Y. MicroRNAs in NF-kappaB signaling. J Mol Cell Biol. 2011;3(3):159–66. doi:10.1093/jmcb/mjr007.

Marco A, Macpherson JI, Ronshaugen M, Griffiths-Jones S. MicroRNAs from the same precursor have different targeting properties. Silence. 2012;3(1):8. doi:10.1186/1758-907X-3-8.

Matranga C, Tomari Y, Shin C, Bartel DP, Zamore PD. Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell. 2005;123(4):607–20. doi:10.1016/j.cell.2005.08.044.

Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008;105(30):10513–8. doi:10.1073/pnas.0804549105.

Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, Gordon S, Hamilton JA, Ivashkiv LB, Lawrence T, Locati M, Mantovani A, Martinez FO, Mege JL, Mosser DM, Natoli G, Saeij JP, Schultze JL, Shirey KA, Sica A, Suttles J, Udalova I, van Ginderachter JA, Vogel SN, Wynn TA. Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity. 2014;41(1):14–20. doi:10.1016/j.immuni.2014.06.008.

Nam D, Ni CW, Rezvan A, Suo J, Budzyn K, Llanos A, Harrison D, Giddens D, Jo H. Partial carotid ligation is a model of acutely induced disturbed flow, leading to rapid endothelial dysfunction and atherosclerosis. Am J Phys Heart Circ Phys. 2009;297 (4):H1535–43. doi:10.1152/ajpheart.00510.2009. 00510.2009 [pii].

Nazari-Jahantigh M, Wei Y, Noels H, Akhtar S, Zhou Z, Koenen RR, Heyll K, Gremse F, Kiessling F, Grommes J, Weber C, Schober A. MicroRNA-155 promotes atherosclerosis by repressing Bcl6 in macrophages. J Clin Invest. 2012a;122(11):4190–202. doi:10.1172/jci61716.

Nazari-Jahantigh M, Wei Y, Schober A. The role of microRNAs in arterial remodelling. Thromb Haemost. 2012b;107(4):611–8. doi:10.1160/th11-12-0826.

Neth P, Nazari-Jahantigh M, Schober A, Weber C. MicroRNAs in flow-dependent vascular remodelling. Cardiovasc Res. 2013;99(2):294–303. doi:10.1093/cvr/cvt096.

O'Connell RM, Rao DS, Chaudhuri AA, Baltimore D. Physiological and pathological roles for microRNAs in the immune system. Nat Rev Immunol. 2010;10(2):111–22. doi:10.1038/nri2708.

O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D. MicroRNA-155 is induced during the macrophage inflammatory response. Proc Natl Acad Sci U S A. 2007;104(5):1604–9. doi:10.1073/pnas.0610731104.

Poissonnier L, Villain G, Soncin F, Mattot V. miR126-5p repression of ALCAM and SetD5 in endothelial cells regulates leucocyte adhesion and transmigration. Cardiovasc Res. 2014;102(3):436–47. doi:10.1093/cvr/cvu040.

Poliseno L, Salmena L, Zhang J, Carver B, Haveman WJ, Pandolfi PP. A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. Nature. 2010;465(7301):1033–8. doi:10.1038/nature09144.

Quinn SR, Mangan NE, Caffrey BE, Gantier MP, Williams BR, Hertzog PJ, McCoy CE, O'Neill LA. The role of Ets2 transcription factor in the induction of microRNA-155 (miR-155) by lipopolysaccharide and its targeting by interleukin-10. J Biol Chem. 2014;289(7):4316–25. doi:10.1074/jbc.M113.522730.

Rayner KJ, Fernandez-Hernando C, Moore KJ. MicroRNAs regulating lipid metabolism in atherogenesis. Thromb Haemost. 2012;107(4):642–7. doi:10.1160/TH11-10-0694.

Rodriguez P, Higueras MA, Gonzalez-Rajal A, Alfranca A, Fierro-Fernandez M, Garcia-Fernandez RA, Ruiz-Hidalgo MJ, Monsalve M, Rodriguez-Pascual F, Redondo JM, de la Pompa JL, Laborda J, Lamas S. The non-canonical NOTCH ligand DLK1 exhibits a novel vascular role as a strong inhibitor of angiogenesis. Cardiovasc Res. 2012;93(2):232–41. doi:10.1093/cvr/cvr296.

Sakurai K, Furukawa C, Haraguchi T, Inada K, Shiogama K, Tagawa T, Fujita S, Ueno Y, Ogata A, Ito M, Tsutsumi Y, Iba H. MicroRNAs miR-199a-5p and -3p target the Brm subunit of SWI/SNF to generate a double-negative feedback loop in a variety of human cancers. Cancer Res. 2011;71(5):1680–9. doi:10.1158/0008-5472.CAN-10-2345.

Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? Cell. 2011;146(3):353–8. doi:10.1016/j.cell.2011.07.014.

Schober A, Nazari-Jahantigh M, Wei Y, Bidzhekov K, Gremse F, Grommes J, Megens RT, Heyll K, Noels H, Hristov M, Wang S, Kiessling F, Olson EN, Weber C. MicroRNA-126-5p promotes endothelial proliferation and limits atherosclerosis by suppressing Dlk1. Nat Med. 2014;20(4):368–76. doi:10.1038/nm.3487.

Sun X, He S, Wara AK, Icli B, Shvartz E, Tesmenitsky Y, Belkin N, Li D, Blackwell TS, Sukhova GK, Croce K, Feinberg MW. Systemic delivery of microRNA-181b inhibits nuclear factor-kappaB activation, vascular inflammation, and atherosclerosis in apolipoprotein E-deficient mice. Circ Res. 2014;114(1):32–40. doi:10.1161/CIRCRESAHA.113.302089.

Tay Y, Rinn J, Pandolfi PP. The multilayered complexity of ceRNA crosstalk and competition. Nature. 2014;505(7483):344–52. doi:10.1038/nature12986.

Tedgui A, Mallat Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. Physiol Rev. 2006;86(2):515–81. doi:10.1152/physrev.00024.2005.

Thum T, Gross C, Fiedler J, Fischer T, Kissler S, Bussen M, Galuppo P, Just S, Rottbauer W, Frantz S, Castoldi M, Soutschek J, Koteliansky V, Rosenwald A, Basson MA, Licht JD, Pena JT, Rouhanifard SH, Muckenthaler MU, Tuschl T, Martin GR, Bauersachs J, Engelhardt S. MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. Nature. 2008;456(7224):980–4. doi:10.1038/nature07511.

Tian FJ, An LN, Wang GK, Zhu JQ, Li Q, Zhang YY, Zeng A, Zou J, Zhu RF, Han XS, Shen N, Yang HT, Zhao XX, Huang S, Qin YW, Jing Q. Elevated microRNA-155 promotes foam cell formation by targeting HBP1 in atherogenesis. Cardiovasc Res. 2014;103(1):100–10. doi:10.1093/cvr/cvu070.

Uchino K, Takeshita F, Takahashi RU, Kosaka N, Fujiwara K, Naruoka H, Sonoke S, Yano J, Sasaki H, Nozawa S, Yoshiike M, Kitajima K, Chikaraishi T, Ochiya T. Therapeutic effects of microRNA-582-5p and -3p on the inhibition of bladder cancer progression. Mol Ther. 2013;21(3):610–9. doi:10.1038/mt.2012.269.

Vickers KC, Remaley AT. Lipid-based carriers of microRNAs and intercellular communication. Curr Opin Lipidol. 2012;23(2):91–7. doi:10.1097/MOL.0b013e328350a425.

Villarroya-Beltri C, Gutierrez-Vazquez C, Sanchez-Cabo F, Perez-Hernandez D, Vazquez J, Martin-Cofreces N, Martinez-Herrera DJ, Pascual-Montano A, Mittelbrunn M, Sanchez-Madrid F. Sumoylated hnRNPA2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs. Nat Commun. 2013;4:2980. doi:10.1038/ncomms3980.

Wagner J, Riwanto M, Besler C, Knau A, Fichtlscherer S, Roxe T, Zeiher AM, Landmesser U, Dimmeler S. Characterization of levels and cellular transfer of circulating lipoprotein-bound microRNAs. Arterioscler Thromb Vasc Biol. 2013;33(6):1392–400. doi:10.1161/atvbaha.112.300741.

Wang S, Aurora AB, Johnson BA, Qi X, McAnally J, Hill JA, Richardson JA, Bassel-Duby R, Olson EN. The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. Dev Cell. 2008;15(2):261–71. doi:10.1016/j.devcel.2008.07.002.

Weber C, Noels H. Atherosclerosis: current pathogenesis and therapeutic options. Nat Med. 2011;17(11):1410–22. doi:10.1038/nm.2538.

Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, Galas DJ, Wang K. The microRNA spectrum in 12 body fluids. Clin Chem. 2010;56(11):1733–41. doi:10.1373/clinchem.2010.147405.

Wei Y, Nazari-Jahantigh M, Chan L, Zhu M, Heyll K, Corbalan-Campos J, Hartmann P, Thiemann A, Weber C, Schober A. The microRNA-342-5p fosters inflammatory macrophage activation through an Akt1- and microRNA-155-dependent pathway during atherosclerosis. Circulation. 2013a;127(15):1609–19. doi:10.1161/CIRCULATIONAHA.112.000736.

Wei Y, Nazari-Jahantigh M, Neth P, Weber C, Schober A. MicroRNA-126, −145, and −155: a therapeutic triad in atherosclerosis? Arterioscler Thromb Vasc Biol. 2013b;33(3):449–54. doi:10.1161/ATVBAHA.112.300279.

Wei Y, Schober A, Weber C. Pathogenic arterial remodeling: the good and bad of microRNAs. Am J Phys Heart Circ Phys. 2013c;304(8):H1050–9. doi:10.1152/ajpheart.00267.2012.

Wolfs IM, Donners MM, de Winther MP. Differentiation factors and cytokines in the atherosclerotic plaque micro-environment as a trigger for macrophage polarisation. Thromb Haemost. 2011;106(5):763–71. doi:10.1160/TH11-05-0320.

Yang X, Du WW, Li H, Liu F, Khorshidi A, Rutnam ZJ, Yang BB. Both mature miR-17-5p and passenger strand miR-17-3p target TIMP3 and induce prostate tumor growth and invasion. Nucleic Acids Res. 2013;41(21):9688–704. doi:10.1093/nar/gkt680.

Zampetaki A, Kiechl S, Drozdov I, Willeit P, Mayr U, Prokopi M, Mayr A, Weger S, Oberhollenzer F, Bonora E, Shah A, Willeit J, Mayr M. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. Circ Res. 2010;107(6):810–7. doi:10.1161/CIRCRESAHA.110.226357. CIRCRESAHA.110.226357 [pii].

Zeng L, Zampetaki A, Margariti A, Pepe AE, Alam S, Martin D, Xiao Q, Wang W, Jin ZG, Cockerill G, Mori K, Li YS, Hu Y, Chien S, Xu Q. Sustained activation of XBP1 splicing leads to endothelial apoptosis and atherosclerosis development in response to disturbed flow. Proc Natl Acad Sci U S A. 2009;106(20):8326–31. doi:10.1073/pnas.0903197106.

Zernecke A, Bidzhekov K, Noels H, Shagdarsuren E, Gan L, Denecke B, Hristov M, Koppel T, Jahantigh MN, Lutgens E, Wang S, Olson EN, Schober A, Weber C. Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. Sci Signal. 2009;2(100):ra81. doi:10.1126/scisignal.2000610.

Zhang Y, Yang P, Sun T, Li D, Xu X, Rui Y, Li C, Chong M, Ibrahim T, Mercatali L, Amadori D, Lu X, Xie D, Li QJ, Wang XF. miR-126 and miR-126* repress recruitment of mesenchymal stem cells and inflammatory monocytes to inhibit breast cancer metastasis. Nat Cell Biol. 2013;15(3):284–94. doi:10.1038/ncb2690.

Zhou J, Li YS, Nguyen P, Wang KC, Weiss A, Kuo YC, Chiu JJ, Shyy JY, Chien S. Regulation of vascular smooth muscle cell turnover by endothelial cell-secreted microRNA-126: role of shear stress. Circ Res. 2013;113(1):40–51. doi:10.1161/CIRCRESAHA.113.280883.

Noncoding RNAs in Cholesterol Metabolism and Atherosclerosis

2

Nathan L. Price and Carlos Fernández-Hernando

Contents

Abstract

Proper maintenance of cellular and plasma cholesterol levels is critical for proper metabolic function and as such is regulated through tightly controlled mechanisms at both the transcriptional and posttranscriptional level. Cardiometabolic diseases, including atherosclerosis, a prominent cause of human morbidity and mortality in western societies (Glass and Witztum 2001; Lusis 2000), are caused in large part by dysregulation of cholesterol and lipid homeostasis. Although

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many environmental and genetic factors are known to contribute to atherogenesis, elevated levels of low-density lipoprotein cholesterol (LDL-C) are the primary risk factors for atherosclerosis and are sufficient to drive the progression of this disease. For this reason, the pathways governing plasma LDL-C levels have been extensively studied, and their modulation has led to effective therapies for the treatment of atherosclerosis.

2.1 Introduction

Proper maintenance of cellular and plasma cholesterol levels is critical for proper metabolic function and as such is regulated through tightly controlled mechanisms at both the transcriptional and posttranscriptional level. Cardiometabolic diseases, including atherosclerosis, a prominent cause of human morbidity and mortality in western societies (Glass and Witztum 2001; Lusis 2000), are caused in large part by dysregulation of cholesterol and lipid homeostasis. Although many environmental and genetic factors are known to contribute to atherogenesis, elevated levels of low-density lipoprotein cholesterol (LDL-C) are the primary risk factors for atherosclerosis and are sufficient to drive the progression of this disease. For this reason, the pathways governing plasma LDL-C levels have been extensively studied, and their modulation has led to effective therapies for the treatment of atherosclerosis.

The primary treatment option for patients with hypercholesterolemia is statins, a class of drugs which competitively inhibit 3-hydroxy-3methylgutaryl-CoA reductase (HMGCR), the rate-limiting enzyme in the cholesterol biosynthesis pathway (Steinberg 2008; Steinberg et al. 2008). Reduced intracellular cholesterol synthesis in the liver, in response to statins, leads to activation of the sterol regulatory element-binding protein (SREBP), which increases the expression of the LDL receptor (LDLR). This in turn promotes LDL-C uptake from the blood and reduces levels of pro-atherogenic lipoproteins in circulation (Brown and Goldstein 1976). Although statin therapies have proven to be effective at reducing cholesterol levels and limiting cardiovascular-related deaths, some patients have poor tolerance for statin therapies, and the majority of patients still experience adverse coronary events despite treatment with statins (Steinberg 2008; Steinberg et al. 2008). As a result, developing novel approaches for lowering cholesterol that can be used alone or in combination with statins has become a major goal for cardiovascular research. Among these, approaches that modulate noncoding RNAs, including microRNAs (miRNAs) and long noncoding RNAs (lncRNAs), have generated a great deal of interest due to their ability to regulate key pathways in cholesterol metabolism and their dysregulation in different disease states (Grundy 2008; Grundy et al. 2004). In this review, we highlight recent work demonstrating the importance of miRNAs and lncRNAs in regulation of cholesterol metabolism and discuss the potential of noncoding RNA-based therapeutic approaches for the treatment of atherosclerosis.

2.2 miRNAs Regulating HDL-C Metabolism and Reverse Cholesterol Transport

In addition to lowering levels of circulating pro-atherogenic lipids, much interest has recently been focused upon developing strategies to promote the removal of cholesterol from arterial macrophages, as a means to limit plaque progression and promote plaque regression. This process, known as reverse cholesterol transport, involves efflux of cholesterol esters from arterial macrophages by the lipid transporters ATP-binding cassette transporter A1 (ABCA1) and ABCG1 onto circulating high-density lipoprotein cholesterol (HDL-C) molecules (Brooks-Wilson et al. 1999; Gelissen et al. 2006; Oram and Vaughan 2000). These cholesterol esters are then transported to the liver where they can be converted into bile acids and removed from the body (Brooks-Wilson et al. 1999; Gelissen et al. 2006; Oram and Vaughan 2000). In recent years, numerous miRNAs have been shown to control numerous aspects of the reverse cholesterol transport pathway, including HDL biogenesis and uptake, cellular cholesterol efflux, and bile acid synthesis and secretion (Fig. 2.1). These include miR-10b, miR-27b, miR-33, miR-96, miR-125a, miR-128a, miR-144, miR-148a, miR-185, miR-223, miR-302a, and miR-455 (De Aguiar Vallim et al. 2013; Goedeke et al. 2015a, b; Ramirez et al. 2013b; Vickers et al. 2014; Wagschal et al. 2015; Wang et al. 2012, 2013). While all of these miRNAs have the

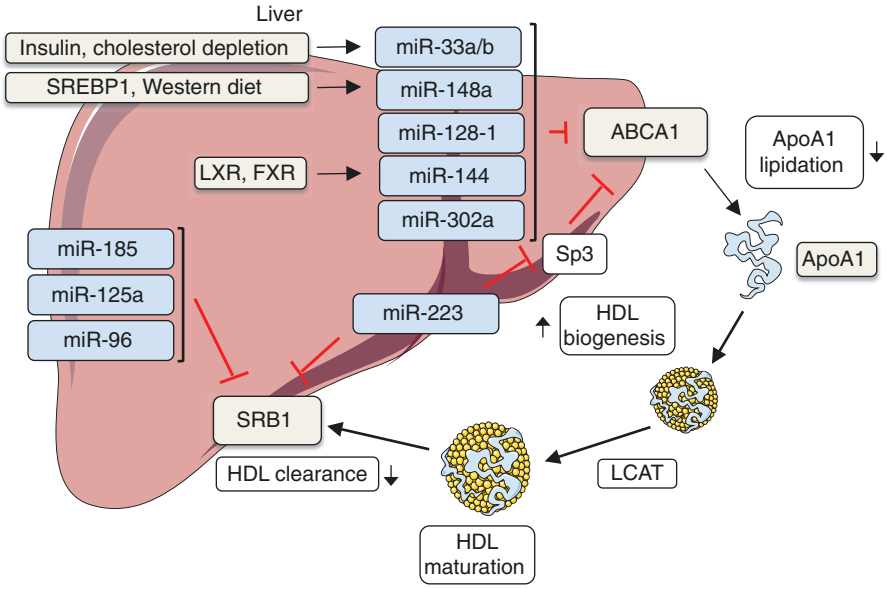

Fig. 2.1 miRNA regulation of HDL-C metabolism. Schematic overview of miRNAs involved in the regulation of HDL-C metabolism. *Blue boxes* highlight miRNAs, which regulate genes that control HDL-C. *ABC* indicates ATP-binding cassette, *SR-BI* scavenger receptor B1, *FXR* farnesoid X receptor, and *LCAT* lecithin–cholesterol acyltransferase (Figure was created using the Servier Medical Art illustration resources (http://www.servier.com))

potential to be important regulators of reverse cholesterol transport and atherogenesis, only a few have thus far been shown to have a significant impact on atherosclerotic plaque progression. Two independent groups have identified miR-144 as an important regulator of ABCA1 expression in both monocyte/macrophages and the liver (De Aguiar Vallim et al. 2013; Ramirez et al. 2013b). These studies further demonstrate that overexpression of miR-144 decreases circulating HDL-C, while inhibition of miR-144 was found to increase circulating HDL-C. Importantly, further work has since demonstrated that administering miR-144 mimics to *ApoE*$^{-/-}$ mice on a pro-atherogenic diet reduces hepatic ABCA1 expression and plasma HDL-C levels leading to increased atherosclerotic plaque formation (Hu et al. 2014). More recently, miR-302a has also been found to control the expression of ABCA1, thereby influencing HDL-C levels. Treatment with inhibitors of miR-302a in vivo was demonstrated to increase hepatic ABCA1 and plasma HDL-C leading to reduce atherosclerosis (Meiler et al. 2015). While most of these miRNAs have not been extensively studied for their role in regulating atherosclerosis, a great deal of work has been done exploring the role of the miR-33 family of miRNAs in regulation of this disease.

2.2.1 miR-33a/miR-33b

The miR-33 family consists of two members, miR-33a and miR-33b. These are intronic miRNAs, which are encoded within the *SREBP2* and *SREBP1 genes*, respectively (Najafi-Shoushtari et al. 2010; Rayner et al. 2010; Marquart et al. 2010; Gerin et al. 2010; Horie et al. 2010). The SREBP1 transcription factor is regulated in response to factors such as insulin and LXR ligands and is primarily responsible for the induction of genes involved in fatty acid synthesis (Horton et al. 2002). SREBP2, on the other hand, is regulated by changes in sterol levels and is the primary factor involved in the induction of genes regulating cholesterol biosynthesis and uptake, thereby allowing cells to carefully regulate their intracellular cholesterol levels (Horton et al. 2002). miR-33a and miR-33b have been demonstrated to be co-transcribed along with their host genes, so conditions that modulate the expression of the *SREBP* genes result in concomitant changes in miR-33 levels (Rayner et al. 2010; Najafi-Shoushtari et al. 2010; Marquart et al. 2010). While the SREBP transcription factors have different target gene specificities, the miR-33 isotypes share the same seed sequence and are therefore believed to target the same set of genes. However, it is not clear whether slight differences in the mature sequence of these miRNAs may alter their affinity for certain targets.

The important role of miR-33 in regulating HDL-C metabolism was first revealed in studies showing that miR-33 could target the cholesterol transporters ABCA1 and ABCG1, both in hepatocytes and macrophages (Najafi-Shoushtari et al. 2010; Rayner et al. 2010; Marquart et al. 2010; Gerin et al. 2010; Horie et al. 2010). Overexpression of miR-33 reduced the expression of ABCA1 and ABCG1 in the liver of mice and decreased plasma HDL-C (Marquart et al. 2010; Najafi-Shoushtari et al. 2010; Rayner et al. 2010; Gerin et al. 2010). Conversely, antisense

oligonucleotides (ASOs) that reduced miR-33 level increased the expression of ABCA1 and ABCG1 and raised plasma HDL-C (Rayner et al. 2010; Najafi-Shoushtari et al. 2010; Marquart et al. 2010). Similarly, genetic loss of miR-33 in mice was reported to increase liver ABCA1 expression and plasma HDL-C (Horie et al. 2010). Importantly, inhibition of miR-33 in nonhuman primates was also demonstrated to significantly increase plasma HDL-C (Rayner et al. 2011a; Rottiers et al. 2013). Additionally, miR-33 was found to regulate hepatic bile acid synthesis through targeting of CYP7a1 (Li et al. *Hepatology* 2013) and secretion via modulation of ABCB11 and ATP8B1 (Allen et al. 2012). Due to its key role in regulating the removal of cholesterol from macrophages, HDL-C biogenesis in the liver, and bile acid metabolism, miR-33 was proposed to be an important regulator of reverse cholesterol transport and the progression of atherosclerosis (Allen et al. 2012; Rayner et al. 2011b). Collectively, these data indicated that ASOs against miR-33 might be a promising therapeutic option to raise HDL-C and treat patients with atherosclerosis.

2.2.2 miR-33a/miR-33b and Atherosclerosis

In response to these initial findings, numerous studies have been performed assessing the impact of miR-33 antagonism on atherosclerosis in mice. The first study of this sort demonstrated that short-term (4-week) administration of miR-33 inhibitors to *Ldlr*$^{-/-}$ mice with established plaques was capable of raising HDL-C levels and promoting atherosclerotic plaque regression (Rayner et al. 2011b). However, another study found that treatment with anti-miR-33 ASOs did not sustain elevated plasma HDL-C levels during a 12-week atherosclerosis progression study, raised plasma triglycerides, and did not have any effect on plaque development (Marquart et al. 2013). Three additional atherosclerosis progression studies using *Ldlr*$^{-/-}$ (Rotllan et al. 2013; Ouimet et al. 2015) and *ApoE*$^{-/-}$ mice (Karunakaran et al. 2015b) reported a decrease in plaque burden in animals treated with miR-33 inhibitors. Surprisingly, although the expression of ABCA1 in the liver and/or macrophages was elevated in mice treated with miR-33 ASOs, HDL-C remained unchanged in these progression studies (Rotllan et al. 2013; Ouimet et al. 2015; Karunakaran et al. 2015b). Together, these findings suggest that the anti-atherosclerotic effects of the ASOs may be the result of direct effects on the plaque rather than alterations in circulating lipids. Consistent with this theory, anti-miR-33 ASOs were found to be efficiently taken up by macrophages in the aortic root of *Ldlr*$^{-/-}$ mice, which could promote ABCA1-/ABCG1-mediated cholesterol efflux. Additionally, it has been reported that anti-miR-33 ASOs decreased inflammatory burden and promoted the recruitment of M2-like macrophages (Rayner et al. 2011b; Ouimet et al. 2015) and T_{reg} cells (Ouimet et al. 2015). Furthermore, bone marrow transplant experiments in which *ApoE*$^{-/-}$ mice were reconstituted with *ApoE*$^{-/-}$ × *miR-33*$^{-/-}$ bone marrow demonstrated that loss of miR-33 in hematopoietic cells caused reduced lipid accumulation in atherosclerotic plaques but did not affect HDL-C levels or total plaque size (Horie et al. 2012). However, whole-body

loss of miR-33 in *ApoE*$^{-/-}$ mice did increase plasma HDL-C and reduce total plaque size (Horie et al. 2012). While the low levels of HDL-C and impaired macrophage cholesterol efflux of *ApoE*$^{-/-}$ mice make this a poor model in which to study effects on reverse cholesterol transport, these findings do indicate that both the liver and macrophages are likely involved in mediating the effects of miR-33 on atherosclerotic plaque formation, although the specific contributions are still not entirely clear.

In addition to its role in regulating lipid metabolism, further studies have showed that miR-33 can also target a number of genes involved in other metabolic processes including fatty acid β-oxidation, insulin signaling, and mitochondrial function (Karunakaran et al. 2015b; Gerin et al. 2010; Davalos et al. 2011; Ramirez et al. 2013a). These findings highlight the complex role of miR-33 in regulating metabolic function and indicate that it may have more diverse functions than have previously been elucidated. Consistent with this, *miR-33*$^{-/-}$ mice on a high-fat diet were found to gain more weight than control animals, leading to more rapid development of hepatic steatosis and insulin resistance (Horie et al. 2013). Similarly, some studies have indicated that prolonged anti-miR-33 therapy may cause unwanted effects such as hypertriglyceridemia (Marquart et al. 2013; Allen et al. 2014; Goedeke et al. 2014) and hepatic steatosis (Goedeke et al. 2014). In contrast, similar long-term miR-33 inhibitor studies showed no change (Rotllan et al. 2013; Ouimet et al. 2015; Horie et al. 2012; Rottiers et al. 2013) or even a decrease (Karunakaran et al. 2015a; Rayner et al. 2011a) in plasma triacylglycerides (TAGs) in mice and nonhuman primates.

Overall, these studies indicate that anti-miR-33 therapies may be a promising approach for developing novel therapies for atheroprotection. However, the multitude of miR-33 targets involved in many different metabolic functions, and the potential for adverse effects of long-term miR-33 inhibition warrant further exploration. Additionally, because there are two isoforms of miR-33 present in humans, but mice and other rodents do not express miR-33b, little is known about the role of this miRNA in regulating atherosclerotic plaque development and overall metabolic function. This is especially important because the host genes for miR-33a and miR-33b (*SREBP2* and *SREBP1*) are regulated by different nutritional and hormonal stimuli and miR-33b is more likely to be altered under conditions of metabolic dysfunction such as diabetes and obesity. Recently, a miR-33b knock-in model was reported (Horie et al. 2014), which should allow further exploration of the role of miR-33b in atherosclerosis and other metabolic diseases.

2.3 miRNAs and LDL-C Metabolism

While the role of miRNAs in regulating HDL-C metabolism has been extensively studied, the importance of miRNAs in controlling LDL-C remains to be fully elucidated. Nevertheless, a number of studies have reported the importance of miRNAs in controlling circulating LDL-C by regulating cholesterol biosynthesis, very-low-density lipoprotein (VLDL) secretion, and LDLR activity (Fig. 2.2).

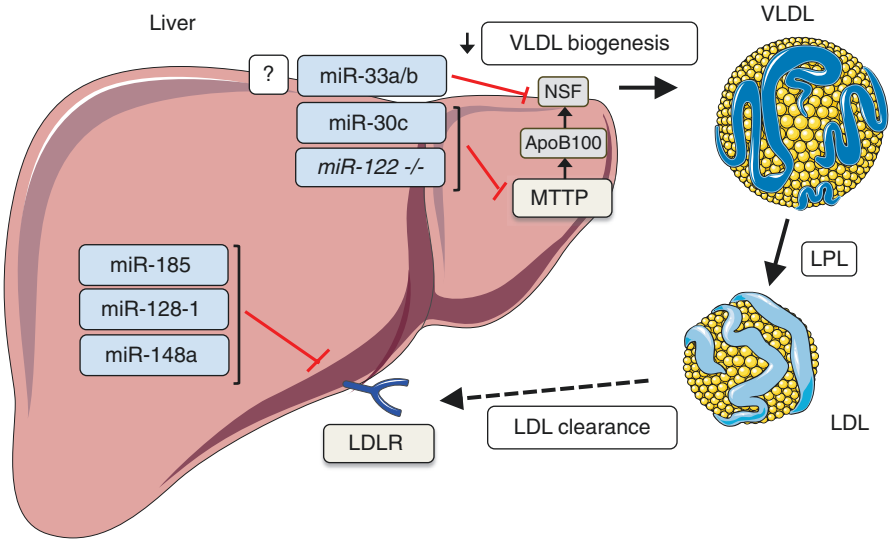

Fig. 2.2 miRNA regulation of LDL-C metabolism. Schematic overview of miRNAs involved in the regulation of LDL-C metabolism. *Blue boxes* highlight miRNAs, which regulate genes that control LDL-C. LDLR indicates low-density lipoprotein receptor, *MTTP* microsomal triglyceride transfer protein, *LPL* lipoprotein lipase, and *NSF* N-ethylmaleimide-sensitive factor (Figure was created using the Servier Medical Art illustration resources (http://www.servier.com))

2.3.1 miRNAs That Regulate Cholesterol Biosynthesis and VLDL Secretion

2.3.1.1 miR-122

The first miRNA identified as a major regulator of cholesterol metabolism was miR-122 (Elmen et al. 2008; Esau et al. 2006; Krutzfeldt et al. 2005). This miRNA is the most abundant miRNA in the liver, accounting for at least 80% of the total miRNA content. Importantly, pharmacological inhibition of miR-122 in mice and nonhuman primates using ASOs or genetic ablation of miR-122 in mice results in a marked reduction of plasma cholesterol levels (Elmen et al. 2008; Esau et al. 2006; Krutzfeldt et al. 2005). Despite these intriguing findings, the molecular mechanisms by which miR-122 controls lipoprotein metabolism remain largely unknown. It is thought that the reduced circulating cholesterol observed in mice treated with miR-122 antagonists is mediated by a combination of different metabolic processes including inhibition of cholesterol and fatty acid biosynthesis, enhanced fatty acid oxidation, and decreased VLDL secretion (Elmen et al. 2008; Esau et al. 2006; Krutzfeldt et al. 2005). However, the direct miR-122 target genes that influence lipid metabolism remain largely unknown. While these findings suggest that the use of miR-122 inhibitors might represent a useful approach to treat dyslipidemia, two recent studies have shown that miR-122 deficiency in mice results in hepatic steatosis, fibrosis, and hepatocellular carcinoma, raising concerns about the potential therapeutic value of miR-122 inhibitors.

2.3.1.2 miR-30c in Lipid Metabolism and Atherosclerosis

In addition to miR-122, miR-30c levels also regulate circulating lipids by regulating the expression of microsomal triglyceride transfer protein (MTTP), an enzyme that lipidates Apo-B and triglycerides and is required for the secretion of plasma lipoproteins that contain Apo-B (Irani et al. 2016; Soh et al. 2013). Moreover, miR-30c inhibits hepatic lipid synthesis. As a result, overexpression of miR-30c using lentiviral constructs or miRNA mimics significantly reduces plasma cholesterol levels and attenuates the progression of atherosclerosis (Irani et al. 2016; Soh et al. 2013). Interestingly the inhibition of MTTP expression and VLDL secretion does not influence the accumulation of lipids in the liver, suggesting that miR-30c therapies might be an interesting approach for treating patients with homozygous hypercholesterolemia.

2.3.1.3 miR-33a/miR-33b

Besides the abovementioned role of miR-33a/miR-33b in regulating HDL-C metabolism and the progression and regression of atherosclerosis, several reports have shown that miR-33 might also control VLDL metabolism (Allen et al. 2014; Goedeke et al. 2014). In this regard, Baldán and colleagues found that chronic inhibition of miR-33 in rodents enhances hepatic VLDL-C. The authors showed that targeting miR-33 in vivo increases the expression of N-ethylmaleimide-sensitive factor (NSF), an ATPase enzyme involved in intracellular trafficking and membrane fusion (Allen et al. 2014). These findings correlate with another study that shows a marked increase in circulating TAG (Goedeke et al. 2014). In agreement with these observations, genetic ablation of miR-33 in mice results in obesity, insulin resistance, hepatosteatosis, and dyslipidemia (Horie et al. 2013). Despite these results, other similar studies in mice and nonhuman primates showed no differences or even a decrease in circulating TAG (Rayner et al. 2011a). The factors that might explain these discrepancies need further investigation.

2.3.1.4 miR-96/miR-182/miR-183

miR-96/miR-182/miR-183 was identified as the most upregulated miRNA in mice fed a chow diet supplemented with a mixture of lovastatin plus ezetimibe (to deplete hepatic cholesterol and enhance cholesterol biosynthesis) compared to mice fed a chow diet supplemented with cholesterol (to increase hepatic cholesterol content and inhibit cholesterol synthesis) (Jeon et al. 2013). The miR-96/miR-182/miR-183 locus is regulated by SREBP and regulates the expression of key proteins that control the maturation and activation of SREBP. miR-96 and miR-182 inhibit the expression of INSIG and FBXW7, proteins that control the retention of the SREBP/SCAP complex in the ER and the degradation nuclear SREBP, respectively (Jeon et al. 2013). As a consequence, overexpression of all three miRNAs enhances cholesterol and fatty acid synthesis in HeLa cells. Further experiments in vivo will be important to define the specific contribution of these miRNAs in regulating hepatic lipid homeostasis and lipoprotein metabolism.

2.3.2 miRNAs That Regulate LDLR Expression and LDL-C Clearance

Several studies have recently identified miRNAs that regulate LDL-C metabolism via posttranscriptional regulation of LDLR. Notably, miR-27a/miR-27b, miR-128-1, miR-130b, miR-148a, miR-185, miR-199a, and miR-301 were shown to directly target the 3'UTR of *LDLR* and modulate LDLR expression in human and mouse hepatic cells (Alvarez et al. 2015; Goedeke et al. 2015b; Jiang et al. 2015; Wagschal et al. 2015). Of these miRNAs, only miR-128-1, miR-148a, and miR-185 significantly altered plasma LDL-C in vivo (Goedeke et al. 2015a; Jiang et al. 2015; Wagschal et al. 2015; Yang et al. 2014). Thus, we will only discuss in greater detail how these miRNAs regulate hepatic lipid metabolism and circulating LDL-C.

2.3.2.1 miR-148a

miR-148a has been recently identified by two independent studies as an important regulator of hepatic LDLR expression and lipoprotein metabolism in a number of mouse models (Goedeke et al. 2015a; Wagschal et al. 2015). In the first study, Goedeke and colleagues developed a high-throughput genome-wide screening assay to systematically identify miRNAs that regulate LDLR activity in human hepatic cells (Goedeke et al. 2015a). From this screen, the authors identified and characterized miR-148a as a negative regulator of LDLR expression and activity. miR-148a is highly expressed in the liver, and its expression is regulated by dietary lipids and SREBP1. Of note, pharmacological inhibition of miR-148a using ASOs lowered plasma LDL-C levels in two different mouse models of hypercholesterolemia (Goedeke et al. 2015a; Wagschal et al. 2015). Surprisingly, targeting miR-148a in vivo also increases hepatic ABCA1 expression and circulating HDL-C. Further experiments demonstrated that *ABCA1* is also a miR-148a target gene. Collectively these studies underscore the therapeutic potential of modulating miR-148a expression to treat dyslipidemias (high plasma LDL-C and low circulating HDL-C).

Several labs independently identified SNPs (rs4722551, rs4719841, and rs6951827) in the promoter region of miR-148a associated with altered plasma TC, LDL-C, and TAG levels (Do et al. 2013; Global Lipids Genetics Consortium et al. 2013; Huan et al. 2015). In particular, a miR-eQTL analysis performed in human livers revealed a strong correlation between SNP status and miR-148a expression (Wagschal et al. 2015). Although the exact mechanism by which these SNPs contribute to altered plasma lipids remains unknown, it could be possible that these genetic variations might influence the regulation of miR-148a expression via SREBP1. However, whether these SNPs affect SREBP1-induced transcription requires further investigation. The role of miR-148a in regulating lipid metabolism is likely to be more complex and not only mediated by its targeting on LDLR and ABCA1. In particular, miR-148a was demonstrated to directly target the 3'UTR of other genes involved in lipid metabolism, including *PGC1a*, *AMPK*, and *INSIG1* (Wagschal et al. 2015; Goedeke et al. 2015a). Taken together, these findings

highlight the importance of miR-148a in regulating lipid metabolism in mice and humans and underscore the therapeutic potential of modulating miR-148a expression to treat dyslipidemias.

2.3.2.2 miR-128-1

Besides miR-148a, Wagschal and colleagues also found a strong association of a number of SNPs in the miR-128-1 gene locus and altered plasma lipid levels (Wagschal et al. 2015). miR-128a regulates the expression of numerous genes associated with multiple metabolic pathways. miR-128a directly targets the 3'UTR of the *LDLR* and *ABCA1*, and its inhibition in mice results in a significant decrease in circulating cholesterol and TAG. In addition to its role in controlling lipoprotein metabolism, targeting miR-128-1 in vivo also improved glucose tolerance and insulin sensitivity. Mechanistically, the authors found that miR-128-1 regulates the expression of the insulin receptor (INSR) and insulin receptor substrate 1 (IRS-1) and downstream phosphorylation levels of Akt. Finally, it was also found that miR-128-1 controls the expression of genes associated with fatty acid synthesis, including the fatty acid synthase (FASN) and SIRT1, an NAD^+-dependent energy sensor and deacetylase that can directly deacetylate and inactivate SREBP1 and thus modulate SREBP1-dependent lipogenesis. Altogether, these observations suggest that miR-128a expression might influence plasma lipid levels by regulating the expression of genes associated with lipid and glucose metabolism. Further studies are necessary to define the specific contribution of miR-128a target genes in different tissues and their impact on regulating lipid and glucose metabolism.

2.3.2.3 miR-185

Several reports have recently shown that miR-185 regulates cholesterol metabolism in vitro and in vivo by regulating the expression of LDLR and SRBI, a hepatic HDL-C receptor that facilitates the uptake of cholesteryl esters from HDL in the liver (Jiang et al. 2015; Wang et al. 2013; Yang et al. 2014). Interestingly, miR-185 also targets KH-type splicing regulatory protein (KSRP), a RNA-binding protein that negatively regulates the expression of the human LDLR (Jiang et al. 2015). These findings suggest that inhibiting miR-185 in vivo might attenuate atherosclerosis by reducing circulating LDL-C and promoting reverse cholesterol transport. Indeed, a recent study demonstrated that inhibition of miR-185 in the atheroprone *ApoE*$^{-/-}$ mice significantly reduced plasma cholesterol levels and attenuated the progression of atherosclerosis (Jiang et al. 2015).

2.4 LncRNAs and Cholesterol Metabolism

While the contribution of miRNAs in regulating lipoprotein metabolism has been deeply studied in the last few years, the role of lncRNAs in controlling cholesterol homeostasis has just started to emerge. Recent studies have demonstrated the important contribution of lncRNAs as key regulators of cholesterol metabolism (Fig. 2.3).

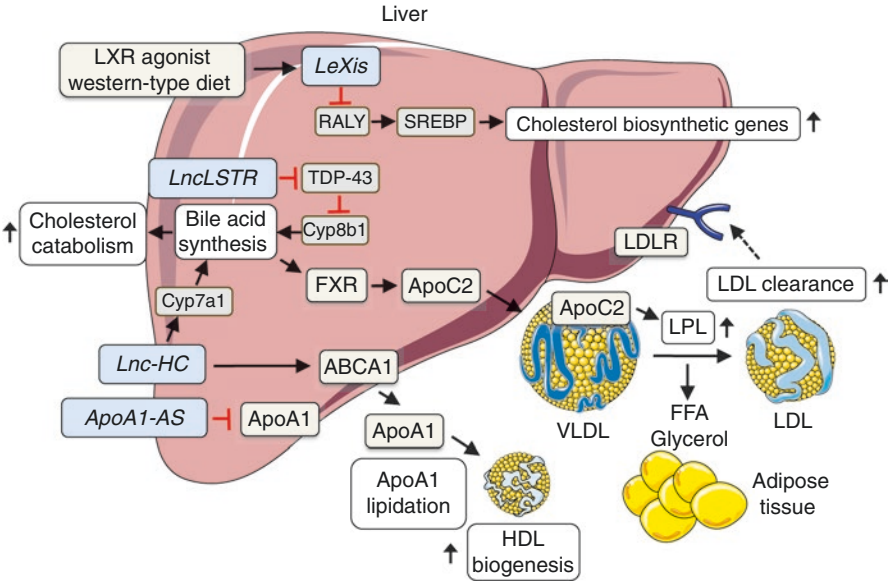

Fig. 2.3 LncRNA regulation of cholesterol metabolism. Schematic overview of lncRNAs involved in the regulation of hepatic cholesterol homeostasis and lipoprotein metabolism. *Blue boxes* highlight lncRNAs, which regulate genes that control cholesterol metabolism. *FXR* indicates farnesoid X receptor, *LeXis* liver-expressed LXR-induced sequence, *LncLSTR* liver-specific triglyceride regulator, *ApoA1-AS* ApoA1 antisense, *LPL* lipoprotein lipase, and *TDP-43* TAR DNA-binding protein 43 (Figure was created using the Servier Medical Art illustration resources (http://www.servier.com))

2.4.1 LeXis

Tontonoz's group has recently identified liver-expressed LXR-induced sequence (*LeXis*), a liver noncoding RNA that is markedly induced in response to LXR agonists and high-fat diet (Sallam et al. 2016). Hepatic *LeXis* overexpression reduces circulating cholesterol, attenuates cholesterol biosynthesis, and inhibits the expression of cholesterol biosynthetic genes. Conversely, genetic ablation of *LeXis* or acute pharmacological inhibition using ASOs enhances the hepatic expression of genes associated with cholesterol biosynthesis, leading to a significant accumulation of cholesterol in the liver. Mechanistically, the authors found that *LeXis* interacts with and influences the binding of RALY to DNA. The authors hypothesize that RALY might cooperate with SREBP2 to control the expression of cholesterol biosynthetic genes. However, further studies are needed to support this hypothesis. Another important question that remains to be addressed is the study of the contribution of *LeXis* in regulating cholesterol metabolism in humans. Importantly, mouse and human genomic comparison revealed a moderate conservation on the *LeXis* genetic locus in a region adjacent to the human *ABCA1* gene.

Further experiments are warranted to determine whether the putative lncRNA annotated in this region (TCONS_00016452) regulates cholesterol metabolism in humans.

2.4.2 Lnc-HC and ApoA1-AS

Other lncRNAs that have been associated to the regulation of cholesterol metabolism include *Lnc-HC* and *ApoA1-AS* (Halley et al. 2014; Lan et al. 2016). *Lnc-HC* is highly expressed in the liver and interacts with hnRNPA2B1 forming a RNA-protein complex, which can then bind to the target mRNAs, *Cyp7a1* and *Abca1* (Lan et al. 2016). Inhibition of *Lnc-HC* increased *Cyp7a1* and *Abca1* expression in hepatocytes, thus promoting cholesterol catabolism. *Lnc-HC* is conserved in humans and rodents and appears to be highly expressed in the liver and fat (Lan et al. 2016). These observations suggest that *Lnc-HC* might play a role in regulating lipid metabolism. However, additional studies in vivo are needed to define the role of *Lnc-HC* in regulating hepatic lipid homeostasis and lipoprotein metabolism. Another interesting finding is the identification of an antisense LncRNA (*ApoA1-AS*) encoded in the apolipoprotein gene cluster that contains four different transcripts including *ApoA1*, *ApoA4*, *ApoA5*, and *ApoC3*. *ApoA1-AS* controls the expression of the apolipoprotein gene cluster epigenetically by recruiting histone-modifying enzymes (Halley et al. 2014). Targeting *ApoA1-AS* using ASOs increases ApoA1 expression in both monkey and human cells and enhances hepatic RNA and protein expression in African green monkeys. While these results are of interest, it is still not known whether the increase in circulating ApoA1 influences plasma lipid levels and/or lipoprotein metabolism.

2.4.3 LncLSTR

Liver-specific triglyceride regulator (LncLSTR) was identified using an unbiased screen aimed to determine lncRNAs highly expressed in the liver (Li et al. 2015). Of note, specific inhibition of LncLSTR leads to a marked reduction in circulating TAG. Mechanistically, LncLSTR depletion increases apoC2 levels, an activator of the lipoprotein lipase (LPL), thus enhancing VLDL and chylomicron catabolism, leading to an increase in plasma TAG clearance (Li et al. 2015). Hepatic LncLSTR expression is regulated by FXR and forms a complex with TDP-43, thus regulating Cyp8b1 expression, a critical enzyme involved in bile acid synthesis.

2.5 Concluding Remarks

Work in recent years has clearly established both miRNAs and lncRNAs as important regulators of lipid metabolism. Since the human genome encodes thousands of lncRNAs, it is expected that in the near future, other lncRNAs will also be identified

as major regulators of lipid metabolism. Despite this exciting future, the analysis and the identification of functional lncRNAs will be challenging because of the modest conservation of these RNA molecules between species. Additionally, a more complete understanding of the mechanisms by which these lncRNAs excerpt their effects and additional studies directly assessing the impact of the lncRNAs on atherosclerotic plaque formation will be needed to properly assess the therapeutic potential of lncRNA-based therapeutic approaches.

On the other hand, a large amount of work has been done demonstrating that miRNAs are capable of targeting key factors regulating lipid metabolism and can have an important impact on the development and progression of atherosclerosis in animal models. As such, miRNA-based therapies may provide useful complimentary approaches for the treatment of atherosclerosis in humans. However, the complicated role of many miRNAs in targeting numerous different genes in different tissues and under different physiologic conditions raises concerns that these therapeutic approaches may result in unintended and possibly detrimental outcomes. These risks are highlighted by the adverse outcomes apparent in some experiments examining inhibition/ablation of miR-33 and miR-122. As such, additional studies including tissue-specific knockouts and careful target gene assessment will be important to understand the full impact of miRNA alterations prior to developing treatment strategies for human patients.

Conflict of Interest The authors declare that they have no conflict of interest.

Human and Animal Rights N/A

References

Allen RM, Marquart TJ, Albert CJ, Suchy FJ, Wang DQ, Ananthanarayanan M, Ford DA, Baldan A. miR-33 controls the expression of biliary transporters, and mediates statin- and diet-induced hepatotoxicity. EMBO Mol Med. 2012;4:882–95.

Allen RM, Marquart TJ, Jesse JJ, Baldan A. Control of very low-density lipoprotein secretion by N-ethylmaleimide-sensitive factor and miR-33. Circ Res. 2014;115:10–22.

Alvarez ML, Khosroheidari M, Eddy E, Done SC. MicroRNA-27a decreases the level and efficiency of the LDL receptor and contributes to the dysregulation of cholesterol homeostasis. Atherosclerosis. 2015;242:595–604.

Brooks-Wilson A, Marcil M, Clee SM, Zhang LH, Roomp K, Van Dam M, Yu L, Brewer C, Collins JA, Molhuizen HO, Loubser O, Ouelette BF, Fichter K, Ashbourne-Excoffon KJ, Sensen CW, Scherer S, Mott S, Denis M, Martindale D, Frohlich J, Morgan K, Koop B, Pimstone S, Kastelein JJ, Genest Jr J, Hayden MR. Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. Nat Genet. 1999;22:336–45.

Brown MS, Goldstein JL. Receptor-mediated control of cholesterol metabolism. Science. 1976;191:150–4.

Davalos A, Goedeke L, Smibert P, Ramirez CM, Warrier NP, Andreo U, Cirera-Salinas D, Rayner K, Suresh U, Pastor-Pareja JC, ESPLUGUES E, Fisher EA, Penalva LO, Moore KJ, Suarez Y, Lai EC, Fernandez-Hernando C. miR-33a/b contribute to the regulation of fatty acid metabolism and insulin signaling. Proc Natl Acad Sci U S A. 2011;108:9232–7.

De Aguiar Vallim T, Tarling E, Kim T, Civelek M, Baldan A, Esau C, Edwards P. MicroRNA-144 regulates hepatic ABCA1 and plasma HDL following activation of the nuclear receptor FXR. Circ Res. 2013;112(12):1602–12.

Do R, Willer CJ, Schmidt EM, Sengupta S, Gao C, Peloso GM, Gustafsson S, Kanoni S, Ganna A, Chen J, Buchkovich ML, Mora S, Beckmann JS, Bragg-Gresham JL, Chang HY, Demirkan A, Den Hertog HM, Donnelly LA, Ehret GB, Esko T, Feitosa MF, Ferreira T, Fischer K, Fontanillas P, Fraser RM, Freitag DF, Gurdasani D, Heikkila K, Hypponen E, Isaacs A, Jackson AU, Johansson A, Johnson T, Kaakinen M, Kettunen J, Kleber ME, Li X, Luan J, Lyytikainen LP, Magnusson PK, Mangino M, Mihailov E, Montasser ME, Muller-Nurasyid M, Nolte IM, O'Connell JR, Palmer CD, Perola M, Petersen AK, Sanna S, Saxena R, Service SK, Shah S, Shungin D, Sidore C, Song C, Strawbridge RJ, Surakka I, Tanaka T, Teslovich TM, Thorleifsson G, Van Den Herik EG, Voight BF, Volcik KA, Waite LL, Wong A, Wu Y, Zhang W, Absher D, Asiki G, Barroso I, Been LF, Bolton JL, Bonnycastle LL, Brambilla P, Burnett MS, Cesana G, Dimitriou M, Doney AS, Doring A, Elliott P, Epstein SE, Eyjolfsson GI, Gigante B, Goodarzi MO, Grallert H, Gravito ML, Groves CJ, Hallmans G, Hartikainen AL, Hayward C, Hernandez D, Hicks AA, Holm H, Hung YJ, Illig T, Jones MR, Kaleebu P, Kastelein JJ, Khaw KT, et al. Common variants associated with plasma triglycerides and risk for coronary artery disease. Nat Genet. 2013;45:1345–52.

Elmen J, Lindow M, Silahtaroglu A, Bak M, Christensen M, Lind-Thomsen A, Hedtjarn M, Hansen JB, Hansen HF, Straarup EM, Mccullagh K, Kearney P, Kauppinen S. Antagonism of microRNA-122 in mice by systemically administered LNA-antimiR leads to up-regulation of a large set of predicted target mRNAs in the liver. Nucleic Acids Res. 2008;36:1153–62.

Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Pear M, Watts L, Booten SL, Graham M, Mckay R, Subramaniam A, Propp S, Lollo BA, Freier S, Bennett CF, Bhanot S, Monia BP. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. Cell Metab. 2006;3:87–98.

Gelissen IC, Harris M, Rye KA, Quinn C, Brown AJ, Kockx M, Cartland S, Packianathan M, Kritharides L, Jessup W. ABCA1 and ABCG1 synergize to mediate cholesterol export to apoA-I. Arterioscler Thromb Vasc Biol. 2006;26:534–40.

Gerin I, Clerbaux LA, Haumont O, Lanthier N, DAS AK, Burant CF, Leclercq IA, Macdougald OA, Bommer GT. Expression of miR-33 from an SREBP2 intron inhibits cholesterol export and fatty acid oxidation. J Biol Chem. 2010;285:33652–61.

Glass CK, Witztum JL. Atherosclerosis. the road ahead. Cell. 2001;104:503–16.

Global Lipids Genetics Consortium, Willer CJ, Schmidt EM, Sengupta S, Peloso GM, Gustafsson S, Kanoni S, Ganna A, Chen J, Buchkovich ML, Mora S, Beckmann JS, Bragg-Gresham JL, Chang HY, Demirkan A, Den Hertog HM, Do R, Donnelly LA, Ehret GB, Esko T, Feitosa MF, Ferreira T, Fischer K, Fontanillas P, Fraser RM, Freitag DF, Gurdasani D, Heikkila K, Hypponen E, Isaacs A, Jackson AU, Johansson A, Johnson T, Kaakinen M, Kettunen J, Kleber ME, Li X, Luan J, Lyytikainen LP, Magnusson PK, Mangino M, Mihailov E, Montasser ME, Muller-Nurasyid M, Nolte IM, O'Connell JR, Palmer CD, Perola M, Petersen AK, Sanna S, Saxena R, Service SK, Shah S, Shungin D, Sidore C, Song C, Strawbridge RJ, Surakka I, Tanaka T, Teslovich TM, Thorleifsson G, Van Den Herik EG, Voight BF, Volcik KA, Waite LL, Wong A, Wu Y, Zhang W, Absher D, Asiki G, Barroso I, Been LF, Bolton JL, Bonnycastle LL, Brambilla P, Burnett MS, Cesana G, Dimitriou M, Doney AS, Doring A, Elliott P, Epstein SE, Eyjolfsson GI, Gigante B, Goodarzi MO, Grallert H, Gravito ML, Groves CJ, Hallmans G, Hartikainen AL, Hayward C, Hernandez D, Hicks AA, Holm H, Hung YJ, Illig T, Jones MR, Kaleebu P, Kastelein JJ, Khaw KT, et al. Discovery and refinement of loci associated with lipid levels. Nat Genet. 2013;45:1274–83.

Goedeke L, Rotllan N, Canfran-Duque A, Aranda JF, Ramirez CM, Araldi E, Lin CS, Anderson NN, Wagschal A, De Cabo R, Horton JD, Lasuncion MA, Naar AM, Suarez Y, Fernandez-Hernando C. MicroRNA-148a regulates LDL receptor and ABCA1 expression to control circulating lipoprotein levels. Nat Med. 2015a;21:1280–9.

Goedeke L, Rotllan N, Ramirez CM, Aranda JF, Canfran-Duque A, Araldi E, Fernandez-Hernando A, Langhi C, De Cabo R, Baldan A, Suarez Y, Fernandez-Hernando C. miR-27b inhibits LDLR and ABCA1 expression but does not influence plasma and hepatic lipid levels in mice. Atherosclerosis. 2015b;243:499–509.

Goedeke L, Salerno A, Ramirez CM, Guo L, Allen RM, Yin X, Langley SR, Esau C, Wanschel A, Fisher EA, Suarez Y, Baldan A, Mayr M, Fernandez-Hernando C. Long-term therapeutic

silencing of miR-33 increases circulating triglyceride levels and hepatic lipid accumulation in mice. EMBO Mol Med. 2014;6:1133–41.

Grundy SM. Promise of low-density lipoprotein-lowering therapy for primary and secondary prevention. Circulation. 2008:117;569–73; discussion 573.

Grundy SM, Cleeman JI, Merz CN, Brewer Jr HB, Clark LT, Hunninghake DB, Pasternak RC, Smith Jr SC, Stone NJ, Coordinating Committee of the National Cholesterol Education Program. Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III Guidelines. J Am Coll Cardiol. 2004;44:720–32.

Halley P, Kadakkuzha BM, Faghihi MA, Magistri M, Zeier Z, Khorkova O, Coito C, Hsiao J, Lawrence M, Wahlestedt C. Regulation of the apolipoprotein gene cluster by a long noncoding RNA. Cell Rep. 2014;6:222–30.

Horie T, Baba O, Kuwabara Y, Chujo Y, Watanabe S, Kinoshita M, Horiguchi M, Nakamura T, Chonabayashi K, Hishizawa M, Hasegawa K, Kume N, Yokode M, Kita T, Kimura T, Ono K. MicroRNA-33 deficiency reduces the progression of atherosclerotic plaque in ApoE−/− mice. J Am Heart Assoc. 2012;1:e003376.

Horie T, Nishino T, BABA O, Kuwabara Y, NAKAO T, NISHIGA M, Usami S, Izuhara M, Nakazeki F, Ide Y, Koyama S, Sowa N, Yahagi N, Shimano H, Nakamura T, Hasegawa K, Kume N, Yokode M, Kita T, Kimura T, Ono K. MicroRNA-33b knock-in mice for an intron of sterol regulatory element-binding factor 1 (Srebf1) exhibit reduced HDL-C in vivo. Sci Rep. 2014;4:5312.

Horie T, Nishino T, Baba O, Kuwabara Y, Nakao T, Nishiga M, Usami S, Izuhara M, Sowa N, Yahagi N, Shimano H, Matsumura S, Inoue K, Marusawa H, Nakamura T, Hasegawa K, Kume N, Yokode M, Kita T, kimura T, Ono K. MicroRNA-33 regulates sterol regulatory element-binding protein 1 expression in mice. Nat Commun. 2013;4:2883.

Horie T, Ono K, Horiguchi M, Nishi H, Nakamura T, Nagao K, Kinoshita M, Kuwabara Y, Marusawa H, Iwanaga Y, Hasegawa K, Yokode M, Kimura T, Kita T. MicroRNA-33 encoded by an intron of sterol regulatory element-binding protein 2 (Srebp2) regulates HDL in vivo. Proc Natl Acad Sci U S A. 2010;107:17321–6.

Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest. 2002;109:1125–31.

Hu YW, Hu YR, Zhao JY, Li SF, Ma X, Wu SG, Lu JB, Qiu YR, Sha YH, Wang YC, Gao JJ, Zheng L, Wang Q. An agomir of miR-144-3p accelerates plaque formation through impairing reverse cholesterol transport and promoting pro-inflammatory cytokine production. PLoS One. 2014;9:e94997.

Huan T, Rong J, Liu C, Zhang X, Tanriverdi K, Joehanes R, Chen BH, Murabito JM, Yao C, Courchesne P, Munson PJ, O'Donnell CJ, Cox N, Johnson AD, Larson MG, Levy D, Freedman JE. Genome-wide identification of microRNA expression quantitative trait loci. Nat Commun. 2015;6:6601.

Irani S, Pan X, Peck BC, Iqbal J, Sethupathy P, Hussain MM. MicroRNA-30c mimic mitigates hypercholesterolemia and atherosclerosis in mice. J Biol Chem. 2016;291:18397–409.

Jeon TI, Esquejo RM, Roqueta-Rivera M, Phelan PE, Moon YA, Govindarajan SS, Esau CC, Osborne TF. An SREBP-responsive microRNA operon contributes to a regulatory loop for intracellular lipid homeostasis. Cell Metab. 2013;18:51–61.

Jiang H, Zhang J, Du Y, Jia X, Yang F, Si S, Wang L, Hong B. microRNA-185 modulates low density lipoprotein receptor expression as a key posttranscriptional regulator. Atherosclerosis. 2015;243:523–32.

Karunakaran D, Richards L, Geoffrion M, Barrette D, Gotfrit RJ, Harper ME, Rayner KJ. Therapeutic inhibition of miR-33 promotes fatty acid oxidation but does not ameliorate metabolic dysfunction in diet-induced obesity. Arterioscler Thromb Vasc Biol. 2015a;35(12):2536–43.

Karunakaran D, Thrush AB, Nguyen MA, Richards L, Geoffrion M, Singaravelu R, Ramphos E, Shangari P, Ouimet M, Pezacki JP, Moore KJ, Perisic L, Maegdefessel L, Hedin U, Harper ME, Rayner KJ. Macrophage mitochondrial energy status regulates cholesterol efflux and is enhanced by anti-miR33 in atherosclerosis. Circ Res. 2015b;117:266–78.

Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, Stoffel M. Silencing of microRNAs in vivo with 'antagomirs'. Nature. 2005;438:685–9.

Lan X, Yan J, Ren J, Zhong B, Li J, Li Y, Liu L, Yi J, Sun Q, Yang X, Sun J, Meng L, Zhu W, Holmdahl R, Li D, Lu S. A novel long noncoding RNA Lnc-HC binds hnRNPA2B1 to regulate expressions of Cyp7a1 and Abca1 in hepatocytic cholesterol metabolism. Hepatology. 2016;64:58–72.

Li T, Francl JM, Boehme S, Chiang JY. Regulation of cholesterol and bile acid homeostasis by the cholesterol 7α-hydroxylase/steroid response element-binding protein 2/microRNA-33a axis in mice. Hepatology. 2013;58(3):1111–21. doi: 10.1002/hep.26427.

Li P, Ruan X, Yang L, Kiesewetter K, Zhao Y, Luo H, Chen Y, Gucek M, Zhu J, Cao H. A liver-enriched long non-coding RNA, lncLSTR, regulates systemic lipid metabolism in mice. Cell Metab. 2015;21:455–67.

Lusis AJ. Atherosclerosis. Nature. 2000;407:233–41.

Marquart TJ, Allen RM, Ory DS, Baldan A. miR-33 links SREBP-2 induction to repression of sterol transporters. Proc Natl Acad Sci U S A. 2010;107:12228–32.

Marquart TJ, Wu J, Lusis AJ, Baldan A. Anti-miR-33 therapy does not alter the progression of atherosclerosis in low-density lipoprotein receptor-deficient mice. Arterioscler Thromb Vasc Biol. 2013;33:455–8.

Meiler S, Baumer Y, Toulmin E, SENG K, Boisvert WA. MicroRNA 302a is a novel modulator of cholesterol homeostasis and atherosclerosis. Arterioscler Thromb Vasc Biol. 2015;35:323–31.

Najafi-Shoushtari SH, Kristo F, Li Y, Shioda T, Cohen DE, Gerszten RE, Naar AM. MicroRNA-33 and the SREBP host genes cooperate to control cholesterol homeostasis. Science. 2010; 238:1566–9.

Oram JF, Vaughan AM. ABCA1-mediated transport of cellular cholesterol and phospholipids to HDL apolipoproteins. Curr Opin Lipidol. 2000;11:253–60.

Ouimet M, Ediriweera HN, Gundra UM, Sheedy FJ, Ramkhelawon B, Hutchison SB, Rinehold K, Van Solingen C, Fullerton MD, Cecchini K, Rayner KJ, Steinberg GR, Zamore PD, Fisher EA, Loke P, Moore KJ. MicroRNA-33-dependent regulation of macrophage metabolism directs immune cell polarization in atherosclerosis. J Clin Invest. 2015;125:4334–48.

Ramirez CM, Goedeke L, Rotllan N, Yoon JH, Cirera-Salinas D, Mattison JA, Suarez Y, De cabo R, Gorospe M, Fernandez-Hernando C. MicroRNA 33 regulates glucose metabolism. Mol Cell Biol. 2013a;33:2891–902.

Ramirez CM, Rotllan N, Vlassov AV, Davalos A, Li M, Goedeke L, Aranda JF, Cirera-Salinas D, Araldi E, Salerno A, Wanschel AC, Zavadil J, Castrillo A, Jungsu K, Suarez Y, Fernandez-Hernando C. Control of cholesterol metabolism and plasma HDL levels by miRNA-144. Circ Res. 2013b;112(12):1592–601.

Rayner KJ, Esau CC, Hussain FN, Mcdaniel AL, Marshall SM, Van Gils JM, Ray TD, Sheedy FJ, Goedeke L, Liu X, khatsenko OG, Kaimal V, Lees CJ, Fernandez-Hernando C, Fisher EA, Temel RE, Moore KJ. Inhibition of miR-33a/b in non-human primates raises plasma HDL and lowers VLDL triglycerides. Nature. 2011a;478:404–7.

Rayner KJ, Sheedy FJ, Esau CC, Hussain FN, Temel RE, Parathath S, Van Gils JM, Rayner AJ, Chang AN, Suarez Y, Fernandez-Hernando C, Fisher EA, Moore KJ. Antagonism of miR-33 in mice promotes reverse cholesterol transport and regression of atherosclerosis. J Clin Invest. 2011b;121:2921–31.

Rayner KJ, Suarez Y, Davalos A, Parathath S, Fitzgerald ML, Tamehiro N, Fisher EA, Moore KJ, Fernandez-Hernando C. miR-33 Contributes to the regulation of cholesterol homeostasis. Science. 2010;238:1570–3.

Rotllan N, Ramirez CM, Aryal B, Esau CC, Fernandez-Hernando C. Therapeutic silencing of microRNA-33 inhibits the progression of atherosclerosis in Ldlr−/− mice--brief report. Arterioscler Thromb Vasc Biol. 2013;33:1973–7.

Rottiers V, Obad S, Petri A, Mcgarrah R, Lindholm MW, Black JC, Sinha S, Goody RJ, Lawrence MS, Delemos AS, Hansen HF, Whittaker S, Henry S, Brookes R, Najafi-Shoushtari SH, Chung RT, Whetstine JR, Gerszten RE, Kauppinen S, Naar AM. Pharmacological inhibition of a

microRNA family in nonhuman primates by a seed-targeting 8-mer antimiR. Sci Transl Med. 2013;5:212ra162.

Sallam T, Jones MC, Gilliland T, Zhang L, Wu X, Eskin A, Sandhu J, Casero D, Vallim TQ, Hong C, Katz M, Lee R, Whitelegge J, Tontonoz P. Feedback modulation of cholesterol metabolism by the lipid-responsive non-coding RNA LeXis. Nature. 2016;534:124–8.

Soh J, IqbAL J, Queiroz J, Fernandez-Hernando C, Hussain MM. MicroRNA-30c reduces hyperlipidemia and atherosclerosis in mice by decreasing lipid synthesis and lipoprotein secretion. Nat Med. 2013;19:892–900.

Steinberg D. The statins in preventive cardiology. N Engl J Med. 2008;359:1426–7.

Steinberg D, Glass CK, Witztum JL. Evidence mandating earlier and more aggressive treatment of hypercholesterolemia. Circulation. 2008;118:672–7.

Vickers KC, Landstreet SR, Levin MG, Shoucri BM, Toth CL, Taylor RC, Palmisano BT, Tabet F, Cui HL, Rye KA, Sethupathy P, Remaley AT. MicroRNA-223 coordinates cholesterol homeostasis. Proc Natl Acad Sci U S A. 2014;111:14518–23.

Wagschal A, Najafi-Shoushtari SH, Wang L, Goedeke L, Sinha S, Delemos AS, Black JC, Ramirez CM, Li Y, Tewhey R, Hatoum I, Shah N, Lu Y, Kristo F, Psychogios N, Vrbanac V, Lu YC, Hla T, De Cabo R, Tsang JS, Schadt E, Sabeti PC, Kathiresan S, Cohen DE, Whetstine J, Chung RT, Fernandez-Hernando C, Kaplan LM, Bernards A, Gerszten RE, Naar AM. Genome-wide identification of microRNAs regulating cholesterol and triglyceride homeostasis. Nat Med. 2015;21:1290–7.

Wang D, Xia M, Yan X, Li D, Wang L, Xu Y, Jin T, Ling W. Gut microbiota metabolism of anthocyanin promotes reverse cholesterol transport in mice via repressing miRNA-10b. Circ Res. 2012;111:967–81.

Wang L, Jia XJ, Jiang HJ, Du Y, Yang F, Si SY, Hong B. MicroRNAs 185, 96, and 223 repress selective high-density lipoprotein cholesterol uptake through posttranscriptional inhibition. Mol Cell Biol. 2013;33:1956–64.

Yang M, Liu W, Pellicane C, Sahyoun C, Joseph BK, Gallo-Ebert C, Donigan M, Pandya D, Giordano C, Bata A, Nickels Jr JT. Identification of miR-185 as a regulator of de novo cholesterol biosynthesis and low density lipoprotein uptake. J Lipid Res. 2014;55:226–38.

MicroRNAs in Diabetes and Its Vascular Complications

3

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Abstract

Diabetes is a complex, metabolic disorder that is characterised by chronic hyperglycaemia and is widely recognised as a major health threat worldwide. The major causes of morbidity and mortality in diabetes result from vascular complications. These can be both microvascular, resulting in retinopathy, nephropathy and neuropathy, and macrovascular, affecting the heart and peripheral vessels. In this chapter, the roles of microRNAs in the development of diabetes are described. In addition, we show how microRNA-mediated mechanisms in diabetes result in an impaired vascular reparative potential, acting via the bone marrow and both stem and progenitor cells. Finally, we discuss the roles of microRNAs in the development of specific microvascular and macrovascular complications in the context of diabetes, and describe the potential of microRNAs as circulating biomarkers of diabetic cardiovascular disease.

3.1 Introduction

Diabetes mellitus (DM) is a complex, multisystem metabolic disorder that is widely recognized as a major health threat of this century (Shaw et al. 2010). The worldwide prevalence is estimated at almost 400 million (International Diabetes Federation 2013). Diabetes is characterized by chronic hyperglycaemia secondary to impaired insulin secretion and/or a decreased responsiveness of target tissues to insulin. The main forms of DM are type 1 DM (T1DM) and type 2 DM (T2DM). DM may also be triggered by pregnancy (gestational DM), the use of drugs and infections (American Diabetes Association 2005). T1DM results from autoimmune pancreatic β-cell destruction, with a consequent failure to produce insulin (Ilonen and Akerblom 1999). T2DM is characterized by peripheral insulin resistance accompanied by insufficient compensatory insulin secretion (Kahn 2001). Although T2DM is far more prevalent (accounting for at least 90% of diabetes), both types can result in complications (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 2003). Many of these complications are vascular in origin and can be microvascular (e.g. nephropathy, retinopathy) or macrovascular (e.g. ischaemic heart disease, peripheral vascular disease) (Winer and Sowers 2004; Brownlee 2001). Cardiovascular disease (CVD) is the major cause of death in diabetes, and the risk of CVD is doubled with diabetes (Emerging Risk Factors Coalition et al. 2010).

The pathophysiology of the vascular complications of diabetes is complex and, to date, not completely understood. Hyperglycaemia-induced vascular damage is the principal overarching mechanism, although coexisting processes are essential. Interestingly, recent studies suggest that microRNAs (miRNAs, miRs) play important roles in this context. As introduced elsewhere in this book, microRNAs are small (~22 nucleotides) noncoding RNA molecules that are synthesized endogenously. They can modulate both physiological and pathological pathways by post-transcriptionally inhibiting the expression of several target genes (van Rooij 2011). MicroRNAs are highly expressed in the vasculature, and their involvement

in angiogenesis and endothelial function is well described (Caporali and Emanueli 2011; Condorelli et al. 2014). Of particular relevance to this chapter, some miRNAs have been associated with the development and/or progression of diabetic vascular complications, although a complete understanding of their actions is still lacking (Shantikumar et al. 2012). It could be hypothesized that miRNAs act as mediators of the damaging effects of hyperglycaemia and/or that they are expressionally dysregulated as a consequence of them. It is noteworthy that other biological factors are at play in diabetes, including alterations in lipid profiles, oxidative stress and an increase in pro-inflammatory cytokines (Tabak et al. 2011), and these factors are likely to be associated with microRNAs.

In this chapter, we review recent findings regarding the role of miRNAs in the pathophysiology of diabetes, but we highlight their actions specifically in microvascular and macrovascular complications. We describe known mechanisms of action of miRNAs in the diabetic milieu on diverse cell types such as pancreatic, adipose, endothelial and cardiac cells. Finally, the potential therapeutic and prognostic value of miRNAs in diabetes and its vascular complications will be discussed.

3.2 MicroRNAs in Diabetes Mellitus

3.2.1 Pancreas

The critical role of miRs in the development and function of pancreatic β-cells became evident when it was demonstrated that deletion of the nuclear ribonuclease III enzyme Dicer in the pancreas of mice led to abnormal β-cell development (Lynn et al. 2007). One of the most abundant miRNAs present in islet cells is miR-375. MiR-375 negatively regulates glucose-stimulated insulin secretion (GSIS). High glucose concentrations reduce the expression of miR-375, and the subsequent derepression of its two main targets myotrophin (Mtpn, involved in insulin granule fusion) and phosphoinositide-dependent protein kinase (PDK1, a component of the phosphatidylinositol 3-kinase signalling cascade) induces insulin secretion (Poy et al. 2004; El Ouaamari et al. 2008). MiR-375 levels are upregulated in the pancreatic islets of *ob/ob* mice (a model of obesity, insulin resistance and T2DM) (Poy et al. 2009), and deletion of miR-375 in these mice reduces the proliferative capacity of the endocrine pancreas and dramatically worsens the diabetic state (Poy et al. 2009). MiR-124a and miR-9 inhibit GSIS by targeting FOXa2 and Onecut-2, respectively, both transcription factors associated with insulin secretion (Plaisance et al. 2006; Puigserver and Rodgers 2006). Recently, in human pancreatic islets from type 2 diabetic patients, miR-187 was found to be upregulated, where it is involved in reducing GSIS through inhibition of homeodomain-interacting protein kinase-3 (HIPK3) (Locke et al. 2014). High levels of miR-34a are found in the islets of T2DM *db/*db mice, and this is associated with a reduced expression of the anti-apoptotic protein BCL2 and an increased β-cell death (Lovis et al. 2008). MiR-335 is upregulated in the islets of Goto-Kakizaki rats (a nonobese model of T2DM) where it targets the exocytotic protein Stxbp1 (Esguerra et al. 2011). A summary of the role of miRNAs in pancreatic β-cell dysfunction is found in Table 3.1.

Table 3.1 MicroRNAs involved in pancreatic β-cell dysfunction

microRNA	Change in diabetes	Target	Functional result	Reference
miR-9	Up	Onecut2	Decreases insulin secretion	Plaisance et al. (2006)
miR-34a	Up	Bcl2	Increases apoptosis	Lovis et al. (2008)
		VAMP2	Decreases insulin secretion	Lovis et al. (2008)
miR-96	Up	Noc2	Decreases insulin secretion	Lovis et al. (2008)
miR-124a	Up	FOXa2	Decreases β-cell maturation	Puigserver and Rodgers (2006)
miR-187	Up	HIPK3	Decreases insulin secretion	Locke et al. (2014)
miR-335	Up	Stxbp1	Decreases insulin secretion	Esguerra et al. (2011)
miR-375	Up	Myotrophin	Decreases insulin secretion	Poy et al. (2004)
		PDK1	Decreases insulin transcription	El Ouaamari et al. (2008)

3.2.2 Adipose Tissue

Insulin resistance describes the failure of target tissues to respond adequately to circulating insulin, and several miRNAs have been found to regulate insulin signalling in these tissues. Adipose tissue in the context of T2DM and obesity is one of the main tissues experiencing acute insulin resistance. It is also an important contributor to the systemic chronic inflammatory state characteristic of diabetes, as macrophage infiltration results in adipocytes releasing pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α) or interleukin-6 (IL-6), and adipokines including leptin and resistin (Jung and Choi 2014). Some miRNAs that are induced during adipogenesis are downregulated in obesity, and this is a potential mechanism for insulin resistance (Xie et al. 2009).

MiR-143 is downregulated in the adipocytes from *ob/ob* mice resulting in increased adipogenesis through the target ERK5, which promotes cell differentiation and proliferation (Xie et al. 2009; Esau et al. 2004). Similar roles in adipogenesis have been attributed to miR-103 and miR-107: they are repressed in adipocytes following TNF-α treatment, and their inhibition enhances insulin signalling, decreases adipocyte size and enhances insulin-stimulated glucose uptake (Xie et al. 2009). MiR-320 is upregulated in insulin-resistant adipocytes, where it decreases insulin sensitivity by targeting p85 (which normally contributes to cell growth by increasing Akt phosphorylation) (Ling et al. 2009). It is well known that the co-occurrence and feedback between inflammation and insulin

resistance in the adipose tissue are crucial in the onset of T2DM. Interestingly, selected miRs may link these two main processes, highlighting their therapeutic potential for preventing insulin resistance. In this context, miR-132, which is upregulated in the omental fat of obese humans (Heneghan et al. 2011), was found to activate NF-kB and subsequently increase the transcription of interleukin-8 and monocyte chemoattractant protein 1 (MCP1) in human adipocytes in vitro (Strum et al. 2009). Conversely, some miRNAs are induced by cytokines, including miR-378, which is induced by TNF-α and IL-6 in matured human adipocytes (Xu et al. 2014), and both miR-221 and miR-222, which are positively correlated with TNF-α levels in the adipose tissue of high-fat diet-fed mice (Parra et al. 2010).

3.2.3 Muscle

Insulin resistance also affects the skeletal muscle in diabetes, and some miRNAs are thought to be differentially expressed in this context. MiR-29a and miR-29b levels are upregulated in the skeletal muscle of GK rats compared to their healthy counterparts (He et al. 2007). Moreover, miR-1 and miR-133 have been shown to respond to insulin, but their regulation in the skeletal muscle of T2DM patients is impaired, which may possibly adversely affect muscle function (Granjon et al. 2009).

3.2.4 Liver

miR-29a, miR-29b, miR-103 and miR-107 expression is increased in diabetic adipose tissue, as previously stated, but they are also upregulated in in the liver of diabetic rats, suggesting a more generalized role in insulin resistance (Trajkovski et al. 2011; Herrera et al. 2010). Another miRNA involved in insulin resistance in the liver is miR-181a. Expression is increased in insulin-resistant cultured hepatocytes, as well as in the liver and in the serum of diabetic patients, and it causes insulin resistance by inhibiting SIRT1 (Zhou et al. 2012), which itself is known to improve insulin sensitivity (Stefanowicz et al. 2015). MiR-130a-3p increases insulin sensitivity in hepatocytes. Levels are decreased in the livers of *db/db* mice, but forced overexpression in this model improved liver steatosis (Xiao et al. 2014).

Taking together, these data suggest that miRNAs may participate in the onset and development of diabetes, having an influence on insulin production, secretion and action and in glucose homeostasis. More information can be found in other reviews (Shantikumar et al. 2012; Chakraborty et al. 2014; Ozcan 2014; Beltrami et al. 2014). A summary of the miRNAs involved in insulin resistance is found in Table 3.2 (Lustig et al. 2014; Najafi-Shoushtari et al. 2010; Ryu et al. 2011; Shi et al. 2007, 2014).

Table 3.2 MicroRNAs involved in insulin resistance

microRNA	Change in diabetes	Target(s)	Functional result	Reference
miR-33a/b	Up	IRS2	Decreased intracellular insulin signalling	Najafi-Shoushtari et al. (2010)
miR-103	Up	Caveolin 1	Increases adipogenesis	Trajkovski et al. (2011)
miR-107	Up	Caveolin 1	Increases adipogenesis	Trajkovski et al. (2011)
miR-126	Up	IRS1	Decreased intracellular insulin signalling	Ryu et al. (2011)
miR-143	Down	ERK5	Increases adipogenesis	Esau et al. (2004)
miR-145	Up	IRS1	Decreased intracellular insulin signalling	Shi et al. (2007)
miR-181a	Up	SIRT1	Increases insulin resistance	Zhou et al. (2012)
miR-221	Up	AdipoR1	Decreases adiponectin signalling	Lustig et al. (2014)
miR-222	Up	ERa	Increases insulin resistance in gestational diabetes	Shi et al. (2014)

3.3 MicroRNAs Modulating the Impact of Diabetes Mellitus on Bone Marrow and on Stem and Progenitor Cells

Several studies show that vascular reparative progenitor cells (PCs) are less abundant in the bone marrow (BM) and peripheral blood (PB) of patients with DM, and alterations of PC invasive and migratory capacities may contribute to the impaired vascular repair in these subjects (Fadini et al. 2010; Fadini et al. 2005; Ferraro et al. 2011; Saito et al. 2012; Spinetti et al. 2013a; Tepper et al. 2002). Studies in animal models suggest that the alteration in the spectrum of circulating cells is secondary to a deregulated control of cell mobilization from the BM (Ferraro et al. 2011; Busik et al. 2009; Krankel et al. 2008; Segal et al. 2006). In line with this, clinical data suggest that the BM of diabetic patients has an impaired capacity to release haematopoietic stem cells following stimulation with granulocyte colony-stimulating factor (G-CSF) (Ferraro et al. 2011). This defect has recently been termed "diabetic stem cell mobilopathy" (DiPersio 2011). Moreover, DM may impinge upon the integrity of stem cells (SCs)/PCs by altering the marrow microvascular microenvironment (Spinetti et al. 2013a). In a mouse model, Oikawa et al. showed that T1DM causes microvascular rarefaction, resulting in critical hypoperfusion, SC depletion at the level of the endosteal niche and altered transendothelial cell trafficking (Oikawa et al. 2010). This experimental study was followed by the first related in-human investigation by our group, demonstrating the presence of microangiopathy in the BM of diabetic patients, with or without peripheral artery disease (Spinetti et al. 2013a). In addition to vascular rarefaction, we reported a reduction

of CD34⁺ PCs in the BM of T2DM patients consequent to induction of apoptosis via downregulation of miR-155 and activation of the FOXO3a/p21/p27 signalling pathway (Spinetti et al. 2013a). The potential functions of miR-155 itself in vascular disease are conflicting, however. There is an upregulation in atherosclerotic lesions (Charo and Ransohoff 2006), but circulating miR-155 is reduced in humans with coronary artery disease (Fichtlscherer et al. 2010) and a haematopoietic deficiency in mice enhances plaque development (Donners et al. 2012). Further in vivo studies are required to delineate its role, but it is clear that miR-155 has a wider role in the inflammatory processes accompanying vascular disease.

MiRNAs are master regulators of haematopoietic and vascular cell function. It is interesting to note that miRNAs exert both an effect on BM cell maturation and on their proangiogenic function. In this respect, recent evidence indicates that miRNAs are hierarchically organized in a circuitry that controls CD34⁺ PC proliferation, viability and differentiation (Georgantas et al. 2007; O'Connell et al. 2010). In addition, miRNAs are key regulators of EC function and angiogenesis and can control postischaemic angiogenesis by acting at different levels (reviewed in (Caporali and Emanueli 2011)). Additionally, miRNAs are important for both maintaining stem cell pluripotency and inducing stem cell vascular differentiation (reviewed in (Howard et al. 2011)). Moreover, we recently showed that miR-132 is essential for the therapeutic proangiogenic actions of pericyte progenitor cells (Katare et al. 2011a). Interestingly, BM PCs produce and secrete proangiogenic and antiangiogenic miRNAs within vesicles (exosomes, microparticles, apoptotic bodies) (Deregibus et al. 2007; Sahoo et al. 2011; Wang and Olson 2009).

Exosomes are small (50–100 nm in diameter) naturally occurring vesicles actively secreted by different cells (e.g. mesenchymal cells, T and B cells, dendritic cells and tumour cells) (Thery et al. 2009; Zhu et al. 2012). Differently from microparticles, which are formed by direct budding of the cellular membrane, exosomes derive from multivesicular endosomes. They can be found in several body fluids (e.g. urine, plasma, milk) and seem to play a role both in physiological processes and in pathology (Thery et al. 2009; Tetta et al. 2011). Exosomes can transfer proteins, lipids, mRNAs and miRNAs to target cells both in a spontaneous and receptor-mediated fashion (Collino et al. 2010; Record et al. 2011; Valadi et al. 2007). Dysfunctional miRNA trafficking via exosomes secreted by CD34⁺ cells may impact on BM diabetic microangiopathy and mobilopathy. When recruited to the ischaemic site, CD34⁺ PCs support neovascularization only in part via incorporation into nascent vessels (Asahara et al. 1997), but mostly by paracrine mechanisms, through the release of soluble factors as well as proangiogenic material packaged in extracellular vesicles (Sahoo et al. 2011; Kumar and Caplice 2010). Interestingly, PC-derived extracellular vesicles have been shown to activate resident endothelial cells (ECs), at least in part through horizontal transfer of miRNAs (Fiordaliso et al. 2000). A recent publication found that circulating CD34⁺ cells isolated from T2DM patients carry less proangiogenic miR-126 in secreted exosomes, with this deficit possibly contributing to the decreased in vitro angiogenic ability of diabetic CD34⁺ PCs (Mocharla et al. 2013). Whether DM alters the

secretion of specific angio-miRNAs by BM CD34⁺ cells via exosomes still needs to be further investigated. In particular, differentially expressed/secreted CD34⁺ miRNAs could both affect BM CD34⁺ PC migration/mobilization and also interfere with BM endothelial cell function.

3.4 MicroRNAs in Diabetic Cardiovascular Complications

DM is associated with vascular complications that represent the major cause of morbidity and mortality. The whole vascular tree is affected. Microangiopathies – angiopathies of the small arterial vessels – result in retinopathy, nephropathy and neuropathy. Macroangiopathies affect the larger arterial vessels, leading to ischaemic heart disease and arteriopathy of the lower limbs and of the supra-aortic trunks. The risk of developing complications is influenced by the duration of DM and genetic factors. Current treatment measures have resulted in a partial reduction in cardiovascular risk, and improved management strategies remain a major need for people with DM.

3.4.1 Microvascular Complications

Diabetic microangiopathy is a leading cause of renal failure, blindness and non-healing foot ulcers.

3.4.1.1 Nephropathy

DM can promote ultrastructural alterations in the glomerular filtration barrier of the kidney that affect its permeability, leading to proteinuria and a decline in glomerular filtration rate. This is known as diabetic nephropathy. Poor glycaemic control and underlying hypertension worsen this condition and accelerate its progression, often resulting in end-stage renal disease (Afkarian et al. 2013). Several miRNAs have been related to the pathobiology of diabetic nephropathy. For example, the increase in transforming growth factor-β (TGF-β) that occurs during diabetic nephropathy and promotes the accumulation of extracellular matrix in the glomerulus is induced by miR-192 (Deshpande et al. 2013). MiR-192 also modulates the expression of miR-200b/c and collagen genes, contributing to glomerular hypertrophy and fibrogenesis (Kato et al. 2007). MiR-21 expression is increased in the kidneys of type 2 diabetic mice (Wang et al. 2013). Under hyperglycaemic conditions, miR-21 inhibits the tumour suppressor gene PTEN and activates Akt and TORC1 pathways, resulting in renal cell hypertrophy and fibrogenesis (Dey et al. 2011). Other miRs upregulated by high glucose levels and related to diabetic nephropathy are miR-195 and miR-377, the former involved in podocyte apoptosis by reducing expression of BCL2 and the latter contributing to fibrogenesis by inducing fibronectin expression (Chen et al. 2011; Wang et al. 2008). Some miRs have been found

to be downregulated in the kidney under diabetic conditions, suggesting that their forced expression could be a therapeutic strategy in this context. One example is the miR-29 family, repressed in proximal tubular cells under hyperglycaemia or TGF-β treatment (Chen et al. 2014a). As miR-29 targets collagens I, III and IV, its downregulation contributes to renal fibrosis by increasing the levels of these proteins (Qin et al. 2011). Vascular endothelial growth factor-A (VEGF-A) plays a key role in maintaining the integrity of the glomerulus. It has been shown that miR-93 targets VEGF-A, and miR-93 downregulation promotes an increase in VEGF-A under hyperglycaemic conditions. Furthermore, inhibiting the VEGF-A rise by forced overexpression of miR-93 improves kidney function in diabetic mice (Long et al. 2010). Thus, miR-39 overexpression could be a therapeutic strategy to prevent kidney dysfunction.

3.4.1.2 Retinopathy

Diabetic retinopathy is the most common microvascular complication. During the course of the disease, it is common to develop alterations in the retina as a consequence of chronic hyperglycaemia (American Diabetes Association 2014). Diabetic retinopathy is characterized by altered vascular permeability in the retina, capillary degeneration and exaggerated neovascularization. Several miRs have been found to be upregulated in the retinas of T1DM rats, including miR-146, miR-155, miR-132 and miR-21 – all of them NF-kB responsive (Kovacs et al. 2011). NF-kB plays an important role in the early pathogenesis of DR by triggering a pro-apoptotic program in retinal pericytes (Kowluru et al. 2003). Therefore, these miRs may reinforce the hyperglycaemia-/inflammatory-mediated vascular damage in the retina. Other upregulated miRs in diabetic endothelial retinal cells are miR-17-5p, miR-18a, miR-20a, miR-21, miR-31 and miR-155, all known to be responsive to VEGF, the most important growth factor involved in diabetic retinopathy (Kovacs et al. 2011). MiR-200b, which targets VEGF-A, is downregulated in the retinas of diabetic rats, and forced overexpression of miR-200b reversed VEGF-mediated vascular permeability (McArthur et al. 2011). A recent study has suggested that the combination of serum miR-21, miR-181c and miR-1179 as biomarkers have the ability to discriminate between proliferative and non-proliferative diabetic retinopathy (Qing et al. 2014). The above evidence highlights the potential role of miRNAs in regulating the inflammatory and neovascularization processes of diabetic retinopathy and their potential early diagnostic value for proliferative disease.

3.4.1.3 Neuropathy

Approximately half of diabetic patients develop neuropathy, consisting of peripheral nerve dysfunction that can manifest in several different forms, including sensory, focal/multifocal and autonomic neuropathies (American Diabetes Association 2014). There is a paucity of studies relating miRNAs to the development of diabetic neuropathy. A recent study suggests that hyperglycaemia downregulates miR-146a

and that this is related to increased neuronal apoptosis by increasing the expression of interleukin-1 receptor-activate kinase (IRAK1) and tumour necrosis factor-associated factor 6 (TRAF6) in a mouse model of diabetic peripheral neuropathy (Wang et al. 2014a). Indeed, several polymorphisms in miR-146a, as well as miR-128, are associated with susceptibility to diabetic neuropathy (Ciccacci et al. 2014).

3.4.2 Cardiac Disease

Heart disease represents the leading cause of death among patients with DM (Stamler et al. 1993). Epidemiological studies indicate that DM is a potent and prevalent risk factor for ischaemic heart disease and heart failure (HF) (Kannel et al. 1974; Redfield et al. 2003). In addition, the coexistence of DM with either myocardial ischaemia or HF is associated with extremely poor outcomes (Beller 2001; Bell 2003a). Although coronary atherosclerosis is frequently implicated in the development of HF in diabetic patients, recent evidence indicates that DM can cause cardiac dysfunction independently of coronary disease or hypertension (Fang et al. 2004; Bell 2003b; Poornima et al. 2006; Marwick 2006). Screening assessment of ventricular function by echocardiography and tissue Doppler revealed the presence of diastolic dysfunction in 25–75% of young, asymptomatic patients with T1DM or T2DM (Zabalgoitia et al. 2001; Boyer et al. 2004; Rajan and Gokhale 2002; Schannwell et al. 2002). T2DM patients seem to be more susceptible than T1DM subjects, possibly due to the cardio-protective action of insulin supplementation in the latter (Schaible et al. 1983). In the *Strong Heart Study* and *Heart Outcomes Prevention Evaluation* (HOPE) Trial, the degree of diastolic dysfunction and the risk of evolution to HF were proportional to the levels of microalbuminuria and presence of microvascular complications (Liu et al. 2003; Arnold et al. 2003). Because microalbuminuria is a marker of endothelial dysfunction in the kidney, it was postulated that parallel impairment of endothelial function in the myocardium may contribute to increased ventricular scarring and stiffness (Poornima et al. 2006). Mechanisms of diabetic cardiomyopathy are complex. Poor glycaemic control, increased oxidative stress, abnormalities in calcium handling and local activation of the renin-angiotensin system concurrently cause cardiomyocyte and microvascular endothelial cell apoptosis and death (Fiordaliso et al. 2000; Frustaci et al. 2000). Reduction of coronary flow reserve, microvascular spasm and reperfusion injury ascribed to endothelial dysfunction and microangiopathy contribute to focal cardiomyocyte loss by apoptosis and replacement fibrosis (Warley et al. 1995). Furthermore, healing mechanisms are impaired in the diabetic heart, including altered paracrine control of reparative angiogenesis (Yoon et al. 2005) and myocyte survival (Kajstura et al. 2001), as well as accelerated ageing of resident cardiac progenitor cells (Rota et al. 2006). A few studies highlight the differential expression of a subset of miRNAs in proangiogenic cells (PACs) in patients with heart disease (Zhang et al. 2011; Meng et al. 2012; Xu et al. 2012).

MiRNAs may control these cell functions at different levels with their altered expression being the cause or the result of the diabetic complication. In particular, the cardiac and skeletal muscle-specific miR-133a may play a role in the development of the disease by controlling genes related to fibrosis. MiR-133a downregulation in the hearts of diabetic mice has been associated with increased TGF-β1 and collagen IV (COL4A1) expression (Chen et al. 2014b). Of note, recent findings suggest miR-133a may be implicated in the control of cardiomyocyte function in diabetes via the targeting of methylation enzymes (Chavali et al. 2012). The muscle-specific miR-1 is upregulated in a rat cardiomyocyte cell line exposed to high glucose, inhibiting the anti-apoptotic action of IGF-1 (Yu et al. 2008). Another study with a similar in vitro model found again that miR-1 was upregulated and negatively regulated heat-shock protein 60 expression (Shan et al. 2010). In a diabetic mouse model, we found that cardiomyopathy was associated with a rise in miR-1 and a concurrent decline in the levels of a target Pim-1, which itself plays a key role in the cardiac response to stressors (Katare et al. 2011b). A different profile is seen in the specific case of established diabetic cardiomyopathy, where miR-1 has been found to be downregulated in affected rats (Yildirim et al. 2013). This was associated with an upregulation of the target junctin1 and a consequent increase in oxidative stress (Yildirim et al. 2013). In humans, a study of left ventricle biopsies demonstrated the differential expression between diabetics and non-diabetics of six miRNAs: miR-34b, miR-34c, miR-199b, miR-210, miR-650 and miR-223. These data were confirmed for miR-199a, miR-199b and miR-210 in vitro in cardiomyocytes and endothelial cells subjected to hypoxia and high glucose (Greco et al. 2012).

MiRNAs can exert their action in the cell of origin, but can also act in a paracrine fashion via release in the circulation where they travel bound to protein but also embedded in extracellular vesicles (see previous Sect. 3.3 for more details). In addition, via extracellular vesicle transport, miRNAs can be transferred directly to neighbouring cells and target mRNA expression in the recipient cell. This relatively newly discovered behaviour of miRNAs is currently attracting interest in the cardiovascular field. A recent report demonstrates that cardiomyocytes isolated from T2DM diabetic rats actually transfer the antiangiogenic miR-320 to endothelial cells and affect their function. This effect was counterbalanced in healthy cardiomyocytes by angiogenic miR-126 transport (Wang et al. 2014b).

A prolonged QT interval is an adverse cardiac feature of diabetes that can result in arrhythmias and is an independent predictor of mortality in DM (Rossing et al. 2001). This occurs as a result of dysfunction of multiple ion channels, predominantly the I_K/HERG (human ether-a-go-go) channel (Xiao et al. 2007). MiR-133 levels are significantly upregulated in the hearts of diabetic rabbits, where it targets HERG (Xiao et al. 2007). This suggests a role for miR133 dysregulation in prolonging the QT interval, and causing the resultant arrhythmias, in diabetes (Xiao et al. 2007).

3.4.3 Peripheral Vascular Disease

In diabetic patients, large-calibre vessels show aggressive macrovascular atherosclerotic disease that accounts for ischaemic complications, including myocardial infarction, peripheral artery disease (PAD) and stroke. PAD can progress to critical limb ischaemia (CLI), a life-threatening condition characterized by pain at rest, tissue loss with ulceration and/or gangrene (Norgren et al. 2007). Once CLI occurs, blood flow (BF) must be restored by either percutaneous angioplasty (PTA) or surgical revascularization. If revascularisation is infeasible, then amputation is inevitable, and this outcome is far more common in diabetic patients (Faglia 2011). A recent report showed that miR-126, which is known to be highly expressed in endothelial cells where it positively regulates angiogenesis (Fish et al. 2008), is downregulated in culture-selected PACs isolated from diabetic patients without vascular complications (Meng et al. 2012). In this respect, we have recently showed that culture-selected PACs generated from peripheral blood mononuclear cells (MNCs) of diabetic patients with CLI express increased levels of antiangiogenic miRNAs. Among those, miR-15a and miR-16 showed anti-migratory and pro-apoptotic activities on PACs. Interestingly, miR-15a and miR-16 were also abundantly present in PB $CD34^+$ cells and other vascular cells and were packaged in cell-secreted exosomes (Spinetti et al. 2013b). In *Homo sapiens*, two miR-15/16 clusters exist: miR-15a/miR-16-1 and miR-15b/miR-16-2 (at 13q14.2 and 3q25.33, respectively). MiR-15a and miR-16 share a portion of their "seed" sequence (i.e. the sequence which binds to the 3'UTR region of the targeted mRNAs) with five other miRNAs, including miR-503 and miR-424 (Caporali and Emanueli 2012). We previously showed that miR-503 impairs angiogenesis in the setting of CLI and diabetes, by directly targeting the cell cycle regulators cyclin E1 and cdc25A (Caporali et al. 2011). Chamorro et al. demonstrated that miR-16 and miR-424 inhibit in vitro endothelial function and angiogenesis by modulating the expression of VEGF-A, KDR and FGF-R1 (fibroblast growth factor receptor-1) (Chamorro-Jorganes et al. 2011). Moreover, Hullinger et al. showed that inhibition of miR-15 protects against cardiac ischaemic injury (Hullinger et al. 2012).

Since miRNAs influence the therapeutic potential of human embryonic stem cell-derived endothelial progenitor cells and pericyte progenitor cells (Katare et al. 2011a; Kane et al. 2012) upon their transplantation in mouse models of peripheral or myocardial ischaemia, it may be feasible in the future to intervene at the miRNA level to augment PAC regenerative potential. In fact, therapeutic stimulation of angiogenesis represents a strategy to support postischaemic BF recovery, wound closure and tissue regeneration. Despite the encouraging evidence from early clinical trials (Assmus et al. 2002; Fadini et al. 2009; Gupta and Losordo 2011; Amann et al. 2008; Sprengers et al. 2010), the regenerative potential of proangiogenic cells derived from patients with either diabetes or tissue ischaemia is reduced, and the underpinning molecular mechanisms have not been clarified (Fadini et al. 2006; Vasa et al. 2001; Heeschen et al. 2004).

A summary of the outcomes of miRNA dysregulation in diabetic cardiac disease and limb ischaemia is found in Fig. 3.1.

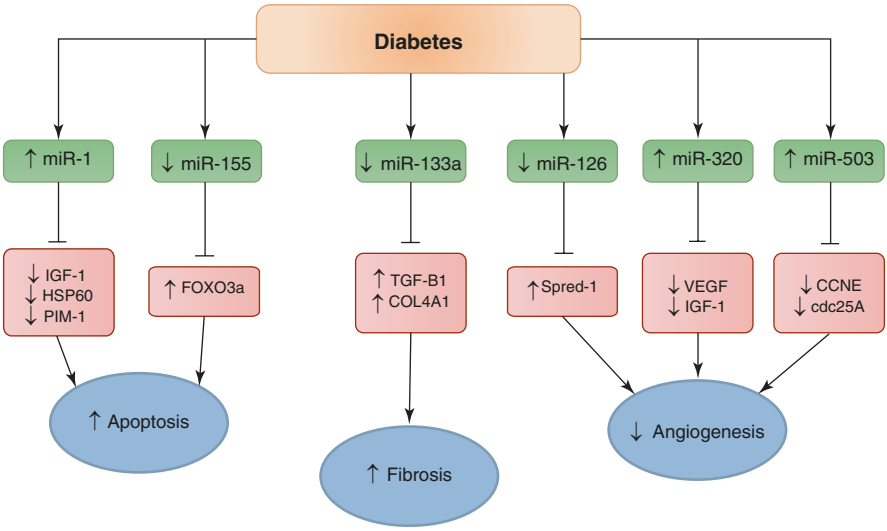

Fig. 3.1 Dysfunction of microRNAs in diabetic cardiovascular disease

3.5 MicroRNA as Circulating Biomarkers of Diabetic Cardiovascular Disease

The extracellular miRNAs in human serum and plasma are known to be relatively stable, reproducible and consistent, allowing them to be of potential value as biomarkers, and the interest toward the use of miRNAs in this area is growing. The role of miRNAs as biomarkers in cardiovascular disease is reviewed elsewhere (Creemers et al. 2012). Additionally, circulating levels of several miRNAs are also modified by diabetes (reviewed in (Shantikumar et al. 2012; Rawal et al. 2014)). Zampetaki et al. described the first plasma microRNA signature in patients with T2DM in a large population cohort. They found five microRNAs (miR-15a, miR-126, miR-320, miR-223, miR-28-3p) to be deregulated in patients with diabetes (Zampetaki et al. 2010). There has been much less study of circulating miRNAs in the specific context of diabetic vascular disease. We found that plasma levels of miR-50, miR-15a and miR-16 were increased in diabetic patients with CLI. The latter two miRNAs correlate with adverse events during 12 months follow-up in T2D patients who underwent PTA to correct CLI (Spinetti et al. 2013b). Another study looking at serum miRNA expression in T2DM patients, with and without vascular complications, found differential expression in patients with microvascular or macrovascular disease. Specifically, they found that miR-31 was significantly upregulated in T2DM patients with microvascular complications (Sebastiani et al. 2013).

These preliminary findings must be confirmed in larger patient populations, including subjects from different ethnic backgrounds and with various comorbidities. Differences in patient characteristics could alter several processes which

determine circulating miRNA values. These processes could include miRNA transcription, maturation, release from cells to the circulation, uptake from the circulation by other cells and excretion. Moreover, the data above leave several questions open regarding circulating miRNAs, including the cellular sources, the stimuli and the mechanisms permitting their extracellular release, their form of transportation and their putative function as local or at-distance communicators (Fichtlscherer et al. 2011).

3.6 Concluding Remarks

In this chapter we have discussed the complex role of microRNAs in the pancreatic dysfunction and insulin resistance that contribute to the development of diabetes, as well as their role in the development of microvascular and macrovascular complications, with special focus on cardiac disease and limb ischaemia. The identification of miRNAs involved in these pathological processes is the first step in the approach for finding improved diagnostic and therapeutic tools. The use of miRNA mimics and inhibitors are an attractive strategy for targeted therapy of diabetic vascular complications.

Conflict of Interest The authors declare that they have no conflict of interest.

References

Afkarian M, Sachs MC, Kestenbaum B, et al. Kidney disease and increased mortality risk in type 2 diabetes. J Am Soc Nephrol JASN. 2013;24(2):302–8.

Amann B, Ludemann C, Ruckert R, et al. Design and rationale of a randomized, double-blind, placebo-controlled phase III study for autologous bone marrow cell transplantation in critical limb ischemia: the BONe Marrow Outcomes Trial in Critical Limb Ischemia (BONMOT-CLI). Vasa. 2008;37(4):319–25.

American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2005;28(Suppl 1):37–42.

American Diabetes Association. Standards of medical care in diabetes – 2014. Diabetes Care. 2014;37(Suppl 1):S14–80.

Arnold JM, Yusuf S, Young J, et al. Prevention of heart failure in patients in the heart outcomes prevention evaluation (HOPE) study. Circulation. 2003;107(9):1284–90.

Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science. 1997;275(5302):964–7.

Assmus B, Schachinger V, Teupe C, et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). Circulation. 2002;106(24):3009–17.

Bell DS. Heart failure: the frequent, forgotten, and often fatal complication of diabetes. Diabetes Care. 2003a;26(8):2433–41.

Bell DS. Diabetic cardiomyopathy. Diabetes Care. 2003b;26(10):2949–51.

Beller GA. Coronary heart disease in the first 30 years of the 21st century: challenges and opportunities: The 33rd Annual James B. Herrick Lecture of the Council on Clinical Cardiology of the American Heart Association. Circulation. 2001;103(20):2428–35.

Beltrami C, Angelini TG, Emanueli C. Noncoding RNAs in diabetes vascular complications. J Mol Cell Cardiol. 2014;89:42–50.
Boyer JK, Thanigaraj S, Schechtman KB, et al. Prevalence of ventricular diastolic dysfunction in asymptomatic, normotensive patients with diabetes mellitus. Am J Cardiol. 2004;93(7):870–5.
Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature. 2001;414(6865):813–20.
Busik JV, Tikhonenko M, Bhatwadekar A, et al. Diabetic retinopathy is associated with bone marrow neuropathy and a depressed peripheral clock. J Exp Med. 2009;206(13):2897–906.
Caporali A, Emanueli C. MicroRNA regulation in angiogenesis. Vasc Pharmacol. 2011;55(4): 79–86.
Caporali A, Emanueli C. MicroRNA-503 and the extended microRNA-16 family in angiogenesis. Trends Cardiovasc Med. 2012;21:162–6.
Caporali A, Meloni M, Vollenkle C, et al. Deregulation of microRNA-503 contributes to diabetes mellitus-induced impairment of endothelial function and reparative angiogenesis after limb ischemia. Circulation. 2011;123(3):282–91.
Chakraborty C, Doss CG, Bandyopadhyay S, et al. Influence of miRNA in insulin signaling pathway and insulin resistance: micro-molecules with a major role in type-2 diabetes. Wiley Interdiscip Rev RNA. 2014;5(5):697–712.
Chamorro-Jorganes A, Araldi E, Penalva LO, et al. MicroRNA-16 and microRNA-424 regulate cell-autonomous angiogenic functions in endothelial cells via targeting vascular endothelial growth factor receptor-2 and fibroblast growth factor receptor-1. Arterioscler Thromb Vasc Biol. 2011;31(11):2595–606.
Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. N Engl J Med. 2006;354(6):610–21.
Chavali V, Tyagi SC, Mishra PK. MicroRNA-133a regulates DNA methylation in diabetic cardiomyocytes. Biochem Biophys Res Commun. 2012;425(3):668–72.
Chen YQ, Wang XX, Yao XM, et al. MicroRNA-195 promotes apoptosis in mouse podocytes via enhanced caspase activity driven by BCL2 insufficiency. Am J Nephrol. 2011;34(6):549–59.
Chen HY, Zhong X, Huang XR, et al. MicroRNA-29b inhibits diabetic nephropathy in db/db mice. Mol Ther. 2014a;22(4):842–53.
Chen S, Puthanveetil P, Feng B, et al. Cardiac miR-133a overexpression prevents early cardiac fibrosis in diabetes. J Cell Mol Med. 2014b;18(3):415–21.
Ciccacci C, Morganti R, Di Fusco D, et al. Common polymorphisms in MIR146a, MIR128a and MIR27a genes contribute to neuropathy susceptibility in type 2 diabetes. Acta Diabetol. 2014;51(4):663–71.
Collino F, Deregibus MC, Bruno S, et al. Microvesicles derived from adult human bone marrow and tissue specific mesenchymal stem cells shuttle selected pattern of miRNAs. PLoS One. 2010;5(7):e11803.
Condorelli G, Latronico MV, Cavarretta E. microRNAs in cardiovascular diseases: current knowledge and the road ahead. J Am Coll Cardiol. 2014;63(21):2177–87.
Creemers EE, Tijsen AJ, Pinto YM. Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease? Circ Res. 2012;110(3):483–95.
Deregibus MC, Cantaluppi V, Calogero R, et al. Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA. Blood. 2007;110(7):2440–8.
Deshpande SD, Putta S, Wang M, et al. Transforming growth factor-beta-induced cross talk between p53 and a microRNA in the pathogenesis of diabetic nephropathy. Diabetes. 2013;62(9):3151–62.
Dey N, Das F, Mariappan MM, et al. MicroRNA-21 orchestrates high glucose-induced signals to TOR complex 1, resulting in renal cell pathology in diabetes. J Biol Chem. 2011;286(29):25586–603.
DiPersio JF. Diabetic stem-cell "mobilopathy". N Engl J Med. 2011;365(26):2536–8.

Donners MM, Wolfs IM, Stoger LJ, et al. Hematopoietic miR155 deficiency enhances atherosclerosis and decreases plaque stability in hyperlipidemic mice. PLoS One. 2012;7(4):e35877.
El Ouaamari A, Baroukh N, Martens GA, et al. miR-375 targets 3'-phosphoinositide-dependent protein kinase-1 and regulates glucose-induced biological responses in pancreatic beta-cells. Diabetes. 2008;57(10):2708–17.
Emerging Risk Factors Coalition, Sarwar N, Gao P, et al. Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. Lancet. 2010;375(9733):2215–22.
Esau C, Kang X, Peralta E, et al. MicroRNA-143 regulates adipocyte differentiation. J Biol Chem. 2004;279(50):52361–5.
Esguerra JL, Bolmeson C, Cilio CM, et al. Differential glucose-regulation of microrna s in pancreatic islets of non-obese type 2 diabetes model Goto-Kakizaki rat. PLoS One. 2011;6(4):e18613.
Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Report of the expert committee on the diagnosis and classification of diabetes mellitus. Diabetes Care. 2003;26 Suppl 1:S5–20.
Fadini GP, Miorin M, Facco M, et al. Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus. J Am Coll Cardiol. 2005;45(9):1449–57.
Fadini GP, Sartore S, Albiero M, et al. Number and function of endothelial progenitor cells as a marker of severity for diabetic vasculopathy. Arterioscler Thromb Vasc Biol. 2006; 26(9):2140–6.
Fadini GP, Agostini C, Avogaro A. Autologous stem cell therapy for peripheral arterial disease meta-analysis and systematic review of the literature. Atherosclerosis. 2009;209(1):10–7.
Fadini GP, Boscaro E, de Kreutzenberg S, et al. Time course and mechanisms of circulating progenitor cell reduction in the natural history of type 2 diabetes. Diabetes Care. 2010;33(5):1097–102.
Faglia E. Characteristics of peripheral arterial disease and its relevance to the diabetic population. Int J Low Extrem Wounds. 2011;10(3):152–66.
Fang ZY, Prins JB, Marwick TH. Diabetic cardiomyopathy: evidence, mechanisms, and therapeutic implications. Endocr Rev. 2004;25(4):543–67.
Ferraro F, Lymperi S, Mendez-Ferrer S, et al. Diabetes impairs hematopoietic stem cell mobilization by altering niche function. Sci Transl Med. 2011;3(104):104ra01.
Fichtlscherer S, De Rosa S, Fox H, et al. Circulating microRNAs in patients with coronary artery disease. Circ Res. 2010;107(5):677–84.
Fichtlscherer S, Zeiher AM, Dimmeler S. Circulating microRNAs: biomarkers or mediators of cardiovascular diseases? Arterioscler Thromb Vasc Biol. 2011;31(11):2383–90.
Fiordaliso F, Li B, Latini R, et al. Myocyte death in streptozotocin-induced diabetes in rats in angiotensin II- dependent. Lab Investig. 2000;80(4):513–27.
Fish JE, Santoro MM, Morton SU, et al. miR-126 regulates angiogenic signaling and vascular integrity. Dev Cell. 2008;15(2):272–84.
Frustaci A, Kajstura J, Chimenti C, et al. Myocardial cell death in human diabetes. Circ Res. 2000;87(12):1123–32.
Georgantas 3rd RW, Hildreth R, Morisot S, et al. CD34+ hematopoietic stem-progenitor cell microRNA expression and function: a circuit diagram of differentiation control. Proc Natl Acad Sci U S A. 2007;104(8):2750–5.
Granjon A, Gustin MP, Rieusset J, et al. The microRNA signature in response to insulin reveals its implication in the transcriptional action of insulin in human skeletal muscle and the role of a sterol regulatory element-binding protein-1c/myocyte enhancer factor 2C pathway. Diabetes. 2009;58(11):2555–64.
Greco S, Fasanaro P, Castelvecchio S, et al. MicroRNA dysregulation in diabetic ischemic heart failure patients. Diabetes. 2012;61(6):1633–41.
Gupta R, Losordo DW. Cell therapy for critical limb ischemia. Circulation Cardiovas Interv. 2011;4(1):2–5.

He A, Zhu L, Gupta N, et al. Overexpression of micro ribonucleic acid 29, highly up-regulated in diabetic rats, leads to insulin resistance in 3 T3-L1 adipocytes. Mol Endocrinol. 2007;21(11):2785–94.

Heeschen C, Lehmann R, Honold J, et al. Profoundly reduced neovascularization capacity of bone marrow mononuclear cells derived from patients with chronic ischemic heart disease. Circulation. 2004;109(13):1615–22.

Heneghan HM, Miller N, McAnena OJ, et al. Differential miRNA expression in omental adipose tissue and in the circulation of obese patients identifies novel metabolic biomarkers. J Clin Endocrinol Metab. 2011;96(5):E846–50.

Herrera BM, Lockstone HE, Taylor JM, et al. Global microRNA expression profiles in insulin target tissues in a spontaneous rat model of type 2 diabetes. Diabetologia. 2010;53(6): 1099–109.

Howard L, Kane NM, Milligan G, et al. MicroRNAs regulating cell pluripotency and vascular differentiation. Vasc Pharmacol. 2011;55(4):69–78.

Hullinger TG, Montgomery RL, Seto AG, et al. Inhibition of miR-15 protects against cardiac ischemic injury. Circ Res. 2012;110(1):71–81.

Ilonen J, Akerblom HK. New technologies and genetics of type 1 diabetes. Diabetes Technol Ther. 1999;1(2):205–7.

International Diabetes Federation, 2013. http://www.idf.org/diabetesatlas. Secondary International Diabetes Federation, 2013. http://www.idf.org/diabetesatlas.

Jung UJ, Choi MS. Obesity and its metabolic complications: the role of adipokines and the relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty liver disease. Int J Mol Sci. 2014;15(4):6184–223.

Kahn SE. Clinical review 135: The importance of beta-cell failure in the development and progression of type 2 diabetes. J Clin Endocrinol Metab. 2001;86(9):4047–58.

Kajstura J, Fiordaliso F, Andreoli AM, et al. IGF-1 overexpression inhibits the development of diabetic cardiomyopathy and angiotensin II-mediated oxidative stress. Diabetes. 2001;50(6):1414–24.

Kane NM, Howard L, Descamps B, et al. Role of microRNAs 99b, 181a, and 181b in the differentiation of human embryonic stem cells to vascular endothelial cells. Stem Cells. 2012;30(4):643–54.

Kannel WB, Hjortland M, Castelli WP. Role of diabetes in congestive heart failure: the Framingham study. Am J Cardiol. 1974;34(1):29–34.

Katare R, Riu F, Mitchell K, et al. Transplantation of human pericyte progenitor cells improves the repair of infarcted heart through activation of an angiogenic program involving microRNA-132. Circ Res. 2011a;109(8):894–906.

Katare R, Caporali A, Zentilin L, et al. Intravenous gene therapy with PIM-1 via a cardiotropic viral vector halts the progression of diabetic cardiomyopathy through promotion of prosurvival signaling. Circ Res. 2011b;108(10):1238–51.

Kato M, Zhang J, Wang M, et al. MicroRNA-192 in diabetic kidney glomeruli and its function in TGF-beta-induced collagen expression via inhibition of E-box repressors. Proc Natl Acad Sci U S A. 2007;104(9):3432–7.

Kovacs B, Lumayag S, Cowan C, et al. microRNAs in early diabetic retinopathy in streptozotocin-induced diabetic rats. Invest Ophthalmol Vis Sci. 2011;52(7):4402–9.

Kowluru RA, Koppolu P, Chakrabarti S, et al. Diabetes-induced activation of nuclear transcriptional factor in the retina, and its inhibition by antioxidants. Free Radic Res. 2003;37(11):1169–80.

Krankel N, Katare RG, Siragusa M, et al. Role of kinin B2 receptor signaling in the recruitment of circulating progenitor cells with neovascularization potential. Circ Res. 2008;103(11): 1335–43.

Kumar AH, Caplice NM. Clinical potential of adult vascular progenitor cells. Arterioscler Thromb Vasc Biol. 2010;30(6):1080–7.

Ling HY, Ou HS, Feng SD, et al. Changes in microRNA profile and effects of miR-320 in insulin-resistant 3T3-L1 adipocytes. Clin Exp Pharmacol Physiol. 2009;36:e32–9.

Liu JE, Robbins DC, Palmieri V, et al. Association of albuminuria with systolic and diastolic left ventricular dysfunction in type 2 diabetes: the Strong Heart Study. J Am Coll Cardiol. 2003;41(11):2022–8.

Locke JM, da Silva XG, Dawe HR, et al. Increased expression of miR-187 in human islets from individuals with type 2 diabetes is associated with reduced glucose-stimulated insulin secretion. Diabetologia. 2014;57(1):122–8.

Long J, Wang Y, Wang W, et al. Identification of microRNA-93 as a novel regulator of vascular endothelial growth factor in hyperglycemic conditions. J Biol Chem. 2010;285(30):23457–65.

Lovis P, Roggli E, Laybutt DR, et al. Alterations in microRNA expression contribute to fatty acid-induced pancreatic beta-cell dysfunction. Diabetes. 2008;57(10):2728–36.

Lustig Y, Barhod E, Ashwal-Fluss R, et al. RNA-binding protein PTB and microRNA-221 coregulate AdipoR1 translation and adiponectin signaling. Diabetes. 2014;63(2):433–45.

Lynn FC, Skewes-Cox P, Kosaka Y, et al. MicroRNA expression is required for pancreatic islet cell genesis in the mouse. Diabetes. 2007;56(12):2938–45.

Marwick TH. Diabetic heart disease. Heart. 2006;92(3):296–300.

McArthur K, Feng B, Wu Y, et al. MicroRNA-200b regulates vascular endothelial growth factor-mediated alterations in diabetic retinopathy. Diabetes. 2011;60(4):1314–23.

Meng S, Cao JT, Zhang B, et al. Downregulation of microRNA-126 in endothelial progenitor cells from diabetes patients, impairs their functional properties, via target gene Spred-1. J Mol Cell Cardiol. 2012;53(1):64–72.

Mocharla P, Briand S, Giannotti G, et al. AngiomiR-126 expression and secretion from circulating CD34+ and CD14+ PBMCs: role for proangiogenic effects and alterations in type 2 diabetics. Blood. 2013;121(1):226–36.

Najafi-Shoushtari SH, Kristo F, Li Y, et al. MicroRNA-33 and the SREBP host genes cooperate to control cholesterol homeostasis. Science. 2010;328(5985):1566–9.

Norgren L, Hiatt WR, Dormandy JA, et al. Inter-society consensus for the management of peripheral arterial disease (TASC II). European Journal of Vascular and Endovascular Surgery. 2007;33(1 Suppl):S1–S75.

O'Connell RM, Chaudhuri AA, Rao DS, et al. MicroRNAs enriched in hematopoietic stem cells differentially regulate long-term hematopoietic output. Proc Natl Acad Sci U S A. 2010;107(32):14235–40.

Oikawa A, Siragusa M, Quaini F, et al. diabetes mellitus induces bone marrow microangiopathy. Arterioscler Thromb Vasc Biol. 2010;30(3):498–508.

Ozcan S. Minireview: microRNA function in pancreatic beta cells. Mol Endocrinol. 2014;28(12):1922–33.

Parra P, Serra F, Palou A. Expression of adipose microRNAs is sensitive to dietary conjugated linoleic acid treatment in mice. PLoS One. 2010;5(9):e13005.

Plaisance V, Abderrahmani A, Perret-Menoud V, et al. MicroRNA-9 controls the expression of Granuphilin/Slp4 and the secretory response of insulin-producing cells. J Biol Chem. 2006;281(37):26932–42.

Poornima IG, Parikh P, Shannon RP. Diabetic cardiomyopathy: the search for a unifying hypothesis. Circ Res. 2006;98(5):596–605.

Poy MN, Eliasson L, Krutzfeldt J, et al. A pancreatic islet-specific microRNA regulates insulin secretion. Nature. 2004;432(7014):226–30.

Poy MN, Hausser J, Trajkovski M, et al. miR-375 maintains normal pancreatic alpha- and beta-cell mass. Proc Natl Acad Sci U S A. 2009;106(14):5813–8.

Puigserver P, Rodgers JT. Foxa2, a novel transcriptional regulator of insulin sensitivity. Nat Med. 2006;12(1):38–9.

Qin W, Chung AC, Huang XR, et al. TGF-beta/Smad3 signaling promotes renal fibrosis by inhibiting miR-29. J Am Soc Nephrol JASN. 2011;22(8):1462–74.

Qing S, Yuan S, Yun C, et al. Serum miRNA biomarkers serve as a fingerprint for proliferative diabetic retinopathy. Cell Physiol Biochem. 2014;34(5):1733–40.

Rajan SK, Gokhale SM. Cardiovascular function in patients with insulin-dependent diabetes mellitus: a study using noninvasive methods. Ann N Y Acad Sci. 2002;958:425–30.
Rawal S, Manning P, Katare R. Cardiovascular microRNAs: as modulators and diagnostic biomarkers of diabetic heart disease. Cardiovasc Diabetol. 2014;13:44.
Record M, Subra C, Silvente-Poirot S, et al. Exosomes as intercellular signalosomes and pharmacological effectors. Biochem Pharmacol. 2011;81(10):1171–82.
Redfield MM, Jacobsen SJ, Burnett Jr JC, et al. Burden of systolic and diastolic ventricular dysfunction in the community: appreciating the scope of the heart failure epidemic. JAMA. 2003;289(2):194–202.
Rossing P, Breum L, Major-Pedersen A, et al. Prolonged QTc interval predicts mortality in patients with Type 1 diabetes mellitus. Diabet Med. 2001;18(3):199–205.
Rota M, LeCapitaine N, Hosoda T, et al. Diabetes promotes cardiac stem cell aging and heart failure, which are prevented by deletion of the p66shc gene. Circ Res. 2006;99(1):42–52.
Ryu HS, Park SY, Ma D, et al. The induction of microRNA targeting IRS-1 is involved in the development of insulin resistance under conditions of mitochondrial dysfunction in hepatocytes. PLoS One. 2011;6(3):e17343.
Sahoo S, Klychko E, Thorne T, et al. Exosomes from human CD34(+) stem cells mediate their proangiogenic paracrine activity. Circ Res. 2011;109(7):724–8.
Saito H, Yamamoto Y, Yamamoto H. Diabetes alters subsets of endothelial progenitor cells that reside in blood, bone marrow, and spleen. Am J Physiol Cell Physiol. 2012;302(6):C892–901.
Schaible TF, Malhotra A, Bauman WA, et al. Left ventricular function after chronic insulin treatment in diabetic and normal rats. J Mol Cell Cardiol. 1983;15(7):445–58.
Schannwell CM, Zimmermann T, Schneppenheim M, et al. Left ventricular hypertrophy and diastolic dysfunction in healthy pregnant women. Cardiology. 2002;97(2):73–8.
Sebastiani G, Nigi L, Spagnuolo I, et al. MicroRNA profiling in sera of patients with type 2 diabetes mellitus reveals an upregulation of miR-31 expression in subjects with microvascular complications. J Biomed Sci Eng. 2013;6(5):58–64.
Segal MS, Shah R, Afzal A, et al. Nitric oxide cytoskeletal-induced alterations reverse the endothelial progenitor cell migratory defect associated with diabetes. Diabetes. 2006; 55(1):102–9.
Shan ZX, Lin QX, Deng CY, et al. miR-1/miR-206 regulate Hsp60 expression contributing to glucose-mediated apoptosis in cardiomyocytes. FEBS Lett. 2010;584(16):3592–600.
Shantikumar S, Caporali A, Emanueli C. Role of microRNAs in diabetes and its cardiovascular complications. Cardiovasc Res. 2012;93(4):583–93.
Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res Clin Pract. 2010;87(1):4–14.
Shi B, Sepp-Lorenzino L, Prisco M, et al. Micro RNA 145 targets the insulin receptor substrate-1 and inhibits the growth of colon cancer cells. J Biol Chem. 2007;282(45):32582–90.
Shi Z, Zhao C, Guo X, et al. Differential expression of microRNAs in omental adipose tissue from gestational diabetes mellitus subjects reveals miR-222 as a regulator of ERalpha expression in estrogen-induced insulin resistance. Endocrinology. 2014;155(5):1982–90.
Spinetti G, Cordella D, Fortunato O, et al. Global remodeling of the vascular stem cell niche in bone marrow of diabetic patients: implication of the microRNA-155/FOXO3a signaling pathway. Circ Res. 2013a;112(3):510–22.
Spinetti G, Fortunato O, Caporali A, et al. MicroRNA-15a and microRNA-16 impair human circulating proangiogenic cell functions and are increased in the proangiogenic cells and serum of patients with critical limb ischemia. Circ Res. 2013b;112(2):335–46.
Sprengers RW, Moll FL, Teraa M, et al. Rationale and design of the JUVENTAS trial for repeated intra-arterial infusion of autologous bone marrow-derived mononuclear cells in patients with critical limb ischemia. J Vasc Surg. 2010;51(6):1564–8.
Stamler J, Vaccaro O, Neaton JD, et al. Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial. Diabetes Care. 1993;16(2):434–44.

Stefanowicz M, Straczkowski M, Karczewska-Kupczewska M. The role of SIRT1 in the pathogenesis of insulin resistance in skeletal muscle. Postepy Hig Med Dosw. 2015;69:63.
Strum JC, Johnson JH, Ward J, et al. MicroRNA 132 regulates nutritional stress-induced chemokine production through repression of SirT1. Mol Endocrinol. 2009;23(11):1876–84.
Tabak O, Gelisgen R, Erman H, et al. Oxidative lipid, protein, and DNA damage as oxidative stress markers in vascular complications of diabetes mellitus. Clin Invest Med. 2011;34(3): E163–71.
Tepper OM, Galiano RD, Capla JM, et al. Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. Circulation. 2002;106(22):2781–6.
Tetta C, Bruno S, Fonsato V, et al. The role of microvesicles in tissue repair. Organogenesis. 2011;7(2):105–15.
Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. Nat Rev Immunol. 2009;9(8):581–93.
Trajkovski M, Hausser J, Soutschek J, et al. MicroRNAs 103 and 107 regulate insulin sensitivity. Nature. 2011;474(7353):649–53.
Valadi H, Ekstrom K, Bossios A, et al. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9(6):654–9.
van Rooij E. The art of microRNA research. Circ Res. 2011;108(2):219–34.
Vasa M, Fichtlscherer S, Aicher A, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ Res. 2001;89(1):e1–7.
Wang S, Olson EN. AngiomiRs--key regulators of angiogenesis. Curr Opin Genet Dev. 2009;19(3):205–11.
Wang Q, Wang Y, Minto AW, et al. MicroRNA-377 is up-regulated and can lead to increased fibronectin production in diabetic nephropathy. FASEB J Off Publ Fed Am Soc Exp Biol. 2008;22(12):4126–35.
Wang J, Gao Y, Ma M, et al. Effect of miR-21 on renal fibrosis by regulating MMP-9 and TIMP1 in kk-ay diabetic nephropathy mice. Cell Biochem Biophys. 2013;67(2):537–46.
Wang L, Chopp M, Szalad A, et al. The role of miR-146a in dorsal root ganglia neurons of experimental diabetic peripheral neuropathy. Neuroscience. 2014a;259:155–63.
Wang X, Huang W, Liu G, et al. Cardiomyocytes mediate anti-angiogenesis in type 2 diabetic rats through the exosomal transfer of miR-320 into endothelial cells. J Mol Cell Cardiol. 2014b;74:139–50.
Warley A, Powell JM, Skepper JN. Capillary surface area is reduced and tissue thickness from capillaries to myocytes is increased in the left ventricle of streptozotocin-diabetic rats. Diabetologia. 1995;38(4):413–21.
Winer N, Sowers JR. Epidemiology of diabetes. J Clin Pharmacol. 2004;44(4):397–405.
Xiao J, Luo X, Lin H, et al. MicroRNA miR-133 represses HERG K+ channel expression contributing to QT prolongation in diabetic hearts. J Biol Chem. 2007;282(17):12363–7.
Xiao F, Yu J, Liu B, et al. A novel function of microRNA 130a-3p in hepatic insulin sensitivity and liver steatosis. Diabetes. 2014;63(8):2631–42.
Xie H, Lim B, Lodish HF. MicroRNAs induced during adipogenesis that accelerate fat cell development are downregulated in obesity. Diabetes. 2009;58(5):1050–7.
Xu Q, Seeger FH, Castillo J, et al. Micro-RNA-34a contributes to the impaired function of bone marrow-derived mononuclear cells from patients with cardiovascular disease. J Am Coll Cardiol. 2012;59(23):2107–17.
Xu LL, Shi CM, Xu GF, et al. TNF-alpha, IL-6, and leptin increase the expression of miR-378, an adipogenesis-related microRNA in human adipocytes. Cell Biochem Biophys. 2014;70(2):771–6.
Yildirim SS, Akman D, Catalucci D, et al. Relationship between downregulation of miRNAs and increase of oxidative stress in the development of diabetic cardiac dysfunction: junctin as a target protein of miR-1. Cell Biochem Biophys. 2013;67(3):1397–408.

Yoon YS, Uchida S, Masuo O, et al. Progressive attenuation of myocardial vascular endothelial growth factor expression is a seminal event in diabetic cardiomyopathy: restoration of microvascular homeostasis and recovery of cardiac function in diabetic cardiomyopathy after replenishment of local vascular endothelial growth factor. Circulation. 2005;111(16):2073–85.

Yu XY, Song YH, Geng YJ, et al. Glucose induces apoptosis of cardiomyocytes via microRNA-1 and IGF-1. Biochem Biophys Res Commun. 2008;376(3):548–52.

Zabalgoitia M, Ismaeil MF, Anderson L, et al. Prevalence of diastolic dysfunction in normotensive, asymptomatic patients with well-controlled type 2 diabetes mellitus. Am J Cardiol. 2001;87(3):320–3.

Zampetaki A, Kiechl S, Drozdov I, et al. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. Circ Res. 2010;107(6):810–7.

Zhang Q, Kandic I, Kutryk MJ. Dysregulation of angiogenesis-related microRNAs in endothelial progenitor cells from patients with coronary artery disease. Biochem Biophys Res Commun. 2011;405(1):42–6.

Zhou B, Li C, Qi W, et al. Downregulation of miR-181a upregulates sirtuin-1 (SIRT1) and improves hepatic insulin sensitivity. Diabetologia. 2012;55(7):2032–43.

Zhu W, Huang L, Li Y, et al. Exosomes derived from human bone marrow mesenchymal stem cells promote tumor growth in vivo. Cancer Lett. 2012;315(1):28–37.

Noncoding RNAs in Ischemic Cardiovascular Disease and Repair Mechanisms

4

Ulf Landmesser and Philipp Jakob

Contents

Abstract

Ischemic cardiomyopathy is frequently a consequence of myocardial infarction resulting in a marked loss of cardiomyocytes, which is replaced by a scar. As endogenous cardiovascular repair mechanisms are not sufficient to compensate for the loss of heart muscle and subsequent adverse remodeling predisposes to development of heart failure, novel experimental therapies are intensely explored to reduce scar size, counteract adverse hypertrophy and promote repair of the heart muscle. These therapies include cell-based therapies, cardiomyocytes renewal or proliferation and trans-differentiation into cardiomyocytes. Importantly, microRNAs evolved as major modulators of pathophysiological mechanisms involved in heart failure progression as well as repair mechanisms. Identification of microRNAs contributing or counteracting these deteriorative mechanisms resulted in novel microRNA-targeted therapies in experimental studies. While this class of non-coding RNAs has already entered clinical translation, another subclass of non-coding RNAs - long-non-coding RNAs (lncRNAs) – is now intensely explored as well. Recent observations indicate that lncRNAs are important regulators of cardiovascular development and mechanisms involved

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in adverse cardiac remodeling and development of cardiomyopathy. Therefore lncRNAs, in addition to microRNAs, may also emerge as novel therapeutic targets in cardiovascular disease.

4.1 Targeting miRNAs to Enhance Cell-Based Therapies

The studies of cell-based therapies using bone marrow-derived cells in patients with acute myocardial infarction (Assmus et al. 2002) triggered a vast number of experimental and clinical investigations using bone marrow-, mesenchymal-, or cardiac-derived stem and progenitor cells (Jakob and Landmesser 2013). Recently, cardiac-derived stem cells were examined in clinical phase I/II trials (Bolli et al. 2011; Makkar et al. 2012). However, while some clinical trials using cell-based therapies after myocardial infarction observed an improvement of left ventricular function or clinical symptoms, others failed to show beneficial effects (Jeevanantham et al. 2012; Fisher et al. 2015; Gyongyosi et al. 2015). Importantly, as the majority of conducted clinical trials included only small number of patients and was mostly underpowered for mortality as an endpoint, a Pan-European clinical phase III trial investigating the effect of cell-based treatment in patients with acute myocardial infarction (MI) (http://www.bami-fp7.eu/) is now being conducted.

The modest effects observed in cell-based therapies may in part be attributable to isolation procedures (Assmus et al. 2010; Seeger et al. 2012), low survival (Li et al. 2009), limited homing (Chavakis and Dimmeler 2011), and highly limited differentiation into contractility contributing cardiomyocytes of the applied cell product (Tongers et al. 2011). Of note, cells derived from patients with cardiovascular diseases show an impaired cardiac repair capacity when compared to cells from healthy subjects (Jakob et al. 2012a; Heeschen et al. 2004). Mechanistic studies have shown that miRNAs are crucially involved in these processes. As a potential mechanism, deregulation of miRNAs in cells with repair capacity was observed (Mocharla et al. 2013; Jakob et al. 2012a; Xu et al. 2012). We and others observed deregulation of miRNA expression in early angiogenic outgrowth cells (EOCs) derived from patients with chronic heart failure (CHF) (Jakob et al. 2012b). Notably, overexpression of miR-126 that was reduced in EOCs from patients with CHF enhanced EOC-mediated cardiac repair capacity in vivo (Jakob et al. 2012b). MiR-126 was previously shown to stimulate angiogenesis (Wang et al. 2008) and is also involved in the prevention of experimental atherosclerosis (Schober et al. 2014). In addition, miR-21, that is upregulated in EOCs from patients with coronary artery disease, impairs their migratory potential through an increase in reactive oxygen species (Fleissner et al. 2010). Another approach is to prevent apoptosis of transplanted cells, a process thought to substantially decrease cardiac repair capacity after cell transplantation due to low survival of transplanted cells. Expression of miR-34a, a pro-apoptotic miRNA, was increased in bone marrow mononuclear cells (BMC) from patients with myocardial infarction (Xu et al. 2012). Pretreatment of BMCs with miR-34a inhibitors improved their capacity to restore cardiac function in a murine infarct model (Xu et al. 2012). Of note, miR-34a is also increased during ageing in the heart (Boon et al. 2013). Hu et al. applied a cocktail consisting of

miR-21, miR-24, and miR-221 to cardiac progenitor cells, which increased their survival after cardiac transplantation in an experimental myocardial infarct model and resulted in a better preserved cardiac function (Hu et al. 2011). Bim, an inducer of apoptosis, was repressed by these three miRNAs (Hu et al. 2011), demonstrating that multiple miRNAs can synergistically repress one target. Hence, miRNAs have the potential to improve impaired cardiac repair capacity of adult stem/progenitor cells, and miRNA modulation of adult stem/progenitor cells may serve as a strategy to enhance cardiac repair processes in cell-based therapies (Fig. 4.1).

As cell isolation procedures are labor intensive and expensive and improvement in cardiac function is mostly related to paracrine mechanisms (Gnecchi et al. 2008; Murry et al. 2004), recent studies focused on compounds released from progenitor/stem cells. In this context, exosomes, small secreted membrane-bound vesicles released from cells, evolve as a potential cell-free therapy for cardioprotection (Vicencio et al. 2015; Chen et al. 2013b). Interestingly, miRNA transferred via exosomes contributes to this intercellular communication system. Intramyocardial delivery of exosomes derived from mouse embryonic stem cells improved LV function after induction of myocardial infarction in mice (Khan et al. 2015). This was

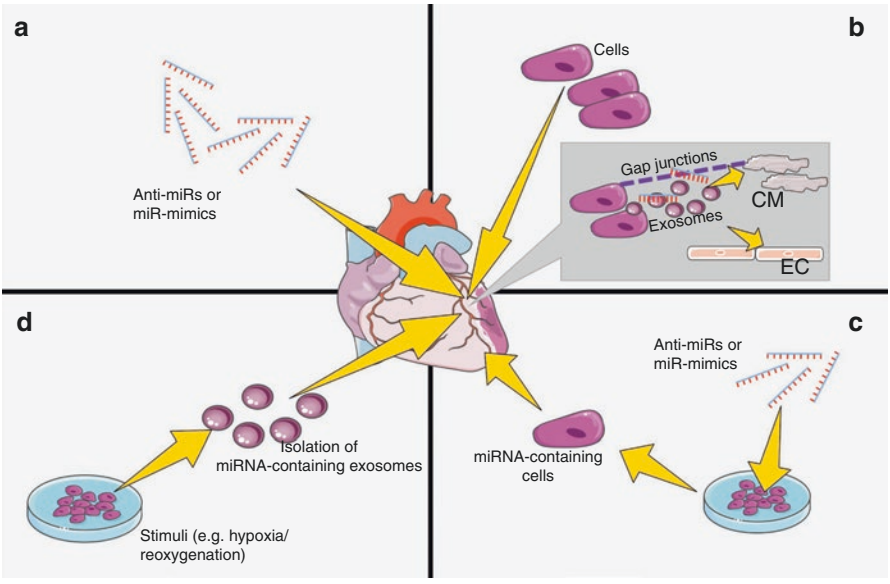

Fig. 4.1 Experimental (and clinical) strategies to improve cardiac function using cells with miRNA-mediated cardiac repair potential, cell-derived miRNA-containing components, or chemically modified synthetic miRNAs. Chemically modified synthetic miRNAs or viral constructs can be delivered directly (local or systemic) for therapeutic manipulation of miRNAs (**a**). Systemic or intramyocardial delivered cells enhance cardiac repair by the release of miRNAs (and other growth factors) to host cells through exosomes and gap junctions (**b**). MiRNA pretreatment of cells may enhance their cardiac repair potential and survival (**c**). MiRNA-containing exosomes of stem/progenitor cells can be isolated and delivered to improve cardiac repair capacity. This cell-free strategy avoids potential side effects that may arise after transplantation of stem cells (**d**). *CM* cardiomyocytes, *EC* endothelial cells, *miR* microRNA

related to an increase in proliferative myocytes and number of cardiac progenitor cells (CPCs, c-kit + cells) in vivo. Of note, miRNA profiling of exosomes revealed an enhanced expression of the cell cycle regulator cluster miR-290. Overexpression of one of the members, miR-294, in CPCs increased proliferation and decreased apoptosis in vitro (Khan et al. 2015). Similarly, hypoxia-induced release of exosomes in CPCs improved LV function in an experimental ischemia-reperfusion model, which is related to an increased exosome content of miRNAs involved in fibrosis pathways (Gray et al. 2015). In addition, exosomes from host tissue after cell therapy may alter function of the applied cell product. Ong et al. showed that CPCs co-delivered with a minicircle plasmid containing hypoxia-inducible factor 1 (HIF-1)-induced endothelial cells to secrete exosomes enriched of miR-126 and miR-210. Uptake of these miRs in CPCs leads to a higher tolerance against hypoxic stress in vitro which in turn enhances survival of CPCs after intramyocardial delivery (Ong et al. 2014). Interestingly, Hosoda et al. (2011) showed that miR-499 may also be transferred via gap junctions from myocytes to cardiac stem cells, thereby promoting differentiation via suppression of differentiation modulators Sox6 and Rod1. MiR-499 is highly expressed in differentiated cardiomyocytes and markedly reduces proliferation rates of cardiomyocyte progenitor cells (Sluijter et al. 2010).

4.2 Targeting miRNAs to Induce a Cardiac Phenotype in Pluripotent Stem Cells

Stimulation of cardiomyocyte lineage commitment was reported in embryonic stem cells (ESCs) and inducible pluripotent stem cells (iPSCs) as a potential strategy to promote cardiac regeneration. Human ESC-derived cardiomyocytes enhanced cardiac function in a rat myocardial infarction model (Laflamme et al. 2007). In addition, integration and survival of human ESC-derived cardiomyocytes after transplantation in nonhuman primates in an experimental myocardial infarct model were reported recently. Re-muscularization of substantial amounts of the infarcted monkey heart was observed, albeit with occurrence of nonfatal ventricular arrhythmias (Chong et al. 2014). Dynamic regulation of miRNA is involved in differentiation of ESCs toward a cardiomyocyte fate. In vitro, induction of miR-1 and miR-499 in the differentiation from human ECS and cardiac progenitor cells toward cardiomyocytes was observed. Forced expression of these miRNAs enhanced differentiation toward a cardiomyocyte fate (Wilson et al. 2010; Sluijter et al. 2010). In vivo, transplantation of hESCs treated with miR-1 mimics improved cardiac function and increased the number of donor-derived cardiomyocytes (Glass and Singla 2011). Of interest, cardiac apoptosis was decreased after treatment (Glass and Singla 2011) suggesting that miR-1 not only facilitates cardiomyocyte differentiation but also contributes to cardioprotection after cardiac injury via paracrine mechanisms. Using miRNA sequencing and bioinformatical analysis in hESCs-derived cardiomyocytes before and after (1 year) cardiac maturation revealed a pronounced upregulation of members of the let-7 family. Overexpression of let-7 in human ESC-CMs accelerated cardiac maturation as shown by enhanced morphological and functional characteristics. Profiling after overexpression of let-7 members showed upregulation of fatty

acid metabolism and downregulation of PI3/AKT/insulin signaling, suggesting that a metabolic switch enhances cardiomyocyte maturation (Kuppusamy et al. 2015). Together, these studies reveal important roles of miRNAs for cardiac lineage commitment and maturation of pluripotent stem cells.

4.3 Targeting miRNAs to Facilitate Cardiac Regenerative Pathways

A limited number of cardiac cells are able or regain the potential to reenter cell cycle (Bergmann et al. 2009). However, this cell renewal cannot compensate for cardiomyocyte loss after acute myocardial infarction or in the progression of chronic heart failure. Therefore, therapeutic strategies to induce cardiac regeneration are currently intensely investigated. As miRNAs are crucially involved in cardiac development, lineage commitment, differentiation, and maturation of cardiomyocytes, miRNAs were recently investigated for their potential to regenerate the heart, either by direct cardiac reprogramming or induction of cardiomyocyte proliferation (Fig. 4.2).

Direct cardiac reprogramming describes a process in which resident cardiac host cells are directly trans-differentiated into cardiomyocytes. Direct reprogramming therefore circumvents the step of dedifferentiation into pluripotent stem cells but

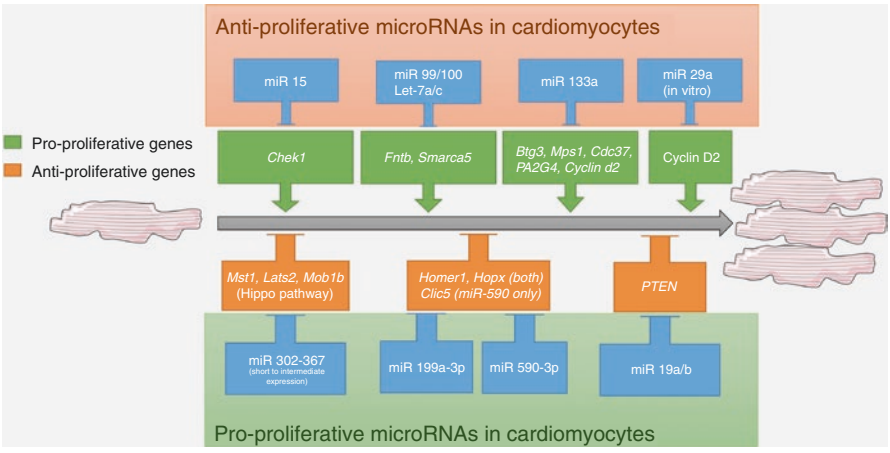

Fig. 4.2 Induction or inhibition of cardiomyocyte proliferation is regulated by miRNAs. Whereas neonatal mice show a robust cardiac repair response after cardiac injury, proliferative capacity of cardiomyocytes is (almost) lost in the adult heart of human and mice. Therapeutic targeting of miRNAs that suppresses genes involved in cell cycle reentry and mitosis results in an increased proliferation of cardiomyocytes. *Chek1* checkpoint kinase 1, *Fntb* beta subunit of farnesyltransferase, *Smarca5* SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily a, member 5, *Btg3* B-cell translocation gene 3, *Mps1* monopolar spindle 1, *Cdc37* cell division cycle 37, *PA2G4* proliferation associated protein, *mst1* mammalian STE20-like protein kinase 1, *Lats2* large tumor suppressor 2, *Mob1b* Mps one binder kinase activator 1B, *Hopx* HOP homeobox, *PTEN* phosphatase and tensin homolog

pursues reprogramming directly from endogenous non-cardiomyocytes (e.g., cardiac fibroblasts) toward functional cardiomyocytes. Ieda et al. (2010) reported recently reprogramming of *mouse* fibroblasts into cardiomyocytes. Delivery of three cardiac transcription factors (Gata4, Mef2c, Tbx5, or GMT) into fibroblasts derived from mouse hearts and skin resulted in cardiomyocyte-like cells with expression of cardiomyocyte-specific promoters and structures. The induction was also observed in a murine myocardial infarction model by using viral transfection of cardiac transcription factors (Qian et al. 2012; Song et al. 2012). Interestingly, the addition of miR-133a to GMT increased the number of directly reprogrammed cardiomyocytes and accelerated onset of beating cells by suppression of fibroblast signatures through SNAI1, a master gene of epithelial-to-mesenchymal transition (Muraoka et al. 2014). Jayawardena et al. (2012) extended these observations by using miRNAs involved in cardiac muscle development. Overexpression of miR-1, miR-133, miR-208, and miR-499 in mouse cardiac fibroblasts drives these cells toward cardiomyocytes with expression of cardiomyocyte markers and functions in vitro. Moreover, genetic tracing methods showed that intramyocardial injection of this set of miRNAs after experimental myocardial infarction converted cardiac fibroblast into cardiomyocytes-like cells (Jayawardena et al. 2012). In a follow-up study (Jayawardena et al. 2015), serial echocardiography in mice undergoing MI and injected with miR-1, miR-133, miR-208, and miR-499 showed an improvement in cardiac function over 3 months as compared to controls. Furthermore, reprogrammed rod-shaped cells exhibited similar physiological properties as mature adult ventricular cardiomyocytes. Fibroblasts from humans are more resistant to reprogramming techniques, as cardiac transcription factors (Gata4, Mef2c, Tbx5, and Hand2) used in mouse fibroblasts (Song et al. 2012) failed to reprogram human fibroblasts (Nam et al. 2013). However, addition of MyocD effectively induced cardiac gene expression. Interestingly, miR-1 and miR-133 together with four transcription factors (Gata4, Hand2, Tbx5, and MyocD) further enhanced reprogramming efficiency toward a cardiomyocyte phenotype (Nam et al. 2013). Though 19% of reprogrammed cells were cardiac troponin T-positive, functional characteristics of mature cardiomyocytes such as upregulation of cardiac genes, calcium transients, and beating cells were rarely observed.

Apart from direct reprogramming, induction of cardiomyocyte proliferation is an alternative different strategy to enhance cardiac function in injured hearts that likely underlies the capacity of zebra fish and postnatal mice to regenerate after cardiac injury. Cardiomyocyte proliferation is recognized for decades to be a prerequisite in embryogenesis and for lower vertebrates. However, Bergmann et al. showed that in adults, a low but relevant count of cardiomyocytes still proliferates (approx. 1% turnover rate/year) (Bergmann et al. 2009), which raises the possibility to enhance cell cycle in mature cardiomyocytes. In experimental studies, proliferation of cardiomyocytes after surgical injury in neonatal mice has been reported (Porrello et al. 2011b). These observations indicate postnatal regeneration of the heart. MiRNAs are required for modulation of proliferative and apoptotic processes in cardiomyocytes, as cardiac deletion of enzymes required in the biogenesis of miRNAs resulted in dilatation of the heart and premature lethality (Chen

et al. 2008; Rao et al. 2009). MiR-1 and miR-133 have been shown to regulate mitotic processes. MiR-1 is specifically expressed in the skeletal and cardiac muscle and consists of two miRNAs, miR-1-1 and miR-1-2 (Zhao et al. 2007). Mice lacking miR-1-2 die early due to ventricular septal defects (Zhao et al. 2007). Adult mice lacking miR-1 will result to overt cardiomyocyte hyperplasia. Molecular studies showed an increased expression of proteins involved in cardiac morphogenesis and development, such as Hand2 (Zhao et al. 2007). In contrast cardiac-specific overexpression of miR-1 leads to decreased ventricular cardiomyocyte proliferation (Zhao et al. 2005). MiR-133a is co-transcribed as a bicistronic construct with miR-1 and involved in cardiac development. Deletion of miR-133a-1/miR-133a-2 causes lethal ventricular septal defects in embryonic and neonatal stages and dilated cardiomyopathy in surviving adult mice (Liu et al. 2008). In these double-mutant mice, a disorganization of sarcomeres and an increased proliferation and apoptosis of cardiomyocytes were detected. Consistently, cell cycle genes were upregulated in double knockout mice. In zebra fish, downregulation of miR-133 was observed after resection of the cardiac apex. Transgenic overexpression of miR-133 suppresses cell cycle genes btg3, cdc37, PA2G4 and mps1, and connexin-43, a gap junction protein required for intercellular communication (Yin et al. 2012), as it was shown for miR-499 (Hosoda et al. 2011). MiR-133a therefore suppresses cardiomyocyte cell cycle and guides differentiation into cardiomyocytes. As changes in spatiotemporal expression of miRNA are observed, a study linked the transient regenerative capacity in postnatal murine hearts (Porrello et al. 2011b) to detect up- and downregulated miRNAs using a microarray approach (Porrello et al. 2011a). MiR-195, a member of the miR-15 family, is highly upregulated in mouse hearts between day 1 and 10 after birth. Delivery of anti-miRs targeting miR-15 family members in neonatal mice increased cardiomyocyte proliferation by de-inhibition of cell cycle genes (Porrello et al. 2011a). Porrello et al. (2013) further investigated the impact of miR-15 on cardiac regeneration after cardiac injury in postnatal mice. Postnatal MI at day 1 resulted in an extensive infarcted area. However, at day 21, a functional recovery can be observed (Porrello et al. 2013). Cardiac-specific overexpression of miR-195 (a member of the miR-15 family) in these mice impaired cardiac regenerative capacity with extensive fibrosis in the infarcted area and decreased proliferating cardiomyocytes (Porrello et al. 2013). Furthermore, pretreatment of postnatal mice with anti-miR-15 improved cardiac function after induction of myocardial infarction in adult mice (Porrello et al. 2013). Of note, transgenic overexpression of miR-195 results in cardiac growth and disassembly of cardiomyocytes (van Rooij et al. 2006), leading to dilated cardiomyopathy. However, inhibition of miR-195 was recently shown to increase elastin deposition in the aorta of mice (Zampetaki et al. 2014). Therefore, the role of miR-195 in cardiac extracellular matrix deposition has to be determined in future studies. Similar to miR-195, miR-29a is upregulated when comparing miRNA array expression data from cardiomyocytes derived from rats at postnatal day 2 when compared to postnatal day 28 (Cao et al. 2013). MiR-29a targets cell cycle genes (CCND2). In vitro, inhibition of miR-29a in neonatal cardiomyocytes enhances cardiomyocyte proliferation (Cao et al. 2013).

However, important regulators of regenerative processes can be missed in mammals when evolutionary conserved mechanisms are not activated upon heart injury. In zebra fish, heart amputation results in a downregulation of miR-99/100 and let--7a/c, which is not observed in mice after MI (Aguirre et al. 2014). However, intramyocardial delivery of an adenovirus encoding for anti-miR-99/100 and anti-let-7a/c in mice undergoing MI improved cardiac function and decreased scar formation. These effects were triggered by an increase in dedifferentiated and proliferation of cardiomyocytes and resembled the regenerative mechanisms observed in zebra fish (Aguirre et al. 2014).

These studies investigated miRNAs with antiproliferative effects on cardiomyocytes. In contrast, a recent study reported that miRNAs can also induce proliferation of cardiomyocytes (Eulalio et al. 2012). A functional high-throughput screening was performed to detect miRNAs involved in cardiomyocyte proliferation. Neonatal cardiomyocytes were transfected with a miRNA library consisting of 875 miRNAs (Eulalio et al. 2012). Remarkably, 204 miRNAs increased neonatal cardiomyocyte proliferation in vitro. Two pro-proliferative miRNAs – miR-199a and miR-590 – were further used for in vivo experiments. Overexpression of these miRNAs in neonatal rats revealed a thicker myocardium and increased cardiomyocyte proliferation. Moreover, intramyocardial overexpression of miR-199a and miR-590 in adult mice undergoing myocardial infarction induced cardiomyocyte proliferation in the peri-infarct area, reduced infarct size, and improved cardiac function (Eulalio et al. 2012).

The role of the miRNA cluster miR-17-92 for cardiac proliferative processes was also investigated. Cardiac-specific deletion of miR-17-92 leads to decreased cardiomyocyte proliferation in postnatal hearts (Chen et al. 2013a). Consistently, overexpression of miR-17-92 in embryonic and postnatal cardiomyocytes increased their proliferative capacity with a thickened myocardium due to hyperplasia. Intriguingly, induced cardiac expression of miR-17-92 in adult mice, where proliferative capacity of cardiomyocytes is almost lost, resulted in an increased cardiomyocyte proliferation. In addition, cardiac overexpression of miR-17-92 preserved cardiac function after myocardial infarction (Chen et al. 2013a). Another study investigated the miR-302-367 cluster in hearts due to its contribution in lung development. Cardiac-specific knockout resulted in decreased embryonic cell proliferation associated with a decreased cardiomyocyte differentiation (Tian et al. 2015). Target analysis after overexpression of miR-302-367 showed suppression of Mst1, Lats2, and Mob1b, which are all acting as contributors of the Hippo signaling pathway upstream of the Yes-associated protein (YAP). Phosphorylation of the transcriptional co-activator YAP results in suppression of cell proliferation. Consistently, transgenic cardiac or systemic transient overexpression of the miR-302-367 enhanced cardiomyocyte proliferation and improved cardiac function in a mouse myocardial infarction model. However, long-term overexpression of miR-302-367, consistent with the role of the Hippo pathway in the regulation of organ growth, leads to dilatation of the left ventricle, which favors a transient application of this miR cluster (Tian et al. 2015). These studies indicate that cell cycle reentry of cardiomyocytes can be induced by administration of pro-proliferative miRNAs.

4.4 MiRNAs in the Development and Progression of Heart Failure

Multiple mechanical and pathological stress triggers and cardiac injury evoke detrimental cardiac remodeling processes in the adult heart leading to chronic heart failure. It is not surprising that miRNAs are also involved in cardiomyocyte pathophysiological mechanisms (including cardiomyocyte hypertrophy, apoptosis, survival, and reactivation of the fetal gene program). However, as the heart comprises other important cell fractions, miRNAs altering functions of cardiac fibroblasts, which trigger extracellular matrix deposition and fibrosis and changes in endothelial-derived miRNAs that regulate angiogenesis, are also important mechanisms for heart failure development and progression. During heart failure development, many fetal genes, which are quiescent in the adult heart, are reactivated (Miyata et al. 2000; Nakao et al. 1997). As activation of gene programs in the fetal heart is regulated by miRNAs, miRNAs inducing fetal genes were also found in experimental and clinical studies. First insights came from a conditional knockout of Dicer, an enzyme needed for processing functional mature miRNAs. Knockout of Dicer led to cardiac remodeling processes and upregulation of fetal gene transcripts (viz., Acta1, Nppb, Myh7, and Nppa) (da Costa Martins et al. 2008). Comparison of miRNAs in experimental hypertrophic cardiomyopathy models showed >12 deregulated miRNAs as compared to sham-operated mice, which showed an overlap when comparing nonfailing versus end-stage heart failure tissues from humans (van Rooij et al. 2006). Seok et al. (2014) observed a downregulation of miR-155 in cardiomyocytes in a pressure overload model. Knockout of miR-155 in mice repressed cardiac hypertrophy, partly by targeting Jarid2, a key transcriptional regulator of cardiac development and function. Formerly, miR-155 was known as an abundantly expressed miRNA in macrophages and monocytes. The loss of miR-155 in macrophages was found to inhibit leukocyte infiltration and protect murine hearts from hypertrophy, mostly by inhibition of pro-inflammatory macrophage-derived factors and downregulation of adhesion molecules (Heymans et al. 2013). These studies imply that targeted therapy of one miRNA may protect different cell types from pathological mechanisms – either by inhibition of paracrine secretion of macrophages or from hypertrophic response in cardiomyocyte after pressure overload.

Comparison of miRNA expression profiles using left ventricular tissue samples from patients with heart failure, nonfailing hearts, and fetal human heart tissues revealed a profound alteration in miRNA expression of heart failure tissues as compared to healthy tissue samples. In addition, up- and downregulated miRNA showed a > 85% coverage when miRNA expression of heart failure tissues was compared with fetal tissue, indicating a close relationship in molecular miRNA-dependent mechanism with the reactivation of fetal gene programs (Thum et al. 2007). Another elegant study performed a deep-sequencing analysis of RNA of human left ventricular tissue samples derived from nonfailing human LV and failing human LV before and after left ventricular assist device (LVAD) support (Yang et al. 2014). More than 147 miRNAs were differentially regulated when comparing nonfailing

with heart failure LV samples; however, only two to five miRNAs returned to normal levels after LVAD support. These observations are consistent with a recent study where only subtle changes in miRNA expression between ischemic and dilated cardiomyopathy were detected, and miRNA profiling did not reveal differences before and after LVAD treatment (Akat et al. 2014). In contrast, >570 lncRNAs were found to be deregulated, mostly of mitochondrial origin, and approximately 10% of these lncRNAs normalized after LVAD support (Yang et al. 2014).

Changes in intracellular calcium handling are critical for heart failure, as they determine cardiac contractility. Gene therapy using an adenoviral vector containing sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2) led to a decrease in clinical symptoms and reverse remodeling in heart failure patients in a small clinical phase II study (Jessup et al. 2011). SERCA2 is a calcium-transporting ATPase, which enables calcium uptake in the sarcoplasmic reticulum during relaxation of cardiomyocytes. Wahlquist et al. (2014) used the 3′-UTR region of SERCA2 as a sensor construct to identify miRNAs targeting SERCA2. MiR-25 markedly inhibited SERCA2 expression and was upregulated in the myocardium derived from patients with heart failure. Treatment with anti-miR-25 in a transaortic constriction (TAC) model in mice blunted progression of cardiac dysfunction and improved survival. Interestingly, in a study investigating experimental Hand2-induced cardiac hypertrophy, inhibition of miR-25 resulted in impaired cardiac function using a TAC model (Dirkx et al. 2013). Although the two studies used the same experimental hypertrophy model, inhibition of miR-25 was started 3 months (Wahlquist et al. 2014) versus 3 days (Dirkx et al. 2013) after TAC operation and cardiac function were assessed at 5.5 months (Wahlquist et al. 2014) as compared to 1 month (Dirkx et al. 2013) after TAC. The findings therefore suggest that miR-25 has different functions in the subacute versus chronic heart failure phase, and miR-25 expression may be dynamic in the course of hypertrophy and heart failure. However, these questions have to be addressed in future studies. Dynamic expression of miRNAs upon a stress trigger has been shown in various studies, such as for miR-212/132 (Ucar et al. 2012), miR-208 (van Rooij et al. 2007), and miR-195 (van Rooij et al. 2006).

Cardiac fibroblasts contribute to adverse remodeling processes and progression of heart failure. MiRNAs have been identified as critical regulators in cardiac fibroblasts, thereby contributing to extracellular matrix modulation and cardiac fibrosis. MiR-29 targets multiple collagens and expression of extracellular matrix proteins. In an experimental model of MI, miR-29 expression was markedly reduced in the infarct region and mostly of fibroblast origin when comparing expression between fibroblasts and cardiomyocytes (van Rooij et al. 2008). Interestingly, transforming growth factor β (TGFβ), a key enhancer of cardiac fibrosis, decreased miR-29 expression in vitro, suggesting that upregulation of TGFβ represses miR-29 expression, resulting in an enhanced deposition of extracellular matrix proteins (van Rooij et al. 2008). MiR-29 downregulation was also identified as a pro-fibrotic mechanism in pulmonary (Cushing et al. 2011) and renal (Qin et al. 2011) fibrosis. However, miR-29 is also involved in the progression of aortic aneurysms. Reduced expression levels of miR-29a and inverse correlation with aortic size have also been

observed in patients with aortic aneurysm (Jones et al. 2011). Another miR-29 family member, miR-29b, was reported to be upregulated in patients and in experimental models of aortic aneurysm (no significant difference of miR-29a expression was observed in this study). Downregulation of miR-29 by delivery of LNA-modified antisense oligonucleotides in vivo resulted in enhanced expression of collagen members and reduction in aortic diameter (Boon et al. 2011). Therefore, whereas overexpression of miR-29 after myocardial infarction may reduce cardiac fibrosis, a reduced matrix deposition in miR-29-treated subjects may lead to the progression of aortic dilatation.

Another prominent fibrosis regulating miRNA is miR-21 that is upregulated in rodent models of ischemia-reperfusion (Roy et al. 2009) and hypertrophy (van Rooij et al. 2006). First shown as a pro-fibrotic miRNA in a pressure overload mouse model, which supports cardiac remodeling by an increase of ERK-MAP kinase activity in cardiac fibroblasts, it was later shown that upregulation of miR-21 after experimental ischemic preconditioning was protective in cardiac myocytes by inhibiting the expression of programmed cell death 4 (PDCD4) (Dong et al. 2009). In addition, sodium sulfide (Na2S), a donor for hydrogen sulfide, which is protective in various injury models of the heart, induces miR-21 in cardiomyocytes, indicating that the observed improvement in survival and decreased infarct size in an ischemia-reperfusion injury model are mediated by miR-21 through an inhibition of inflammasome function (Toldo et al. 2014). As miR-21 is expressed in both cardiomyocytes and cardiac fibroblasts, miRNA-mediated cell-to-cell communication was recently investigated. In this study, miR-21-3p (the star strand of miR-21, which is supposedly degraded) showed a high abundance in fibroblast-secreted exosomes and uptake of miR-21-3p in cardiomyocytes that resulted in cardiomyocyte hypertrophy via downregulation of SH3 domain-containing protein 2 (SORBS2) and PDZ and LIM domain 5 (PDLIM5) (Bang et al. 2014). These experimental studies imply that one miRNA may exert different functions in different cell types within the heart. In addition, miR-21-3p, a star strand that was earlier thought to be degraded, may also act as a functional miRNA. Moreover, the observation that miR-21 is not needed to induce cardiac hypertrophy in a knockout model and an experimental model using a different anti-miR (8-mer LNA miR-21) (Patrick et al. 2010) and different cardiac heart failure models leading to divergent findings of miR-21 action suggests that spatiotemporal expression of miRNAs and different methodological approaches are important determinants of miRNA functional activity.

4.5 Long Noncoding RNAs in Cardiovascular Disease

Long noncoding RNAs (lncRNAs) consist of a new group of noncoding RNAs emerging as genetic modifiers in cardiovascular disease. Whereas miRNAs belong to the short RNAs (i.e., <200 nucleotides), lncRNAs harbor more than 200 nucleotides. In addition, other than miRNAs, lncRNA interaction is not (almost) exclusively dependent on mRNA translational repression or mRNA degradation but rather activates or silences gene transcription through chromatin regulation and

transcriptional modulation (Geisler and Coller 2013; Wahlestedt 2013). In addition, transcriptional modulation of lncRNAs acts by *cis* (at the site of synthesis) or *trans* (at many different sites) fashion (Wahlestedt 2013; Mercer and Mattick 2013). LncRNA are not well preserved between species, and around 30% are specifically detected in primates (Derrien et al. 2012). Initial studies were performed in the oncology field, where investigated lncRNA was linked to cancer diseases (Gutschner et al. 2013; Yap et al. 2010). Some of these lncRNAs now gain also attention in the cardiovascular field, such as MALAT1 and ANRIL (Vausort et al. 2014; Burd et al. 2010). As gene transcription regulators, lncRNAs, like miRNAs, are involved in the regulation of cardiac development. One such lncRNA is Fendrr. Fendrr knockout models have been shown to impair embryonic cardiomyocyte proliferation, leading to hypoplasia and impaired cardiac function, resulting in embryonic death (Grote et al. 2013). Mechanistic studies observed that Fendrr modifies chromatin regulation through the binding of PRC2 and TrxG/MLL complexes (Grote et al. 2013). Deletion of Fendrr in another knockout model (Sauvageau et al. 2013) resulted in perinatal lethality. Ventricular septal defects and unstructured vessels were observed. In addition, reduced expression of Fendrr was observed in mutants of an endothelial-specific knockout model of Forkhead Box transcription factor F1 (FOXF1), a critical factor for vascular development (Ren et al. 2014). Another lncRNA involved in heart development is Braveheart, which is expressed in mice but lacks an orthologue in other species (Klattenhoff et al. 2013). Braveheart was found to interact in a gene network upstream of Mesp1 and is needed for activation of cardiac transcription factors, which drive mesodermal cells toward a cardiovascular phenotype (Klattenhoff et al. 2013). Braveheart was also detected as a cardiac-enriched lncRNA in a study investigating lncRNA expression in different mouse tissues (hearts, livers, and skin cells) using RNA sequencing (Matkovich et al. 2014). In adult mouse hearts, 152 lncRNAs showed high expression levels. Out of these lncRNAs, 48 lncRNAs are enriched in the heart as compared to liver and skin cells. Furthermore, RNA sequencing in cardiomyocytes and fibroblasts from adult mouse hearts indicates that most of these lncRNAs are enriched in the cardiomyocyte cell fraction (Matkovich et al. 2014). RNA sequencing allows also to search for differentially regulated lncRNA in cardiovascular disease models. Pedrazzini's research group investigated alterations in cardiac lncRNA profiles after myocardial infarction in mice (Ounzain et al. 2015). Analysis of the RNA sequencing revealed 988 annotated lncRNAs but also identified 1521 novel lncRNAs, of which 60% are heart specific according to computational analysis. Importantly, human orthologues were found in 73% of novel lncRNAs detected in mouse. Downregulation of an unannotated novel lncRNA, NovInc6, was further shown in patients with dilated cardiomyopathy, in concert with suppression of a predicted target Nkx2-5, a key transcription factor of cardiac development and cardiac gene program. Using a similar approach, Zangrando et al. (2014) screened for differentially expressed lncRNAs 24 h after induction of MI in C57/BL6 mice using an Agilent microarray with 55,681 probes. Ten and twenty lncRNAs were down- and upregulated more than twofold, with NR_028427 (named myocardial infarction-associated transcript 1 (MIRT1)) and ENSMUST000001005122 (named MIRT2) showing the highest fold changes

between groups. As a trend toward a correlation with LV remodeling parameters was observed, computational analysis of genes involved in remodeling processes identified strong correlation with 18 (for MIRT 1) and 17 (for MIRT 2) remodeling genes. However, no orthologues of MIRT1 and MIRT2 exist in humans.

As experimental models significantly affect lncRNA levels, it is of interest whether therapeutic interventions result in changes of lncRNA expression in humans. Yang et al. (2014) investigated differential expression of lncRNA in ischemic and nonischemic cardiomyopathy before and after LVAD support using RNA sequencing. Interestingly, cluster analysis revealed lncRNA signatures discriminating between ischemic and nonischemic cardiomyopathy. After LVAD support, a higher percentage of lncRNA show improved or normalized levels as compared to miRNA and mRNA expression profiles. LncRNA expression profiles furthermore were able to distinguish between before and after LVAD treatment, which indicates that lncRNA is involved in signaling pathways leading to reverse remodeling after LVAD support (Yang et al. 2014). Changes in transcriptome, including lncRNA expression, not only occur in the heart. A recent report by Deveaux's group (Vausort et al. 2014) assumed that MI alters lncRNA levels in the blood drawn from these patients. In a large sample group, five pre-specified lncRNAs associated with cardiovascular disease processes (hypoxia-inducible factor 1A antisense RNA 2 (aHIF), ANRIL, potassium voltage-gated channel, KQT-like subfamily, member 1 opposite strand/antisense transcript 1 (KCNQ1OT1), MIAT, and MALAT1) were analyzed in patients with acute MI and presumably healthy subjects. Levels of aHIF, MALAT1, and KCNQ1OT1 were higher, and expression of ANRIL is lower in patients with MI as compared to healthy subjects. Expression analysis of the five lncRNAs in subpopulations of mononuclear cells (in healthy subjects) showed that the distribution pattern of lncRNAs differs in the subpopulations. In addition, ANRIL and KCNQ1OT1 added prognostic information to a clinical model for LV dysfunction (LVEF <40%) at 4-month follow-up.

Genetic variants have been observed as predictors of cardiovascular diseases. Variations on chromosome 9p21 (Samani et al. 2007; Ye et al. 2008) increase the susceptibility of cardiovascular disease. Single nucleotide polymorphisms (SNPs) within this genomic region are associated with coronary artery disease and premature myocardial infarction (Abdullah et al. 2008; Samani et al. 2007). Interestingly chromosome 9p21 harbors the lncRNA ANRIL (antisense noncoding RNA at the ink4 locus or CDKN2BAS (antisense to CDKN2B)). Recently, ANRIL expression was correlated with variants associated with a higher risk for coronary artery disease, suggesting that ANRIL regulates chromatin modulation of coronary artery disease susceptibility genes like the INK/ARF locus (Burd et al. 2010). Overexpression of ANRIL in monocytic cell lines increased proliferation, cell adhesion, and blunts apoptosis (Holdt et al. 2013), potential mechanisms that trigger atherosclerosis. This raises the possibility that at least some lncRNAs are the missing link between SNP and risk of MI and CAD. Another example of SNPs causing a risk of myocardial infarction is the discovery of myocardial infarction associated transcript (MIAT). Subjects with a SNP in exon 5 of MIAT show a higher susceptibility for myocardial infarction in a large-scale case-control association study.

Numerous reports observed that MIAT is involved in splicing efficiency, which may explain the findings of the aforementioned study (Aprea et al. 2013; Tsuiji et al. 2011). Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1)/NEAT2 is another nuclear lncRNA with splicing ability (Hutchinson et al. 2007) and was found to enhance proliferation in human diploid fibroblasts and HeLa cells via this mechanism. However, in endothelial cells, where MALAT1 silencing impairs endothelial cell proliferation, expression of splicing-related genes is not altered, but cyclins and kinases were downregulated (Michalik et al. 2014). Together, these studies expand the knowledge of silencing and activation of gene networks in cardiovascular research and introduce lncRNA as new regulators in the complex molecular understanding. As lncRNAs are crucially involved in key features of cardiac injury, such as apoptosis, inflammation, impaired angiogenesis, and device treatment that lead to a change in lncRNA expression, lncRNAs may provide a future diagnostic and therapeutic clinical tool.

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Conflict of Interest Philipp Jakob has received research grants from Bayer Healthcare (Grants4Targets).

Compliance with Ethical Standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

References

Abdullah KG, Li L, Shen GQ, Hu Y, Yang Y, MacKinlay KG, Topol EJ, Wang QK. Four SNPS on chromosome 9p21 confer risk to premature, familial CAD and MI in an American Caucasian population (GeneQuest). Ann Hum Genet. 2008;72(Pt 5):654–7. doi:10.1111/j.1469-1809.2008.00454.x.

Aguirre A, Montserrat N, Zacchigna S, Nivet E, Hishida T, Krause MN, Kurian L, Ocampo A, Vazquez-Ferrer E, Rodriguez-Esteban C, Kumar S, Moresco JJ, Yates 3rd JR, Campistol JM, Sancho-Martinez I, Giacca M, Izpisua Belmonte JC. In vivo activation of a conserved microRNA program induces mammalian heart regeneration. Cell Stem Cell. 2014;15(5):589–604. doi:10.1016/j.stem.2014.10.003.

Akat KM, Moore-McGriff D, Morozov P, Brown M, Gogakos T, Correa Da Rosa J, Mihailovic A, Sauer M, Ji R, Ramarathnam A, Totary-Jain H, Williams Z, Tuschl T, Schulze PC. Comparative RNA-sequencing analysis of myocardial and circulating small RNAs in human heart failure and their utility as biomarkers. Proc Natl Acad Sci U S A. 2014;111(30):11151–6. doi:10.1073/pnas.1401724111.

Aprea J, Prenninger S, Dori M, Ghosh T, Monasor LS, Wessendorf E, Zocher S, Massalini S, Alexopoulou D, Lesche M, Dahl A, Groszer M, Hiller M, Calegari F. Transcriptome sequencing during mouse brain development identifies long non-coding RNAs functionally

involved in neurogenic commitment. EMBO J. 2013;32(24):3145–60. doi:10.1038/emboj.2013.245.

Assmus B, Schachinger V, Teupe C, Britten M, Lehmann R, Dobert N, Grunwald F, Aicher A, Urbich C, Martin H, Hoelzer D, Dimmeler S, Zeiher AM. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). Circulation. 2002;106(24):3009–17.

Assmus B, Tonn T, Seeger FH, Yoon CH, Leistner D, Klotsche J, Schachinger V, Seifried E, Zeiher AM, Dimmeler S. Red blood cell contamination of the final cell product impairs the efficacy of autologous bone marrow mononuclear cell therapy. J Am Coll Cardiol. 2010;55(13):1385–94. doi:10.1016/j.jacc.2009.10.059.

Bang C, Batkai S, Dangwal S, Gupta SK, Foinquinos A, Holzmann A, Just A, Remke J, Zimmer K, Zeug A, Ponimaskin E, Schmiedl A, Yin X, Mayr M, Halder R, Fischer A, Engelhardt S, Wei Y, Schober A, Fiedler J, Thum T. Cardiac fibroblast-derived microRNA passenger strand-enriched exosomes mediate cardiomyocyte hypertrophy. J Clin Invest. 2014;124(5):2136–46. doi:10.1172/JCI70577.

Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabe-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisen J. Evidence for cardiomyocyte renewal in humans. Science. 2009;324(5923):98–102. doi:10.1126/science.1164680.

Bolli R, Chugh AR, D'Amario D, Loughran JH, Stoddard MF, Ikram S, Beache GM, Wagner SG, Leri A, Hosoda T, Sanada F, Elmore JB, Goichberg P, Cappetta D, Solankhi NK, Fahsah I, Rokosh DG, Slaughter MS, Kajstura J, Anversa P. Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial. Lancet. 2011;378(9806):1847–57. doi:10.1016/S0140-6736(11)61590-0.

Boon RA, Seeger T, Heydt S, Fischer A, Hergenreider E, Horrevoets AJ, Vinciguerra M, Rosenthal N, Sciacca S, Pilato M, van Heijningen P, Essers J, Brandes RP, Zeiher AM, Dimmeler S. MicroRNA-29 in aortic dilation: implications for aneurysm formation. Circ Res. 2011;109(10):1115–9. doi:10.1161/CIRCRESAHA.111.255737.

Boon RA, Iekushi K, Lechner S, Seeger T, Fischer A, Heydt S, Kaluza D, Treguer K, Carmona G, Bonauer A, Horrevoets AJ, Didier N, Girmatsion Z, Biliczki P, Ehrlich JR, Katus HA, Muller OJ, Potente M, Zeiher AM, Hermeking H, Dimmeler S. MicroRNA-34a regulates cardiac ageing and function. Nature. 2013;495(7439):107–10. doi:10.1038/nature11919.

Burd CE, Jeck WR, Liu Y, Sanoff HK, Wang Z, Sharpless NE. Expression of linear and novel circular forms of an INK4/ARF-associated non-coding RNA correlates with atherosclerosis risk. PLoS Genet. 2010;6(12):e1001233. doi:10.1371/journal.pgen.1001233.

Cao X, Wang J, Wang Z, Du J, Yuan X, Huang W, Meng J, Gu H, Nie Y, Ji B, Hu S, Zheng Z. MicroRNA profiling during rat ventricular maturation: a role for miR-29a in regulating cardiomyocyte cell cycle re-entry. FEBS Lett. 2013;587(10):1548–55. doi:10.1016/j.febslet.2013.01.075.

Chavakis E, Dimmeler S. Homing of progenitor cells to ischemic tissues. Antioxid Redox Signal. 2011;15(4):967–80. doi:10.1089/ars.2010.3582.

Chen JF, Murchison EP, Tang R, Callis TE, Tatsuguchi M, Deng Z, Rojas M, Hammond SM, Schneider MD, Selzman CH, Meissner G, Patterson C, Hannon GJ, Wang DZ. Targeted deletion of Dicer in the heart leads to dilated cardiomyopathy and heart failure. Proc Natl Acad Sci U S A. 2008;105(6):2111–6. doi:10.1073/pnas.0710228105.

Chen J, Huang ZP, Seok HY, Ding J, Kataoka M, Zhang Z, Hu X, Wang G, Lin Z, Wang S, Pu WT, Liao R, Wang DZ. mir-17-92 cluster is required for and sufficient to induce cardiomyocyte proliferation in postnatal and adult hearts. Circ Res. 2013a;112(12):1557–66. doi:10.1161/CIRCRESAHA.112.300658.

Chen L, Wang Y, Pan Y, Zhang L, Shen C, Qin G, Ashraf M, Weintraub N, Ma G, Tang Y. Cardiac progenitor-derived exosomes protect ischemic myocardium from acute ischemia/reperfusion injury. Biochem Biophys Res Commun. 2013b;431(3):566–71. doi:10.1016/j.bbrc.2013.01.015.

Chong JJ, Yang X, Don CW, Minami E, Liu YW, Weyers JJ, Mahoney WM, Van Biber B, Palpant NJ, Gantz JA, Fugate JA, Muskheli V, Gough GM, Vogel KW, Astley CA, Hotchkiss CE, Baldessari A, Pabon L, Reinecke H, Gill EA, Nelson V, Kiem HP, Laflamme MA, Murry

CE. Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts. Nature. 2014; doi:10.1038/nature13233.

Cushing L, Kuang PP, Qian J, Shao F, Wu J, Little F, Thannickal VJ, Cardoso WV, Lu J. miR-29 is a major regulator of genes associated with pulmonary fibrosis. Am J Respir Cell Mol Biol. 2011;45(2):287–94. doi:10.1165/rcmb.2010-0323OC.

da Costa Martins PA, Bourajjaj M, Gladka M, Kortland M, van Oort RJ, Pinto YM, Molkentin JD, De Windt LJ. Conditional dicer gene deletion in the postnatal myocardium provokes spontaneous cardiac remodeling. Circulation. 2008;118(15):1567–76. doi:10.1161/CIRCULATIONAHA.108.769984.

Derrien T, Johnson R, Bussotti G, Tanzer A, Djebali S, Tilgner H, Guernec G, Martin D, Merkel A, Knowles DG, Lagarde J, Veeravalli L, Ruan X, Ruan Y, Lassmann T, Carninci P, Brown JB, Lipovich L, Gonzalez JM, Thomas M, Davis CA, Shiekhattar R, Gingeras TR, Hubbard TJ, Notredame C, Harrow J, Guigo R. The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. Genome Res. 2012;22(9):1775–89. doi:10.1101/gr.132159.111.

Dirkx E, Gladka MM, Philippen LE, Armand AS, Kinet V, Leptidis S, El Azzouzi H, Salic K, Bourajjaj M, da Silva GJ, Olieslagers S, van der Nagel R, de Weger R, Bitsch N, Kisters N, Seyen S, Morikawa Y, Chanoine C, Heymans S, Volders PG, Thum T, Dimmeler S, Cserjesi P, Eschenhagen T, da Costa Martins PA, De Windt LJ. Nfat and miR-25 cooperate to reactivate the transcription factor Hand2 in heart failure. Nat Cell Biol. 2013;15(11):1282–93. doi:10.1038/ncb2866.

Dong S, Cheng Y, Yang J, Li J, Liu X, Wang X, Wang D, Krall TJ, Delphin ES, Zhang C. MicroRNA expression signature and the role of microRNA-21 in the early phase of acute myocardial infarction. J Biol Chem. 2009;284(43):29514–25. doi:10.1074/jbc.M109.027896.

Eulalio A, Mano M, Dal Ferro M, Zentilin L, Sinagra G, Zacchigna S, Giacca M. Functional screening identifies miRNAs inducing cardiac regeneration. Nature. 2012;492(7429):376–81. doi:10.1038/nature11739.

Fisher SA, Doree C, Mathur A, Martin-Rendon E. Meta-analysis of cell therapy trials for patients with heart failure – an update. Circ Res. 2015; doi:10.1161/CIRCRESAHA.116.304386.

Fleissner F, Jazbutyte V, Fiedler J, Gupta SK, Yin X, Xu Q, Galuppo P, Kneitz S, Mayr M, Ertl G, Bauersachs J, Thum T. Short communication: asymmetric dimethylarginine impairs angiogenic progenitor cell function in patients with coronary artery disease through a microRNA-21-dependent mechanism. Circ Res. 2010;107(1):138–43. doi:10.1161/CIRCRESAHA.110.216770.

Geisler S, Coller J. RNA in unexpected places: long non-coding RNA functions in diverse cellular contexts. Nat Rev Mol Cell Biol. 2013;14(11):699–712. doi:10.1038/nrm3679.

Glass C, Singla DK. MicroRNA-1 transfected embryonic stem cells enhance cardiac myocyte differentiation and inhibit apoptosis by modulating the PTEN/Akt pathway in the infarcted heart. Am J Physiol Heart Circ Physiol. 2011;301(5):H2038–49. doi:10.1152/ajpheart.00271.2011.

Gnecchi M, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res. 2008;103(11):1204–19. doi:10.1161/CIRCRESAHA.108.176826.

Gray WD, French KM, Ghosh-Choudhary S, Maxwell JT, Brown ME, Platt MO, Searles CD, Davis ME. Identification of therapeutic covariant microRNA clusters in hypoxia-treated cardiac progenitor cell exosomes using systems biology. Circ Res. 2015;116(2):255–63. doi:10.1161/CIRCRESAHA.116.304360.

Grote P, Wittler L, Hendrix D, Koch F, Wahrisch S, Beisaw A, Macura K, Blass G, Kellis M, Werber M, Herrmann BG. The tissue-specific lncRNA Fendrr is an essential regulator of heart and body wall development in the mouse. Dev Cell. 2013;24(2):206–14. doi:10.1016/j.devcel.2012.12.012.

Gutschner T, Hammerle M, Eissmann M, Hsu J, Kim Y, Hung G, Revenko A, Arun G, Stentrup M, Gross M, Zornig M, MacLeod AR, Spector DL, Diederichs S. The noncoding RNA MALAT1 is a critical regulator of the metastasis phenotype of lung cancer cells. Cancer Res. 2013;73(3):1180–9. doi:10.1158/0008-5472.CAN-12-2850.

Gyongyosi M, Wojakowski W, Lemarchand P, Lunde K, Tendera M, Bartunek J, Marban E, Assmus B, Henry TD, Traverse JH, Moye LA, Surder D, Corti R, Huikuri H, Miettinen J,

Wohrle J, Obradovic S, Roncalli J, Malliaras K, Pokushalov E, Romanov A, Kastrup J, Bergmann MW, Atsma DE, Diederichsen A, Edes I, Benedek I, Benedek T, Pejkov H, Nyolczas N, Pavo N, Bergler-Klein J, Pavo IJ, Sylven C, Berti S, Navarese EP, Maurer G, Investigators A. Meta-Analysis of Cell-based CaRdiac stUdiEs (ACCRUE) in patients with acute myocardial infarction based on individual patient data. Circ Res. 2015;116(8):1346–60. doi:10.1161/CIRCRESAHA.116.304346.

Heeschen C, Lehmann R, Honold J, Assmus B, Aicher A, Walter DH, Martin H, Zeiher AM, Dimmeler S. Profoundly reduced neovascularization capacity of bone marrow mononuclear cells derived from patients with chronic ischemic heart disease. Circulation. 2004;109(13):1615–22. doi:10.1161/01.CIR.0000124476.32871.E3.

Heymans S, Corsten MF, Verhesen W, Carai P, van Leeuwen RE, Custers K, Peters T, Hazebroek M, Stoger L, Wijnands E, Janssen BJ, Creemers EE, Pinto YM, Grimm D, Schurmann N, Vigorito E, Thum T, Stassen F, Yin X, Mayr M, de Windt LJ, Lutgens E, Wouters K, de Winther MP, Zacchigna S, Giacca M, van Bilsen M, Papageorgiou AP, Schroen B. Macrophage microRNA-155 promotes cardiac hypertrophy and failure. Circulation. 2013;128(13):1420–32. doi:10.1161/CIRCULATIONAHA.112.001357.

Holdt LM, Hoffmann S, Sass K, Langenberger D, Scholz M, Krohn K, Finstermeier K, Stahringer A, Wilfert W, Beutner F, Gielen S, Schuler G, Gabel G, Bergert H, Bechmann I, Stadler PF, Thiery J, Teupser D. Alu elements in ANRIL non-coding RNA at chromosome 9p21 modulate atherogenic cell functions through trans-regulation of gene networks. PLoS Genet. 2013;9(7):e1003588. doi:10.1371/journal.pgen.1003588.

Hosoda T, Zheng H, Cabral-da-Silva M, Sanada F, Ide-Iwata N, Ogorek B, Ferreira-Martins J, Arranto C, D'Amario D, del Monte F, Urbanek K, D'Alessandro DA, Michler RE, Anversa P, Rota M, Kajstura J, Leri A. Human cardiac stem cell differentiation is regulated by a mircrine mechanism. Circulation. 2011;123(12):1287–96. doi:10.1161/CIRCULATIONAHA.110.982918.

Hu S, Huang M, Nguyen PK, Gong Y, Li Z, Jia F, Lan F, Liu J, Nag D, Robbins RC, Wu JC. Novel MicroRNA prosurvival cocktail for improving engraftment and function of cardiac progenitor cell transplantation. Circulation. 2011;124(11 Suppl):S27–34. doi:10.1161/CIRCULATIONAHA.111.017954.

Hutchinson JN, Ensminger AW, Clemson CM, Lynch CR, Lawrence JB, Chess A. A screen for nuclear transcripts identifies two linked noncoding RNAs associated with SC35 splicing domains. BMC Genomics. 2007;8:39. doi:10.1186/1471-2164-8-39.

Ieda M, Fu JD, Delgado-Olguin P, Vedantham V, Hayashi Y, Bruneau BG, Srivastava D. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. Cell. 2010;142(3):375–86. doi:10.1016/j.cell.2010.07.002.

Jakob P, Landmesser U. Current status of cell-based therapy for heart failure. Curr Heart Fail Rep. 2013;10(2):165–76. doi:10.1007/s11897-013-0134-z.

Jakob P, Doerries C, Briand S, Mocharla P, Krankel N, Besler C, Mueller M, Manes C, Templin C, Baltes C, Rudin M, Adams H, Wolfrum M, Noll G, Ruschitzka F, Luscher T, Landmesser U. Loss of angiomiR-126 and 130a in angiogenic early outgrowth cells from patients with chronic heart failure: role for impaired in vivo neovascularization and cardiac repair capacity. Circulation. 2012a; doi:10.1161/CIRCULATIONAHA.112.093906.

Jakob P, Doerries C, Briand S, Mocharla P, Krankel N, Besler C, Mueller M, Manes C, Templin C, Baltes C, Rudin M, Adams H, Wolfrum M, Noll G, Ruschitzka F, Luscher TF, Landmesser U. Loss of angiomiR-126 and 130a in angiogenic early outgrowth cells from patients with chronic heart failure: role for impaired in vivo neovascularization and cardiac repair capacity. Circulation. 2012b;126(25):2962–75. doi:10.1161/CIRCULATIONAHA.112.093906.

Jayawardena TM, Egemnazarov B, Finch EA, Zhang L, Payne JA, Pandya K, Zhang Z, Rosenberg P, Mirotsou M, Dzau VJ. MicroRNA-mediated in vitro and in vivo direct reprogramming of cardiac fibroblasts to cardiomyocytes. Circ Res. 2012;110(11):1465–73. doi:10.1161/CIRCRESAHA.112.269035.

Jayawardena TM, Finch EA, Zhang L, Zhang H, Hodgkinson CP, Pratt RE, Rosenberg PB, Mirotsou M, Dzau VJ. MicroRNA induced cardiac reprogramming in vivo: evidence for

mature cardiac myocytes and improved cardiac function. Circ Res. 2015;116(3):418–24. doi:10.1161/CIRCRESAHA.116.304510.

Jeevanantham V, Butler M, Saad A, Abdel-Latif A, Zuba-Surma EK, Dawn B. Adult bone marrow cell therapy improves survival and induces long-term improvement in cardiac parameters: a systematic review and meta-analysis. Circulation. 2012;126(5):551–68. doi:10.1161/CIRCULATIONAHA.111.086074.

Jessup M, Greenberg B, Mancini D, Cappola T, Pauly DF, Jaski B, Yaroshinsky A, Zsebo KM, Dittrich H, Hajjar RJ, Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease Investigators. Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID): a phase 2 trial of intracoronary gene therapy of sarcoplasmic reticulum Ca2 + −ATPase in patients with advanced heart failure. Circulation. 2011;124(3):304–13. doi:10.1161/CIRCULATIONAHA.111.022889.

Jones JA, Stroud RE, O'Quinn EC, Black LE, Barth JL, Elefteriades JA, Bavaria JE, Gorman 3rd JH, Gorman RC, Spinale FG, Ikonomidis JS. Selective microRNA suppression in human thoracic aneurysms: relationship of miR-29a to aortic size and proteolytic induction. Circ Cardiovasc Genet. 2011;4(6):605–13. doi:10.1161/CIRCGENETICS.111.960419.

Khan M, Nickoloff E, Abramova T, Johnson J, Verma SK, Krishnamurthy P, Mackie AR, Vaughan E, Garikipati VN, Benedict CL, Ramirez V, Lambers E, Ito A, Gao E, Misener S, Qin G, Luongo TS, Elrod JW, Houser SR, Koch WJ, Kishore R. Embryonic stem cell-derived exosomes promote endogenous repair mechanisms and enhance cardiac function following myocardial infarction. Circ Res. 2015; doi:10.1161/CIRCRESAHA.117.305990.

Klattenhoff CA, Scheuermann JC, Surface LE, Bradley RK, Fields PA, Steinhauser ML, Ding H, Butty VL, Torrey L, Haas S, Abo R, Tabebordbar M, Lee RT, Burge CB, Boyer LA. Braveheart, a long noncoding RNA required for cardiovascular lineage commitment. Cell. 2013;152(3):570–83. doi:10.1016/j.cell.2013.01.003.

Kuppusamy KT, Jones DC, Sperber H, Madan A, Fischer KA, Rodriguez ML, Pabon L, Zhu WZ, Tulloch NL, Yang X, Sniadecki NJ, Laflamme MA, Ruzzo WL, Murry CE, Ruohola-Baker H. Let-7 family of microRNA is required for maturation and adult-like metabolism in stem cell-derived cardiomyocytes. Proc Natl Acad Sci U S A. 2015;112(21):E2785–94. doi:10.1073/pnas.1424042112.

Laflamme MA, Chen KY, Naumova AV, Muskheli V, Fugate JA, Dupras SK, Reinecke H, Xu C, Hassanipour M, Police S, O'Sullivan C, Collins L, Chen Y, Minami E, Gill EA, Ueno S, Yuan C, Gold J, Murry CE. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol. 2007;25(9):1015–24. doi:10.1038/nbt1327.

Li Z, Lee A, Huang M, Chun H, Chung J, Chu P, Hoyt G, Yang P, Rosenberg J, Robbins RC, Wu JC. Imaging survival and function of transplanted cardiac resident stem cells. J Am Coll Cardiol. 2009;53(14):1229–40. doi:10.1016/j.jacc.2008.12.036.

Liu N, Bezprozvannaya S, Williams AH, Qi X, Richardson JA, Bassel-Duby R, Olson EN. microRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart. Genes Dev. 2008;22(23):3242–54. doi:10.1101/gad.1738708.

Makkar RR, Smith RR, Cheng K, Malliaras K, Thomson LE, Berman D, Czer LS, Marban L, Mendizabal A, Johnston PV, Russell SD, Schuleri KH, Lardo AC, Gerstenblith G, Marban E. Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial. Lancet. 2012;379(9819):895–904. doi:10.1016/S0140-6736(12)60195-0.

Matkovich SJ, Edwards JR, Grossenheider TC, de Guzman SC, Dorn 2nd GW. Epigenetic coordination of embryonic heart transcription by dynamically regulated long noncoding RNAs. Proc Natl Acad Sci U S A. 2014;111(33):12264–9. doi:10.1073/pnas.1410622111.

Mercer TR, Mattick JS. Structure and function of long noncoding RNAs in epigenetic regulation. Nat Struct Mol Biol. 2013;20(3):300–7. doi:10.1038/nsmb.2480.

Michalik KM, You X, Manavski Y, Doddaballapur A, Zornig M, Braun T, John D, Ponomareva Y, Chen W, Uchida S, Boon RA, Dimmeler S. Long noncoding RNA MALAT1 regulates

endothelial cell function and vessel growth. Circ Res. 2014;114(9):1389–97. doi:10.1161/CIRCRESAHA.114.303265.

Miyata S, Minobe W, Bristow MR, Leinwand LA. Myosin heavy chain isoform expression in the failing and nonfailing human heart. Circ Res. 2000;86(4):386–90.

Mocharla P, Briand S, Giannotti G, Dorries C, Jakob P, Paneni F, Luscher T, Landmesser U. AngiomiR-126 expression and secretion from circulating CD34(+) and CD14(+) PBMCs: role for proangiogenic effects and alterations in type 2 diabetics. Blood. 2013;121(1):226–36. doi:10.1182/blood-2012-01-407106.

Muraoka N, Yamakawa H, Miyamoto K, Sadahiro T, Umei T, Isomi M, Nakashima H, Akiyama M, Wada R, Inagawa K, Nishiyama T, Kaneda R, Fukuda T, Takeda S, Tohyama S, Hashimoto H, Kawamura Y, Goshima N, Aeba R, Yamagishi H, Fukuda K, Ieda M. MiR-133 promotes cardiac reprogramming by directly repressing Snai1 and silencing fibroblast signatures. EMBO J. 2014;33(14):1565–81. doi:10.15252/embj.201387605.

Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, Pasumarthi KB, Virag JI, Bartelmez SH, Poppa V, Bradford G, Dowell JD, Williams DA, Field LJ. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature. 2004;428(6983):664–8. doi:10.1038/nature02446.

Nakao K, Minobe W, Roden R, Bristow MR, Leinwand LA. Myosin heavy chain gene expression in human heart failure. J Clin Invest. 1997;100(9):2362–70. doi:10.1172/JCI119776.

Nam YJ, Song K, Luo X, Daniel E, Lambeth K, West K, Hill JA, DiMaio JM, Baker LA, Bassel-Duby R, Olson EN. Reprogramming of human fibroblasts toward a cardiac fate. Proc Natl Acad Sci U S A. 2013;110(14):5588–93. doi:10.1073/pnas.1301019110.

Ong SG, Lee WH, Huang M, Dey D, Kodo K, Sanchez-Freire V, Gold JD, Wu JC. Cross talk of combined gene and cell therapy in ischemic heart disease: role of exosomal microRNA transfer. Circulation. 2014;130(11 Suppl 1):S60–9. doi:10.1161/CIRCULATIONAHA.113.007917.

Ounzain S, Micheletti R, Beckmann T, Schroen B, Alexanian M, Pezzuto I, Crippa S, Nemir M, Sarre A, Johnson R, Dauvillier J, Burdet F, Ibberson M, Guigo R, Xenarios I, Heymans S, Pedrazzini T. Genome-wide profiling of the cardiac transcriptome after myocardial infarction identifies novel heart-specific long non-coding RNAs. Eur Heart J. 2015;36(6):353–368a. doi:10.1093/eurheartj/ehu180.

Patrick DM, Montgomery RL, Qi X, Obad S, Kauppinen S, Hill JA, van Rooij E, Olson EN. Stress-dependent cardiac remodeling occurs in the absence of microRNA-21 in mice. J Clin Invest. 2010;120(11):3912–6. doi:10.1172/JCI43604.

Porrello ER, Johnson BA, Aurora AB, Simpson E, Nam YJ, Matkovich SJ, Dorn 2nd GW, van Rooij E, Olson EN. MiR-15 family regulates postnatal mitotic arrest of cardiomyocytes. Circ Res. 2011a;109(6):670–9. doi:10.1161/CIRCRESAHA.111.248880.

Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, Olson EN, Sadek HA. Transient regenerative potential of the neonatal mouse heart. Science. 2011b;331(6020):1078–80. doi:10.1126/science.1200708.

Porrello ER, Mahmoud AI, Simpson E, Johnson BA, Grinsfelder D, Canseco D, Mammen PP, Rothermel BA, Olson EN, Sadek HA. Regulation of neonatal and adult mammalian heart regeneration by the miR-15 family. Proc Natl Acad Sci U S A. 2013;110(1):187–92. doi:10.1073/pnas.1208863110.

Qian L, Huang Y, Spencer CI, Foley A, Vedantham V, Liu L, Conway SJ, Fu JD, Srivastava D. In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes. Nature. 2012;485(7400):593–8. doi:10.1038/nature11044.

Qin W, Chung AC, Huang XR, Meng XM, Hui DS, Yu CM, Sung JJ, Lan HY. TGF-beta/Smad3 signaling promotes renal fibrosis by inhibiting miR-29. J Am Soc Nephrol JASN. 2011;22(8):1462–74. doi:10.1681/ASN.2010121308.

Rao PK, Toyama Y, Chiang HR, Gupta S, Bauer M, Medvid R, Reinhardt F, Liao R, Krieger M, Jaenisch R, Lodish HF, Blelloch R. Loss of cardiac microRNA-mediated regulation leads to dilated cardiomyopathy and heart failure. Circ Res. 2009;105(6):585–94. doi:10.1161/CIRCRESAHA.109.200451.

Ren X, Ustiyan V, Pradhan A, Cai Y, Havrilak JA, Bolte CS, Shannon JM, Kalin TV, Kalinichenko VV. FOXF1 transcription factor is required for formation of embryonic vasculature by regulating VEGF signaling in endothelial cells. Circ Res. 2014;115(8):709–20. doi:10.1161/CIRCRESAHA.115.304382.

Roy S, Khanna S, Hussain SR, Biswas S, Azad A, Rink C, Gnyawali S, Shilo S, Nuovo GJ, Sen CK. MicroRNA expression in response to murine myocardial infarction: miR-21 regulates fibroblast metalloprotease-2 via phosphatase and tensin homologue. Cardiovasc Res. 2009;82(1):21–9. doi:10.1093/cvr/cvp015.

Samani NJ, Erdmann J, Hall AS, Hengstenberg C, Mangino M, Mayer B, Dixon RJ, Meitinger T, Braund P, Wichmann HE, Barrett JH, Konig IR, Stevens SE, Szymczak S, Tregouet DA, Iles MM, Pahlke F, Pollard H, Lieb W, Cambien F, Fischer M, Ouwehand W, Blankenberg S, Balmforth AJ, Baessler A, Ball SG, Strom TM, Braenne I, Gieger C, Deloukas P, Tobin MD, Ziegler A, Thompson JR, Schunkert H, WTCCC and the Cardiogenics Consortium. Genomewide association analysis of coronary artery disease. N Engl J Med. 2007;357(5):443–53. doi:10.1056/NEJMoa072366.

Sauvageau M, Goff LA, Lodato S, Bonev B, Groff AF, Gerhardinger C, Sanchez-Gomez DB, Hacisuleyman E, Li E, Spence M, Liapis SC, Mallard W, Morse M, Swerdel MR, D'Ecclessis MF, Moore JC, Lai V, Gong G, Yancopoulos GD, Frendewey D, Kellis M, Hart RP, Valenzuela DM, Arlotta P, Rinn JL. Multiple knockout mouse models reveal lncRNAs are required for life and brain development. eLife. 2013;2:e01749. doi:10.7554/eLife.01749.

Schober A, Nazari-Jahantigh M, Wei Y, Bidzhekov K, Gremse F, Grommes J, Megens RT, Heyll K, Noels H, Hristov M, Wang S, Kiessling F, Olson EN, Weber C. MicroRNA-126-5p promotes endothelial proliferation and limits atherosclerosis by suppressing Dlk1. Nat Med. 2014;20(4):368–76. doi:10.1038/nm.3487.

Seeger FH, Rasper T, Fischer A, Muhly-Reinholz M, Hergenreider E, Leistner DM, Sommer K, Manavski Y, Henschler R, Chavakis E, Assmus B, Zeiher AM, Dimmeler S. Heparin disrupts the CXCR4/SDF-1 axis and impairs the functional capacity of bone marrow-derived mononuclear cells used for cardiovascular repair. Circ Res. 2012;111(7):854–62. doi:10.1161/CIRCRESAHA.112.265678.

Seok HY, Chen J, Kataoka M, Huang ZP, Ding J, Yan J, Hu X, Wang DZ. Loss of MicroRNA-155 protects the heart from pathological cardiac hypertrophy. Circ Res. 2014;114(10):1585–95. doi:10.1161/CIRCRESAHA.114.303784.

Sluijter JP, van Mil A, van Vliet P, Metz CH, Liu J, Doevendans PA, Goumans MJ. MicroRNA-1 and -499 regulate differentiation and proliferation in human-derived cardiomyocyte progenitor cells. Arterioscler Thromb Vasc Biol. 2010;30(4):859–68. doi:10.1161/ATVBAHA.109.197434.

Song K, Nam YJ, Luo X, Qi X, Tan W, Huang GN, Acharya A, Smith CL, Tallquist MD, Neilson EG, Hill JA, Bassel-Duby R, Olson EN. Heart repair by reprogramming non-myocytes with cardiac transcription factors. Nature. 2012;485(7400):599–604. doi:10.1038/nature11139.

Thum T, Galuppo P, Wolf C, Fiedler J, Kneitz S, van Laake LW, Doevendans PA, Mummery CL, Borlak J, Haverich A, Gross C, Engelhardt S, Ertl G, Bauersachs J. MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure. Circulation. 2007;116(3):258–67. doi:10.1161/CIRCULATIONAHA.107.687947.

Tian Y, Liu Y, Wang T, Zhou N, Kong J, Chen L, Snitow M, Morley M, Li D, Petrenko N, Zhou S, Lu M, Gao E, Koch WJ, Stewart KM, Morrisey EE. A microRNA-Hippo pathway that promotes cardiomyocyte proliferation and cardiac regeneration in mice. Sci Transl Med. 2015;7(279):279ra238. doi:10.1126/scitranslmed.3010841.

Toldo S, Das A, Mezzaroma E, Chau VQ, Marchetti C, Durrant D, Samidurai A, Van Tassell BW, Yin C, Ockaili RA, Vigneshwar N, Mukhopadhyay ND, Kukreja RC, Abbate A, Salloum FN. Induction of microRNA-21 with exogenous hydrogen sulfide attenuates myocardial ischemic and inflammatory injury in mice. Circ Cardiovasc Genet. 2014;7(3):311–20. doi:10.1161/CIRCGENETICS.113.000381.

Tongers J, Losordo DW, Landmesser U. Stem and progenitor cell-based therapy in ischaemic heart disease: promise, uncertainties, and challenges. Eur Heart J. 2011;32(10):1197–206. doi:10.1093/eurheartj/ehr018.

Tsuiji H, Yoshimoto R, Hasegawa Y, Furuno M, Yoshida M, Nakagawa S. Competition between a noncoding exon and introns: Gomafu contains tandem UACUAAC repeats and associates with splicing factor-1. Genes Cells Ddevoted Mol Cellular Mechanisms. 2011;16(5):479–90. doi:10.1111/j.1365-2443.2011.01502.x.

Ucar A, Gupta SK, Fiedler J, Erikci E, Kardasinski M, Batkai S, Dangwal S, Kumarswamy R, Bang C, Holzmann A, Remke J, Caprio M, Jentzsch C, Engelhardt S, Geisendorf S, Glas C, Hofmann TG, Nessling M, Richter K, Schiffer M, Carrier L, Napp LC, Bauersachs J, Chowdhury K, Thum T. The miRNA-212/132 family regulates both cardiac hypertrophy and cardiomyocyte autophagy. Nat Commun. 2012;3:1078. doi:10.1038/ncomms2090.

van Rooij E, Sutherland LB, Liu N, Williams AH, McAnally J, Gerard RD, Richardson JA, Olson EN. A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. Proc Natl Acad Sci U S A. 2006;103(48):18255–60. doi:10.1073/pnas.0608791103.

van Rooij E, Sutherland LB, Qi X, Richardson JA, Hill J, Olson EN. Control of stress-dependent cardiac growth and gene expression by a microRNA. Science. 2007;316(5824):575–9. doi:10.1126/science.1139089.

van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, Hill JA, Olson EN. Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc Natl Acad Sci U S A. 2008;105(35):13027–32. doi:10.1073/pnas.0805038105.

Vausort M, Wagner DR, Devaux Y. Long noncoding RNAs in patients with acute myocardial infarction. Circ Res. 2014;115(7):668–77. doi:10.1161/CIRCRESAHA.115.303836.

Vicencio JM, Yellon DM, Sivaraman V, Das D, Boi-Doku C, Arjun S, Zheng Y, Riquelme JA, Kearney J, Sharma V, Multhoff G, Hall AR, Davidson SM. Plasma exosomes protect the myocardium from ischemia-reperfusion injury. J Am Coll Cardiol. 2015;65(15):1525–36. doi:10.1016/j.jacc.2015.02.026.

Wahlestedt C. Targeting long non-coding RNA to therapeutically upregulate gene expression. Nat Rev Drug Discov. 2013;12(6):433–46. doi:10.1038/nrd4018.

Wahlquist C, Jeong D, Rojas-Munoz A, Kho C, Lee A, Mitsuyama S, van Mil A, Park WJ, Sluijter JP, Doevendans PA, Hajjar RJ, Mercola M. Inhibition of miR-25 improves cardiac contractility in the failing heart. Nature. 2014;508(7497):531–5. doi:10.1038/nature13073.

Wang S, Aurora AB, Johnson BA, Qi X, McAnally J, Hill JA, Richardson JA, Bassel-Duby R, Olson EN. The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. Dev Cell. 2008;15(2):261–71. doi:10.1016/j.devcel.2008.07.002. S1534–5807(08)00281–5 [pii].

Wilson KD, Hu S, Venkatasubrahmanyam S, Fu JD, Sun N, Abilez OJ, Baugh JJ, Jia F, Ghosh Z, Li RA, Butte AJ, Wu JC. Dynamic microRNA expression programs during cardiac differentiation of human embryonic stem cells: role for miR-499. Circ Cardiovasc Genet. 2010;3(5):426–35. doi:10.1161/CIRCGENETICS.109.934281.

Xu Q, Seeger FH, Castillo J, Iekushi K, Boon RA, Farcas R, Manavski Y, Li YG, Assmus B, Zeiher AM, Dimmeler S. Micro-RNA-34a contributes to the impaired function of bone marrow-derived mononuclear cells from patients with cardiovascular disease. J Am Coll Cardiol. 2012;59(23):2107–17. doi:10.1016/j.jacc.2012.02.033.

Yang KC, Yamada KA, Patel AY, Topkara VK, George I, Cheema FH, Ewald GA, Mann DL, Nerbonne JM. Deep RNA sequencing reveals dynamic regulation of myocardial noncoding RNAs in failing human heart and remodeling with mechanical circulatory support. Circulation. 2014;129(9):1009–21. doi:10.1161/CIRCULATIONAHA.113.003863.

Yap KL, Li S, Munoz-Cabello AM, Raguz S, Zeng L, Mujtaba S, Gil J, Walsh MJ, Zhou MM. Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a. Mol Cell. 2010;38(5):662–74. doi:10.1016/j.molcel.2010.03.021.

Ye S, Willeit J, Kronenberg F, Xu Q, Kiechl S. Association of genetic variation on chromosome 9p21 with susceptibility and progression of atherosclerosis: a population-based, prospective study. J Am Coll Cardiol. 2008;52(5):378–84. doi:10.1016/j.jacc.2007.11.087.

Yin VP, Lepilina A, Smith A, Poss KD. Regulation of zebrafish heart regeneration by miR-133. Dev Biol. 2012;365(2):319–27. doi:10.1016/j.ydbio.2012.02.018.

Zampetaki A, Attia R, Mayr U, Gomes RS, Phinikaridou A, Yin X, Langley SR, Willeit P, Lu R, Fanshawe B, Fava M, Barallobre-Barreiro J, Molenaar C, So PW, Abbas A, Jahangiri M, Waltham M, Botnar R, Smith A, Mayr M. Role of miR-195 in aortic aneurysmal disease. Circ Res. 2014;115(10):857–66. doi:10.1161/CIRCRESAHA.115.304361.

Zangrando J, Zhang L, Vausort M, Maskali F, Marie PY, Wagner DR, Devaux Y. Identification of candidate long non-coding RNAs in response to myocardial infarction. BMC Genomics. 2014;15:460. doi:10.1186/1471-2164-15-460.

Zhao Y, Samal E, Srivastava D. Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. Nature. 2005;436(7048):214–20. doi:10.1038/nature03817.

Zhao Y, Ransom JF, Li A, Vedantham V, von Drehle M, Muth AN, Tsuchihashi T, McManus MT, Schwartz RJ, Srivastava D. Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. Cell. 2007;129(2):303–17. doi:10.1016/j.cell.2007.03.030.

5 Circulating microRNAs as Novel Biomarkers in Cardiovascular Disease: Basic and Technical Principles

Anna Zampetaki and Manuel Mayr

Contents

Abstract

The presence of stable endogenous microRNA pools in the circulation sparked interest in their potential use as biomarkers of cardiovascular disease. Several studies demonstrated the presence of microRNAs in the vesicles and in the non-vesicular fraction, mainly residing in protein and lipoprotein complexes. There is a need to identify novel biomarkers that can not only improve the established cardiovascular risk scores and identify individuals at high risk but also gain new mechanistic insights. Early studies on microRNA biomarkers for a wide range of cardiovascular pathologies were predominately conducted in small cohorts and

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provided the first evidence for their putative value. As these findings await confirmation from larger cohorts, several pre-analytical and analytical variables must be determined, and protocols for sample preparation, RNA quantification and data processing must be standardised before one can assess the value of circulating microRNAs for clinical practice. Here, we will review the technical challenges in assessing microRNA profiles and discuss the hurdles in establishing circulating microRNAs as reliable cardiovascular biomarkers.

5.1 Introduction

Multiple lines of evidence suggest that the mammalian genome is pervasively transcribed. The recent discovery that protein-coding genes comprise only 1% of the genome despite the fact that the majority of the bases are associated with at least one primary transcript led to a new appreciation of the functional significance of noncoding RNAs (Ecker et al. 2012; Sabin et al. 2013). Based mainly on their size, two important classes have been identified: long noncoding and small noncoding RNAs.

5.2 MicroRNA Biogenesis and Function

MicroRNAs (miRNAs) are among the best characterised small noncoding RNAs, generated from longer precursors by RNA pol II as primary transcripts with the typical 5′ cap structures and poly(A) tails. These primary miRNAs that comprise one or more hairpin structures with the characteristic long stem and a terminal loop are recognised and processed by the microprocessor complex. The RNase III endonuclease Drosha cleaves the double-stranded stem region to generate the precursor miRNA that is then exported to the cytosol. Here, it undergoes a second round of cleavage by another RNase III endonuclease, Dicer, that removes the terminal loop and generates a 20–22 nucleotide double-stranded miRNA complex. Recent evidence suggests that both strands may be stable and function as mature miRNAs (Indolfi and Curcio 2014). Intriguingly, miRNAs biogenesis can be subjected to an autoregulatory feedback loop as is the case for mature let-7 that can induce its own maturation, a critical observation particularly in the diseased heart (Zisoulis et al. 2012). Additionally, using transgenic expression of pre-microRNAs for miR-378 and miR-499 in the heart, it was revealed that these cardiac miRNAs can indirectly regulate several cardiac miRNAs (Matkovich et al. 2013).

MiRNAs exert their function mainly through hybridization to their target mRNAs (Bartel 2009), although binding to DNA in promoter regions has also been reported (Zardo et al. 2012). The mature miRNA strand is loaded to the RNA-induced silencing complex (RISC) by binding to the Argonaute protein (Ago) and directs the

complex to its target mRNA. This binding occurs with imperfect complementarity and results in transcript degradation through deadenylation and repression of translation. Although the details of miRNA-mRNA recognition are still elusive, it is thought that the seed region, nucleotides 2–8, shows the strongest affinity and is critical for the interaction.

Most miRNAs display a highly conserved sequence among mammals (Liu and Olson 2010). Their hybridization-based function and the multiplicity of their targets could prevent evolutionary drift and perhaps explain their highly conserved sequence. As far as their function is concerned, miRNAs seem to be an evolutionary tool developed to reinforce system robustness and ensure that gene expression occurs both at desirable levels and with appropriate timing (Inui et al. 2010). MiRNAs are largely considered to be the fine tuners of gene expression that exert mild effects under basal conditions and have more pronounced responses after stress. They form regulatory networks that target multiple effectors of the same signalling pathway and thus elicit system-wide biological responses. Co-targeting miRNA networks that regulate the same transcript are also common (Zampetaki and Mayr 2012). Diverse mechanisms of action have been identified. MiRNAs may facilitate signal mediation and modulation and may participate in both positive and negative feedback loops mediating phenotypic switch or signal resolution (Mendell and Olson 2012).

5.3 Circulating miRNAs

The presence of miRNAs in biological fluids has been well established. Blood cells are major contributors to circulating miRNAs, and perturbations in their number can alter the circulating miRNA pool (Pritchard et al. 2012; Tonge and Gant 2016). Platelets in particular have been shown to be key contributors as miRNA shedding from platelets has a prominent effect on the circulating miRNA profile, and the levels of many circulating miRNAs are directly related to the platelet activation state (Kaudewitz et al. 2016; Willeit et al. 2013; Zampetaki et al. 2012). Importantly, upon tissue injury, a robust release of tissue-enriched miRNAs is observed in the circulation. A rapid increase in miR-122 levels is detected after liver damage (Wang et al. 2009), while some cardiac miRNAs, e.g. miR-1 and miR-208b, are usually undetectable in the circulation but can be reliably quantified following cardiac injury (Widera et al. 2011). Additionally, circulating miRNA signatures have been proposed to associate with the onset of micro- and macrovascular complications of diabetes (Wang et al. 2016; Zampetaki et al. 2010, 2016) and risk of cardiovascular events (Zampetaki et al. 2012) implying that circulating miRNA fingerprints may have an added value that goes beyond disease diagnosis but also include risk prediction. Intriguingly, if distinct miRNA signatures were to be identified for specific pathologies, circulating miRNA panels may serve as novel biomarkers of disease.

5.3.1 miRNAs in Vesicles

Accumulating evidence supports the notion that extracellular miRNAs exhibit remarkable stability (Bertoia et al. 2015). It is assumed that this occurs though their presence in vesicles, protein and lipoprotein complexes. The release of cellular vesicles has emerged as a novel mechanism of intercellular communication. Most cells in response to stimulation or under basal conditions will form and secrete vesicles through a variety of processes. The released vesicles that may differ in size and content can transfer proteins, miRNAs or mRNA and can target recipient cells through membrane fusion, endocytosis or receptor-mediated binding (Loyer et al. 2014).

5.3.1.1 Apoptotic Bodies

Apoptotic bodies (ABs) are the largest vesicles (>1 μm) that are released in the circulation upon cell apoptosis. ABs have been implicated in tissue repair and regeneration, and microarray analysis revealed that the miRNA content reflects their cellular origin (Zernecke et al. 2009). The transfer of miR-126 residing in ABs to endothelial cells in a mouse model of atherosclerosis had a protective effect mainly through the regulation of CXCL12 production and the recruitment of progenitor cells that promoted tissue repair (Zernecke et al. 2009). This effect was abolished when ABs from miR-126 null mice were applied indicating that miR-126 mediated the protective response.

5.3.1.2 Microparticles

Plasma membrane blebbing results in the formation of microparticles (MP) that may vary in size from 100 to 1000 nm. MPs contain chemokines, receptors, lipids as well as an mRNA and an miRNA pool. Quantitative differences in the miRNA content of vesicles in response to different release of stimuli suggest that the vesicle formation is an active process. Platelets are a major source of MPs in the circulation. Several lines of evidence highlight an important role for platelet MPs in cardiovascular diseases. These microvesicles carry miRNAs that can have a paracrine effect and modulate vascular endothelial cell function. For example, Laffont et al. (2013) demonstrated a transfer of platelet MP-derived Ago2/miR-223 complexes to endothelial cells. These complexes were functional and could regulate gene expression in the recipient cells as demonstrated by the altered expression of endogenous targets at the mRNA and protein level. In response to advanced glycation end products, platelet MPs were also shown to promote endothelial cell apoptosis by delivering miR-223 into endothelial cells and inhibiting protein expression of the endogenous IGF-1 receptor (Pan et al. 2014). On a different note, the miR-223 transfer to naïve monocytes via macrophage-derived MPs can induce differentiation to macrophages, while silencing miR-223 impaired survival in monocytes (Ismail et al. 2013). Besides platelets, endothelial MPs were shown to limit apoptosis and enhance vascular repair by delivering miR-126 to recipient cells. Increased miR-126 levels inhibited sprouty-related, EVH1 domain-containing protein 1 (SPRED1) and led to an increase in reendothelialization in vitro. Under hyperglycemic conditions, lower

expression of miR-126 was observed, and this protective effect was largely abolished. Interestingly, miR-126 levels in circulating MPs were reduced in diabetic patients (Jansen et al. 2013). MPs also seem to mediate the intercellular communication between monocytes and endothelial cells in the angiogenic process. The miR-150 content of monocytic MPs was reported to promote angiogenesis in vitro and in vivo, although the mechanisms involved are still not well understood (Li et al. 2013; Zhang et al. 2010).

5.3.1.3 Exosomes

Exosomes are the smallest vesicles (50–90 nm) and are released constitutively or in response to cell activation by fusion of multivesicular bodies with the plasma membrane. Thus, exosomes have an endosomal origin that is reflected in their molecular composition (Mittelbrunn and Sanchez-Madrid 2012; Thery et al. 2002). Numerous studies have confirmed the presence of an miRNA and mRNA pool in exosomes, and a novel mechanism of genetic exchange between cells was proposed (Valadi 2007). In the vasculature, endothelial-derived exosomes were shown to mediate transfer of miR-143 and miR-145 to smooth muscle cells and elicit an atheroprotective response (Hergenreider et al. 2012), while an exosome-mediated cross talk between endothelial cells was recently reported to result in the delivery of miR-214 to recipient endothelial cells, an essential step to suppress senescence and stimulate migration and angiogenesis (Van Balkom et al. 2013).

In the heart, a novel paracrine mechanism of miRNA exchange between cardiac fibroblasts and cardiomyocytes was uncovered. Intriguingly, it was mediated by the star strand of the miRNA duplex. Exosome mediated transfer of miR-21-targeted sorbin and SH3 domain-containing protein 2 (SORBS2) and PDZ and LIM domain 5 (PDLIM5) expressions in the recipient cardiomyocytes and that led to cardiac hypertrophy. Remarkably, cardiomyocyte-derived exosomes can control endothelial cell proliferation, migration and angiogenic potential. MiR-320 transfer was proposed to mediate this response, with exosomes from cardiomyocytes in a rat model of type 2 diabetes eliciting an inhibitory effect on angiogenesis (Wang et al. 2014).

5.3.2 miRNAs in Protein Complexes

Using differential centrifugation and size exclusion chromatography, Arroyo et al. (Arroyo et al. 2011) demonstrated the presence of miRNA and Ago2 complexes in the circulation that were not encapsulated in membrane-bound vesicles. Surprisingly, two populations of circulating miRNAs were identified. While the majority of the miRNAs were copurified with cell-free Ago2 complexes, there was a minority of miRNAs such as let-7a that associated predominantly with vesicles (Arroyo et al. 2011). Apart from Ago2, other members of the Argonaute family (Ago1) were also reported to associate with miRNAs in the circulation (Turchinovich et al. 2011). Nucleophosmin (NPM1), an RNA-binding protein, was also identified in the conditioned medium of fibroblasts to form complexes that protect miRNAs from degradation, although the mechanism of action is not well understood (Wang et al. 2010).

5.3.3 miRNAs in Lipoprotein Complexes

The presence of a stable miRNA pool in lipoprotein complexes in the circulation has also been described. Within the high-density lipoprotein (HDL), miRNA complexes were detected, and delivery to recipient cells was shown to occur in a scavenger receptor class B type I-dependent manner (Vickers et al. 2011). A follow-up study demonstrated delivery of functional miRNAs that regulate gene expression in the recipient cells. HDL-mediated transport of miR-223 to endothelial cells was shown to suppress the expression of intercellular adhesion molecule 1(ICAM-1). The transport of miRNA could account for the anti-inflammatory properties of HDL at least partially (Tabet et al. 2014). Interestingly, Wagner et al. reported that no transfer of miRNAs via HDL complexes to endothelial cells could be detected (Wagner et al. 2013), suggesting this transport mechanism is not as universal as initially thought and may occur only under defined conditions.

5.4 Quantification of Circulating miRNAs

The possibility of utilising circulating miRNAs to develop novel prognostic and diagnostic tests is attractive, as their expression is stable, and miRNAs can be amplified and detected with high sensitivity and specificity (Fig. 5.1)**.** Moreover, multiple miRNAs can be assessed in a single experiment (Engelhardt 2012). However, there are several issues that need to be considered to ensure accurate quantification (Fig. 5.2). Sample preparation and data normalisation are the two most critical determinants of quantification accuracy. In this chapter, we will focus

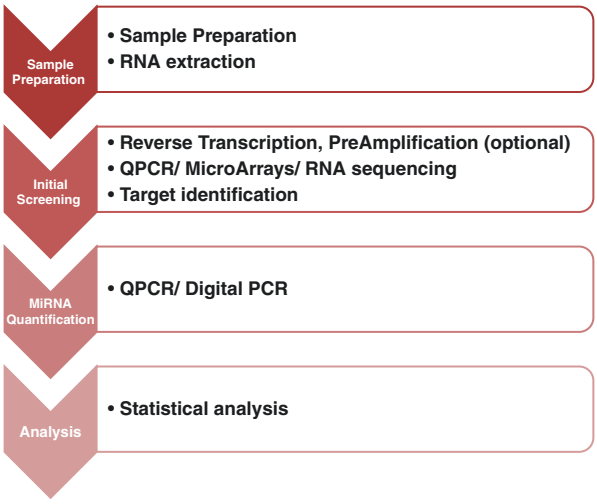

Fig. 5.1 Experimental workflow for the quantification of circulating miRNAs

Step	Sample Preparation	Initial Screening (Discovery Phase)	MiRNA quantification (Validation Phase)	Analysis
Options	• Various biological fluids (e.g. serum, plasma, urine) can be analysed • Chaotropic salt and spin columns are available	• MicroArray-based screening • Amplification-based screening • High throughput sequencing	• QPCR with or without pre-amplification. • Digital PCR	• Advanced statistical analysis • L1-penalized regression analysis • Network inference algorithms
Critical Considerations	• Removal of all cellular components is essential. • Avoid sample dilution • Avoid introduction of contaminants that may interfere with downstream applications • Consider introducing exogenous synthetic miRNAs as spike-in normalization controls • Consider the use of a carrier to improve consistency in RNA extraction	• Selection of well defined control and case groups • Consider potential confounding factors, including medication • Input RNA requirements • Concentration and purity of RNA • Identification of known miRNAs versus discovery of novel miRNAs • Costs • Absolute versus relative quantification • Selection of adequate normalization controls	• Selection of well-matched cases and controls • Consider potential confounding factors, including medication • Absolute versus relative quantification • Selection of adequate normalization controls	• Address the high colinearity and high dimensionality of miRNA expression in biological fluids • Risk of overfitting of data • Validation in independent cohorts

Fig. 5.2 Considerations for the quantification of circulating miRNAs

on the challenges in the preparation of blood specimens that are used to generate plasma (supernatant of the blood collected in the presence of an anticoagulant) or serum (supernatant of coagulated blood depleted of fibrinogen).

5.4.1 Sample Preparation

5.4.1.1 Sample Processing and Handling

Pre-analytical variations in sample preparation can compromise the integrity of the circulating miRNAs and the accuracy of the quantification (Kroh et al. 2010; Mcdonald et al. 2011). Removal of all cellular components is an essential and crucial step, as measurements of circulating miRNAs can be confounded by the miRNAs of haematopoietic cells (Duttagupta et al. 2011; Pritchard et al. 2012). The presence of residual platelets in these preparations is a concern. Platelet contamination may persist in using the standard laboratory protocol for plasma preparation, and hence additional steps of centrifugation are required to eliminate this cellular fraction. This modified protocol was shown to be effective even in samples stored for several years (Cheng et al. 2013).

The choice of anticoagulant can also interfere with miRNA detection. Besides antiplatelet therapy (Cavarretta et al. 2013; De Boer et al. 2013; Kaudewitz et al. 2013; Willeit et al. 2013), intravenous administration of heparin is a confounding factor for miRNA measurements, in particular in patients with cardiovascular disease undergoing percutaneous interventions. Heparin is highly negatively charged and not removed during conventional RNA extraction. It has a dose-dependent inhibitory effect on polymerase chain reaction (PCR) in part due to its binding to magnesium ions (Garcia et al. 2002; Willems et al. 1993; Yokota et al. 1999). Detailed studies demonstrated that heparin has a more pronounced effect on the

exogenous *Caenorhabditis elegans* (*C. elegans*) spike-in control compared to the endogenous miRNAs, suggesting that alternative ways of normalisation should be applied (Kaudewitz et al. 2013). Citrated or EDTA plasma preparations seem to be a better option, although the induction of haemolysis maybe a concern for citrated plasma (Cui et al. 2011). Apart from platelet activation, erythrocyte haemolysis is an issue, in particular for miRNAs like miR-16 and miR-451 that are highly expressed in red blood cells. Several studies have highlighted the good correlation between the degree of haemolysis and the abundance of these miRNAs in the circulation (Kirschner et al. 2011; Mcdonald et al. 2011).

5.4.1.2 RNA Isolation

Extraction of total RNA from biological fluids can be challenging, as typically these samples are rich in protein and lipids and low in RNA content. Nevertheless, several protocols were developed that include lysis using a chaotropic salt, such as guanidinium thiocyanate that simultaneously solubilises the biological material and denatures proteins followed by a solid phase extraction procedure on silica columns. Removal of proteins and lipids results in elution of high-quality RNA (Turchinovich et al. 2011). Recently, commercially available products that can be used to isolate RNAs smaller than 1000 nucleotides have been introduced specifically for RNA isolation from biofluids. Based on spin columns that exploit the use of resins and thus do not require phenol or chloroform extraction, these protocols can be applied to obtain high-quality RNA. However, despite the improved purity, all the extraction methods have low RNA yield. Thus, assessing the concentration using OD260 absorbance is inaccurate. Therefore, a fixed volume of eluted RNA is typically used for all downstream applications (Zampetaki et al. 2010). The efficiency of RNA extraction can improve with the inclusion of a carrier, such as glycogen or bacteriophage MS2 (Andreasen et al. 2010; Turchinovich et al. 2011). Perhaps more importantly, in large clinical studies, the use of a carrier may improve the consistency of RNA extraction across samples.

5.4.2 Data Normalisation

Normalisation is a crucial step to overcome technical variability. It is a major potential source of error in assessing levels of circulating miRNAs. As the origin of extracellular miRNAs may vary and several cell types contribute to the circulating miRNA pool, there is no single endogenous control that can be used for normalisation. Two main strategies were employed: the use of an exogenous spike-in control (Fichtlscherer et al. 2010) and the global miRNA measurement that is usually represented by the Ct average of a panel of endogenous miRNAs (Zampetaki et al. 2012). The exogenous spike-in control involves the introduction of a synthetic miRNA during RNA extraction after the addition of denaturing solution, typically derived from *C. elegans*. These spike-in oligonucleotides can be quantified alongside the endogenous miRNAs. Although these exogenous controls can account for the differences in the volumes handled during RNA extraction, they are not part of any

vesicles or protein or lipoprotein complexes, and thus variation in the extraction efficiency from different biological matrices cannot be accounted for. Additionally, they may differ in their susceptibility to reagents interfering with reactions during quantification, e.g. heparin (Kaudewitz et al. 2013). Hence, exogenous spike-in controls should ideally be combined with endogenous miRNA controls to ensure the robustness of the findings. Several endogenous miRNAs that are detectable in all samples, display low dispersion of expression levels and are not associated with the studied disease, have been used for normalisation purposes (D'alessandra et al. 2010; Goren et al. 2012; Ji et al. 2009; Tijsen et al. 2010; Zampetaki et al. 2010). The drawback of this approach is that the selected miRNA may actually correlate with another pathological condition and therefore be unsuitable as a universal control. While specific miRNAs may vary in expression, their overall abundance should provide a more reliable measure of the RNA content, analogous to the total protein content being used as a normalisation control for microvesicles (Zhang et al. 2010). The statistical analysis is an additional challenge. The remarkably high colinearity among circulating miRNAs requires the application of more advanced statistical and bioinformatics approaches that evaluate miRNA networks to gain the most information from the high-dimensional and highly correlated miRNA data (Zampetaki et al. 2011).

5.4.3 Comprehensive Profiling of Circulating miRNAs

A range of platforms for miRNA screening were developed. In principle, three different methods are in use: microarray hybridization, amplification-based technologies and high-throughput sequencing. Circulating miRNAs can also be (semi) quantified using these technologies, although input requirements render some of the platforms unsuitable for larger clinical studies. Compared to longer transcripts, quantification of miRNAs requires specific modifications. Mature miRNAs have a small size and display relatively low abundance and unequal melting temperatures. Additionally, modifications of the detection methods that enable quantification of the mature miRNAs but not the primary and precursor miRNA as well as the discrimination of closely related miRNA families that exhibit high sequence similarity are essential. A detailed comparative study of the different platforms demonstrated significant variation in terms of reproducibility, sensitivity, accuracy and specificity and concluded that each method has its strengths and weaknesses (Mestdagh et al. 2014).

5.4.3.1 Microarray-Based miRNA Profiling

Microarrays are an established high-throughput method that can facilitate the assessment of multiple known miRNAs in a cost-effective and timely manner. However, due to the short length of miRNAs, optimal probe design for miRNA microarrays is an important consideration, and critical modifications have to be applied to ensure specificity and accuracy of the signal. The capture probes, synthetic oligonucleotides or cDNA fragments that display high specificity and affinity

for individual transcripts, have to be adapted to facilitate binding to miRNAs. Due to the short length of miRNAs, probes' melting temperatures (T_m) may vary between 45 and 74°C. At a medium hybridization temperature, capture probes with lower T_m values will yield lower signals, while capture probes with higher T_m values will display impaired nucleotide discrimination and lower specificity (Wang et al. 2007). Thus, a single hybridization temperature is suboptimal for most miRNA targets unless the probe length is adjusted accordingly, so that high specificity of detection is obtained for closely related mature miRNAs. The enzymatic labelling had little bias as it includes attachment of a single fluorophore-labelled nucleotide to the 3′ end of each miRNA with high yield and minimal sample manipulation. Hybridization to the microarray is carried out under conditions that result in near-equilibrium binding and high hybridization yields for most miRNAs (Wang et al. 2007). This platform was shown to be accurate, although some variability can be observed at low concentrations (Ach et al. 2008; Sah et al. 2010). No fractionation or amplification is required, and 100 ng of total RNA input has been successfully used to assess the miRNA profile in plasma samples (Wang et al. 2009).

The inclusion of locked nucleic acids (LNA) in the capture probes can further increase the sensitivity and specificity of detection. LNAs are nucleotide analogues that are constrained in the ideal conformation for Watson-Crick binding and enable more rapid and stable pairing with the complementary nucleotide. This approach resulted in a significantly higher accuracy. By modifying the LNA content and length of probes, their T_m can be adjusted to facilitate stringent hybridization conditions while maintaining equal affinity. Hence, miRNA profiling is possible with as little as 30 ng of total RNA (Castoldi et al. 2008, 2006). LNA miRNA arrays have been applied to screen plasma samples from hypertensive patients (Li et al. 2011).

A totally different concept was applied in bead-based chips, where a single miRNA-specific oligo (MSO) was used to assess each miRNA on the panel. RNA samples were polyadenylated, reverse transcribed to cDNA; MSOs were hybridised to the sample, and a solid phase primer extension step was included to increase the sensitivity. Following amplification of the extended products and fluorescence labelling, these unique MSO sequences were utilised to identify the specific miRNA content in the sample (Chen et al. 2008). The address sequence from each MSO was used to hybridise specific miRNA products to specific locations on the BeadArray substrate for readout. The BeadArray Reader measured the signal intensity at each address location corresponding to the quantity of the respective miRNA in the original samples. A total of 100–200 ng of total RNA from each sample was sufficient for this quantification. This method improved accuracy and can be modified to include additional miRNA capture beads to the mixture, allowing detection of newly discovered miRNAs (Jay et al. 2007; Lu et al. 2005). However, it required enrichment of small RNAs by fractionation, a step that may introduce bias (Liu et al. 2008). It may also be prone to false-positive results as indicated by a study that utilised this platform for screening differentially expressed miRNAs in a cohort of heart failure patients (Tijsen et al. 2010). In the following extensive validation, the authors reported several discrepancies between the levels of miRNA expression as measured by microarrays and qPCR.

High sensitivity in the screening has also been obtained using a combination of conventional hybridization and an elongation step. The isolated RNA was labelled using the 'klenow' fragment of DNA polymerase I that is added in the channels of a biochip for specific elongation and labelling of hybridised miRNAs (Vorwerk et al. 2008). This eliminates the need for a labelling step prior to the hybridization and reduces the RNA requirements to as little as 20 ng of total RNA. This method was successfully applied for screening of circulating miRNAs in patients with coronary artery disease (Fichtlscherer et al. 2010).

5.4.3.2 Amplification-Based Arrays

The microarray options listed above typically display lower sensitivity than quantitative PCR (qPCR). This observation led to the development of amplification-based arrays, whereby RNA was reverse-transcribed and quantification of miRNA content was performed using quantitative PCR. Three different methods of reverse transcription have been applied. The use of an miRNA-specific stem-loop reverse transcription (RT) primer and a pool of RT primers in a multiplex reaction is particularly popular. The RT primer/ miRNA complexes that are formed display extremely high specificity due to the presence of the stem-loop structure. The cDNA that is generated following extension at the 3′ end of the miRNA is then loaded on a 384-well fluidic card that contains a panel of qPCR primers and TaqMan probes for the miRNAs of interest. A qPCR reaction is run on a thermocycler, and the amplification values are calculated for the entire set of miRNAs. To increase the sensitivity of the assay, a preamplification step can be included. This platform has been widely applied in screening circulating miRNAs, and its sensitivity was further demonstrated in assessing the miRNA pools in the HDL fraction that harbour very low miRNA content (Mitchell et al. 2008; Vickers et al. 2011; Zampetaki et al. 2010). Additionally, it has higher sensitivity than hybridization-based assays as demonstrated by the lower false-positive rate of differential miRNA expression in the qPCR arrays compared to microarrays (Chen et al. 2009).

Alternatively, stem-loop RT primers can be substituted with an LNA-based system of reverse transcription (Arroyo et al. 2011) that includes addition of a poly(A) tail and the use of a polyT primer with a 3′ degenerate anchor and a 5′ universal tag. Subsequently, the cDNA template can be amplified using miRNA-specific LNA forward and reverse primers in a SYBR Green-based qPCR reaction that can provide higher sensitivity (Jensen et al. 2011; Mestdagh et al. 2014). Similar approaches are also available in other platforms with the omission of LNA analogues and the use of specifically formulated buffers to enhance specificity of the reaction or reliance on adapter ligation at the 5′ end and universal RT primer at the 3′ end to ensure accuracy and specificity in a single reaction.

5.4.3.3 High-Throughput Sequencing

Next-generation sequencing (NGS) is a powerful platform that enables the profiling of known sequences and also the discovery of novel small noncoding RNAs with unprecedented sensitivity (Creighton et al. 2009; Metzker 2010; Tam et al. 2014). Differential expression of miRNAs and importantly detection of novel

miRNAs or sequence variations (isomiRs) can be obtained (Ozsolak and Milos 2011). Initially, adapters specifically modified to target miRNAs and other small RNAs that have a 3′ hydroxyl group are ligated to each end of the RNA molecules, and an RT reaction is used to generate single-stranded cDNA that is then PCR amplified. Sequencing data of libraries generated by RNA ligases revealed secondary preferences of these enzymes. A strong bias towards certain small RNAs has been reported thereby preventing determination of absolute numbers of small RNAs (Linsen et al. 2009). This bias was systematic and highly reproducible and was shown to be independent of the sequencing platform but strongly determined by the method of small RNA library preparation (Tang et al. 2013). The technical error in small libraries can be reduced with the introduction of primers containing index sequences during the PCR amplification of the cDNA. Gel purification to prepare a library product for subsequent cluster generation will follow (Sorefan et al. 2012). Recently, improved protocols that require lower RNA input were developed. This is particularly important for circulating miRNAs as they display low abundance. Nevertheless, in most cases, 2–3 ml of plasma or serum must be processed for each sample, limiting the use of this method to the discovery phase in a limited number of pooled samples (Li et al. 2012; Nielsen et al. 2012), rather than applying it routinely in clinical cohorts. Further improvements may involve combination of microfluidic technology (Streets et al. 2014) and high-throughput sequencing and could offer more feasible solutions for larger studies. Recently, NGS workflows designed and optimised specifically for serum and plasma samples that utilise a smaller volume of input material, as little as 500 μl, became commercially available. However, one should exercise caution as these platforms have not been extensively tested. In addition, the overall cost is still substantially higher compared to other platforms, and bioinformatics support for the computational analysis and interpretation is required. Normalisation of the sequencing data can be challenging as a highly abundant miRNA in a sequencing sample may affect the number of sequencing reads that are available for detection and quantification for other miRNAs in the sample. The abundance of a given target can be represented as a percent of total reads, provided that the distribution of miRNA reads does not change substantially. The addition of a spike-in synthetic control during library preparation is an alternative approach. A good correlation between the input material and output reads, a reduction in systematic error and improved accuracy of base quality scores has been demonstrated (Jiang et al. 2011; Zook et al. 2012).

Inter-assay studies demonstrated that the various miRNA profiling technologies display significant differences in specificity, sensitivity, dynamic range and accuracy. On several occasions, poor correlation was observed between the diverse platforms suggesting that the interpretation of population studies performed using different technologies would be hampered by these technical limitations (Ach et al. 2008; Git et al. 2010; Jensen et al. 2011; Mestdagh et al. 2014; Tam et al. 2014). Hence, there is an apparent need for advances in technology and bioinformatics to establish a ‘universal standard’ that can accurately monitor the miRNA profile in the circulation and track expression differences with high sensitivity.

5.5 Quantification of Circulating miRNAs

5.5.1 Quantitative Real-Time PCR

Quantitative real-time PCR (qPCR) remains the gold standard for validating the data derived from miRNA profiling experiments. Several characteristics of the mature miRNAs required the establishment of novel protocols for reverse transcription. The mature miRNAs are small in size, lack a poly(A) tail and encompass sequences that are present in the precursor transcripts and in the genomic DNA. Additionally, the reverse transcription system should be highly sensitive and able to discriminate among related miRNAs that only differ in a single nucleotide.

The use of stem-loop primers and the TaqMan technology led to the development of a very sensitive and accurate platform for miRNA validation. The stem-loop structure forms an RT primer/mature miRNA chimaera that extends the 3′ end of the miRNA. The derived cDNA is the template for a TaqMan qPCR reaction that includes an miRNA-specific forward primer, a reverse primer and a dye-labelled TaqMan probe (Chen et al. 2005; Mestdagh et al. 2008). Compared to conventional linear primers, stem-loop RT primers show better specificity and sensitivity, probably due to the base stacking and spatial constraint of the stem-loop structure. This may also prevent binding to double-stranded genomic DNA molecules (Chen et al. 2005). Multiplexing assays that include pools of stem-loop primers are now available and can be applied to facilitate the simultaneous reverse transcription of multiple mature miRNAs. An additional step of preamplification of the RT product consisting of 10–14 cycles of PCR amplification can be included to increase the sensitivity of detection. A forward primer specifically designed for the target miRNA and a reverse universal primer are used in the preamplification reaction. Although there is an excellent correlation in the expression levels for the most abundant miRNAs obtained with or without preamplification, some variation is observed for the low-abundant miRNAs (Mestdagh et al. 2008). The directionality of the differential gene expression though is consistent, indicating that preamplification products are suitable for the analysis of relative miRNA expression. Further studies confirmed that the systematic bias does not prohibit the comparison of relative miRNA levels between samples (Linsen et al. 2009).

Besides stem-loop primers, polyadenylation of the mature miRNAs by poly(A) polymerase has been successfully applied for the reverse transcription of mature miRNAs. Oligo-dT primers that have a 3′ degenerate anchor and a universal tag sequence on the 5′ end are subsequently used to obtain the cDNA. This system is used in combination with SYBR Green to enable sensitive and specific quantification of mature miRNAs by qPCR. The combination of polyadenylation and the addition of a universal tag eliminate the risk of detecting genomic DNA (Tijsen et al. 2010; Wang et al. 2009, 2010). In a similar manner, LNA analogues can be introduced to the primers to increase specificity and detection efficiency. In this case, cDNA is synthesised using an miRNA-specific RT primer and a sensitive stable reverse transcriptase. Subsequently, the cDNA is amplified by qPCR using SYBR Green. LNA be incorporated in miRNA-specific primers in both the RT and

qPCR reaction (Arroyo et al. 2011; Bryant et al. 2012; Jensen et al. 2011). This can increase PCR sensitivity and amplification efficiency and is particularly useful in multiplexing to specifically target the mature miRNAs of interest (Ballantyne et al. 2008). However, care must be taken with the design of LNA primers, as the number and position of LNA bases can substantially influence the outcome of the amplification and result in false positives (Latorra et al. 2003; Levin et al. 2006).

5.5.2 Digital PCR

Absolute quantification of nucleic acids can be determined using digital PCR (dPCR). This method is based on partitioning the sample into many replicate reactions at a limiting dilution resulting in one or zero molecules in each. Digital PCR is an endpoint measurement that provides the ability to quantify nucleic acids without the use of standard curves. This has many advantages over qPCR as it eliminates the need for normalisation controls and is more robust against reagents that may interfere with the efficiency of the PCR reaction (Vogelstein and Kinzler 1999). Droplet digital polymerase chain reaction (ddPCR), the latest technology of dPCR that measures absolute quantities of nucleic acid molecules encapsulated in discrete, volumetrically defined, water-in-oil droplet partitions, was also applied for circulating the miRNAs and the miRNA content of exosomes (Chevillet et al. 2014; Pinheiro et al. 2012). A systematic comparison of ddPCR and qPCR performance for the quantification of miRNAs was performed using synthetic miRNAs as well as sera from 20 patients with advance prostate cancer and 20 age-matched healthy controls (Hindson et al. 2013). The diagnostic sensitivity and specificity of the ddPCR were demonstrated in a platform that used a 20 μl reaction loaded into an eight-channel droplet generation cartridge, each channel dispensed into 20,000 droplets. Discrimination between droplets that did not contain the target (negative) and those that did (positives) was achieved by applying a global fluorescence amplitude threshold. The two methods displayed comparable sensitivity, but ddPCR had greater precision and higher reproducibility than qPCR.

5.6 Future Directions

Despite the encouraging early findings, the clinical potential of circulating miRNAs as novel diagnostic and prognostic tools with clinical utility remains to be determined. Several technical aspects need to be resolved, and robust protocols need to be established that can overcome errors introduced by pre-analytical differences in sample preparation and processing. Detailed evaluation of the different profiling technologies in terms of sensitivity, accuracy and reproducibility will allow a more standardised execution of miRNA measurements. Notwithstanding the potential challenges, circulating miRNAs may offer unique insights into the pathophysiology of cardiovascular diseases and enable the identification of novel pathogenic mechanisms that include miRNAs.

Conflict of Interest Mayr is named inventor on patents for miRNA biomarkers.

Statements on Human and Animal Rights Studies that include human participants have been approved by appropriate institutional and/or national research ethics committee and have been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

References

Ach RA, Wang H, Curry B. Measuring microRNAs: comparisons of microarray and quantitative PCR measurements, and of different total RNA prep methods. BMC Biotechnol. 2008;8:69. doi:10.1186/1472-6750-8-69.

Andreasen D, Fog JU, Biggs W, et al. Improved microRNA quantification in total RNA from clinical samples. Methods. 2010;50:S6–9. doi:10.1016/j.ymeth.2010.01.006.

Arroyo JD, Chevillet JR, Kroh EM, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A. 2011;108:5003–8. doi:10.1073/pnas.1019055108.

Ballantyne KN, Van Oorschot RA, Mitchell RJ. Locked nucleic acids in PCR primers increase sensitivity and performance. Genomics. 2008;91:301–5. doi:10.1016/j.ygeno.2007.10.016.

Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136:215–33. doi:10.1016/j.cell.2009.01.002.

Bertoia ML, Bertrand KA, Sawyer SJ, et al. Reproducibility of circulating microRNAs in stored plasma samples. PLoS One. 2015;10:e0136665. doi:10.1371/journal.pone.0136665.

Bryant RJ, Pawlowski T, Catto JW, et al. Changes in circulating microRNA levels associated with prostate cancer. Br J Cancer. 2012;106:768–74. doi:10.1038/bjc.2011.595.

Castoldi M, Schmidt S, Benes V, et al. miChip: an array-based method for microRNA expression profiling using locked nucleic acid capture probes. Nat Protoc. 2008;3:321–9. doi:10.1038/nprot.2008.4.

Castoldi M, Schmidt S, Benes V, et al. A sensitive array for microRNA expression profiling (miChip) based on locked nucleic acids (LNA). RNA. 2006;12:913–20. doi:10.1016/j.cardfail.2016.04.016.

Cavarretta E, Chiariello GA, Condorelli G. Platelets, endothelium, and circulating microRNA-126 as a prognostic biomarker in cardiovascular diseases: per aspirin ad astra. Eur Heart J. 2013;34:3400–2. doi:10.1093/eurheartj/eht032.

Chen C, Ridzon DA, Broomer AJ, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 2005;33:e179.

Chen J, Lozach J, Garcia EW, et al. Highly sensitive and specific microRNA expression profiling using BeadArray technology. Nucleic Acids Res. 2008;36:e87. doi:10.1093/nar/gkn387.

Chen Y, Gelfond JA, Mcmanus LM, et al. Reproducibility of quantitative RT-PCR array in miRNA expression profiling and comparison with microarray analysis. BMC Genomics. 2009;10:407. doi:10.1186/1471-2164-10-407.

Cheng HH, Yi HS, Kim Y, et al. Plasma processing conditions substantially influence circulating microRNA biomarker levels. PLoS One. 2013;8:e64795. doi:10.1371/journal.pone.0064795.

Chevillet JR, Kang Q, Ruf IK, et al. Quantitative and stoichiometric analysis of the microRNA content of exosomes. Proc Natl Acad Sci U S A. 2014;111:14888–93. doi:10.1073/pnas.1408301111.

Creighton CJ, Reid JG, Gunaratne PH. Expression profiling of microRNAs by deep sequencing. Brief Bioinform. 2009;10:490–7. doi:10.1093/bib/bbp019.

Cui W, Ma J, Wang Y, et al. Plasma miRNA as biomarkers for assessment of total-body radiation exposure dosimetry. PLoS One. 2011;6:e22988. doi:10.1371/journal.pone.0022988.

D'alessandra Y, Devanna P, Limana F, et al. Circulating microRNAs are new and sensitive biomarkers of myocardial infarction. Eur Heart J. 2010;31:2765–73. doi:10.1093/eurheartj/ehq167.

De Boer HC, Van Solingen C, Prins J, et al. Aspirin treatment hampers the use of plasma microRNA-126 as a biomarker for the progression of vascular disease. Eur Heart J. 2013;34:3451–7. doi:10.1093/eurheartj/eht007.

Duttagupta R, Jiang R, Gollub J, et al. Impact of cellular miRNAs on circulating miRNA biomarker signatures. PLoS One. 2011;6:e20769. doi:10.1371/journal.pone.0020769.

Ecker JR, Bickmore WA, Barroso I, et al. Genomics: ENCODE explained. Nature. 2012;489:52–5. doi:10.1038/489052a.

Engelhardt S. Small RNA biomarkers come of age. J Am Coll Cardiol. 2012;60:300–3. doi:10.1016/j.jacc.2012.04.018.

Fichtlscherer S, De Rosa S, Fox H, et al. Circulating microRNAs in patients with coronary artery disease. Circ Res. 2010;107:677–84. doi:10.1161/CIRCRESAHA.109.215566.

Garcia ME, Blanco JL, Caballero J, et al. Anticoagulants interfere with PCR used to diagnose invasive aspergillosis. J Clin Microbiol. 2002;40:1567–8.

Git A, Dvinge H, Salmon-Divon M, et al. Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression. RNA. 2010;16:991–1006. doi:10.1261/rna.1947110.

Goren Y, Kushnir M, Zafrir B, et al. Serum levels of microRNAs in patients with heart failure. Eur J Heart Fail. 2012;14:147–54. doi:10.1093/eurjhf/hfr155.

Hergenreider E, Heydt S, Treguer K, et al. Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs. Nat Cell Biol. 2012;14:249–56. doi:10.1038/ncb2441.

Hindson CM, Chevillet JR, Briggs HA, et al. Absolute quantification by droplet digital PCR versus analog real-time PCR. Nat Methods. 2013;10:1003–5. doi:10.1038/nmeth.2633.

Indolfi C, Curcio A. Stargazing microRNA maps a new miR-21 star for cardiac hypertrophy. J Clin Invest. 2014;124:1896–8. doi:10.1172/JCI75801.

Inui M, Martello G, Piccolo S. MicroRNA control of signal transduction. Nat Rev Mol Cell Biol. 2010;11:252–63. doi:10.1038/nrm2868.

Ismail N, Wang Y, Dakhlallah D, et al. Macrophage microvesicles induce macrophage differentiation and miR-223 transfer. Blood. 2013;121:984–95. doi:10.1182/blood-2011-08-374793.

Jansen F, Yang X, Hoelscher M, et al. Endothelial microparticle-mediated transfer of MicroRNA-126 promotes vascular endothelial cell repair via SPRED1 and is abrogated in glucose-damaged endothelial microparticles. Circulation. 2013;128:2026–38. doi:10.1161/CIRCULATIONAHA.113.001720.

Jay C, Nemunaitis J, Chen P, et al. miRNA profiling for diagnosis and prognosis of human cancer. DNA Cell Biol. 2007;26:293–300.

Jensen SG, Lamy P, Rasmussen MH, et al. Evaluation of two commercial global miRNA expression profiling platforms for detection of less abundant miRNAs. BMC Genomics. 2011;12:435. doi:10.1186/1471-2164-12-435.

Ji X, Takahashi R, Hiura Y, et al. Plasma miR-208 as a biomarker of myocardial injury. Clin Chem. 2009;55:1944–9. doi:10.1373/clinchem.2009.125310.

Jiang L, Schlesinger F, Davis CA, et al. Synthetic spike-in standards for RNA-seq experiments. Genome Res. 2011;21:1543–51. doi:10.1101/gr.121095.111.

Kaudewitz D, Lee R, Willeit P, et al. Impact of intravenous heparin on quantification of circulating microRNAs in patients with coronary artery disease. Thromb Haemost. 2013;110:609–15. doi:10.1160/TH13-05-0368.

Kaudewitz D, Skroblin P, Bender LH, et al. Association of microRNAs and YRNAs with platelet function. Circ Res. 2016;118:420–32. doi:10.1161/CIRCRESAHA.114.305663.

Kirschner MB, Kao SC, Edelman JJ, et al. Haemolysis during sample preparation alters microRNA content of plasma. PLoS One. 2011;6:e24145. doi:10.1371/journal.pone.0024145.

Kroh EM, Parkin RK, Mitchell PS, et al. Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). Methods. 2010;50:298–301. doi:10.1016/j.ymeth.2010.01.032.

Laffont B, Corduan A, Ple H, et al. Activated platelets can deliver mRNA regulatory Ago2*microRNA complexes to endothelial cells via microparticles. Blood. 2013;122:253–61. doi:10.1182/blood-2013-03-492801.

Latorra D, Arar K, Hurley JM. Design considerations and effects of LNA in PCR primers. Mol Cell Probes. 2003;17:253–9.

Levin JD, Fiala D, Samala MF, et al. Position-dependent effects of locked nucleic acid (LNA) on DNA sequencing and PCR primers. Nucleic Acids Res. 2006;34:e142.

Li H, Guo L, Wu Q, et al. A comprehensive survey of maternal plasma miRNAs expression profiles using high-throughput sequencing. Clin Chim Acta. 2012;413:568–76. doi:10.1016/j.cca.2011.11.026.

Li J, Zhang Y, Liu Y, et al. Microvesicle-mediated transfer of microRNA-150 from monocytes to endothelial cells promotes angiogenesis. J Biol Chem. 2013;288:23586–96. doi:10.1074/jbc.M113.489302.

Li S, Zhu J, Zhang W, et al. Signature microRNA expression profile of essential hypertension and its novel link to human cytomegalovirus infection. Circulation. 2011;124:175–84. doi:10.1161/CIRCULATIONAHA.110.012237.

Linsen SE, De Wit E, Janssens G, et al. Limitations and possibilities of small RNA digital gene expression profiling. Nat Methods. 2009;6:474–6. doi:10.1038/nmeth0709-474.

Liu CG, Calin GA, Volinia S, et al. MicroRNA expression profiling using microarrays. Nat Protoc. 2008;3:563–78. doi:10.1038/nprot.2008.14.

Liu N, Olson EN. MicroRNA regulatory networks in cardiovascular development. Dev Cell. 2010;18:510–25. doi:10.1016/j.devcel.2010.03.010.

Loyer X, Vion AC, Tedgui A, et al. Microvesicles as cell-cell messengers in cardiovascular diseases. Circ Res. 2014;114:345–53. doi:10.1161/CIRCRESAHA.113.300858.

Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. Nature. 2005;435:834–8.

Matkovich SJ, Hu Y, Dorn 2nd GW. Regulation of cardiac microRNAs by cardiac microRNAs. Circ Res. 2013;113:62–71. doi:10.1161/CIRCRESAHA.113.300975.

Mcdonald JS, Milosevic D, Reddi HV, et al. Analysis of circulating microRNA: preanalytical and analytical challenges. Clin Chem. 2011;57:833–40. doi:10.1373/clinchem.2010.157198.

Mendell JT, Olson EN. MicroRNAs in stress signaling and human disease. Cell. 2012;148:1172–87. doi:10.1016/j.cell.2012.02.005.

Mestdagh P, Feys T, Bernard N, et al. High-throughput stem-loop RT-qPCR miRNA expression profiling using minute amounts of input RNA. Nucleic Acids Res. 2008;36:e143. doi:10.1093/nar/gkn725.

Mestdagh P, Hartmann N, Baeriswyl L, et al. Evaluation of quantitative miRNA expression platforms in the microRNA quality control (miRQC) study. Nat Methods. 2014;11:809–15. doi:10.1038/nmeth.3014.

Metzker ML. Sequencing technologies - the next generation. Nat Rev Genet. 2010;11:31–46. doi:10.1038/nrg2626.

Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008;105:10513–8. doi:10.1073/pnas.0804549105.

Mittelbrunn M, Sanchez-Madrid F. Intercellular communication: diverse structures for exchange of genetic information. Nat Rev Mol Cell Biol. 2012;13:328–35. doi:10.1038/nrm3335.

Nielsen LB, Wang C, Sorensen K, et al. Circulating levels of microRNA from children with newly diagnosed type 1 diabetes and healthy controls: evidence that miR-25 associates to residual beta-cell function and glycaemic control during disease progression. Exp Diabetes Res. 2012;2012:896362. doi:10.1155/2012/896362.

Ozsolak F, Milos PM. RNA sequencing: advances, challenges and opportunities. Nat Rev Genet. 2011;12:87–98. doi:10.1038/nrg2934.

Pan Y, Liang H, Liu H, et al. Platelet-secreted microRNA-223 promotes endothelial cell apoptosis induced by advanced glycation end products via targeting the insulin-like growth factor 1 receptor. J Immunol. 2014;192:437–46. doi:10.4049/jimmunol.1301790.

Pinheiro LB, Coleman VA, Hindson CM, et al. Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. Anal Chem. 2012;84:1003–11. doi:10.1021/ac202578x.

Pritchard CC, Kroh E, Wood B, et al. Blood cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies. Cancer Prev Res. 2012;5:492–7. doi:10.1158/1940-6207.CAPR-11-0370.

Sabin LR, Delas MJ, Hannon GJ. Dogma derailed: the many influences of RNA on the genome. Mol Cell. 2013;49:783–94. doi:10.1016/j.molcel.2013.02.010.

Sah S, Mccall MN, Eveleigh D, et al. Performance evaluation of commercial miRNA expression array platforms. BMC Res Notes. 2010;3:80. doi:10.1186/1756-0500-3-80.

Sorefan K, Pais H, Hall AE, et al. Reducing ligation bias of small RNAs in libraries for next generation sequencing. Silence. 2012;3:4. doi:10.1186/1758-907X-3-4.

Streets AM, Zhang X, Cao C, et al. Microfluidic single-cell whole-transcriptome sequencing. Proc Natl Acad Sci U S A. 2014;111:7048–53. doi:10.1073/pnas.1402030111.

Tabet F, Vickers KC, Cuesta Torres LF, et al. HDL-transferred microRNA-223 regulates ICAM-1 expression in endothelial cells. Nat Commun. 2014;5:3292. doi:10.1038/ncomms4292.

Tam S, De Borja R, Tsao MS, et al. Robust global microRNA expression profiling using next-generation sequencing technologies. Laboratory investigation. J Tech Met Pathol. 2014;94:350–8. doi:10.1038/labinvest.2013.157.

Tang DT, Plessy C, Salimullah M, et al. Suppression of artifacts and barcode bias in high-throughput transcriptome analyses utilizing template switching. Nucleic Acids Res. 2013;41:e44. doi:10.1093/nar/gks1128.

Thery C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. Nat Rev Immunol. 2002;2:569–79.

Tijsen AJ, Creemers EE, Moerland PD, et al. MiR423-5p as a circulating biomarker for heart failure. Circ Res. 2010;106:1035–9. doi:10.1161/CIRCRESAHA.110.218297.

Tonge DP, Gant TW. What is normal? Next generation sequencing-driven analysis of the human circulating miRNAOme. BMC Mol Biol. 2016;17:4. doi:10.1186/s12867-016-0057-9.

Turchinovich A, Weiz L, Langheinz A, et al. Characterization of extracellular circulating microRNA. Nucleic Acids Res. 2011;39:7223–33. doi:10.1093/nar/gkr254.

Valadi H. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9:654–9.

Van Balkom BW, De Jong OG, Smits M, et al. Endothelial cells require miR-214 to secrete exosomes that suppress senescence and induce angiogenesis in human and mouse endothelial cells. Blood. 2013;121:3997–4006. doi:10.1182/blood-2013-02-478925.

Vickers KC, Palmisano BT, Shoucri BM, et al. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nat Cell Biol. 2011;13:423–33. doi:10.1038/ncb2210.

Vogelstein B, Kinzler KW. Digital PCR. Proc Natl Acad Sci U S A. 1999;96:9236–41.

Vorwerk S, Ganter K, Cheng Y, et al. Microfluidic-based enzymatic on-chip labeling of miRNAs. New Biotechnol. 2008;25:142–9. doi:10.1016/j.nbt.2008.08.005.

Wagner J, Riwanto M, Besler C, et al. Characterization of levels and cellular transfer of circulating lipoprotein-bound microRNAs. Arterioscler Thromb Vasc Biol. 2013;33:1392–400. doi:10.1161/ATVBAHA.112.300741.

Wang C, Wan S, Yang T, et al. Increased serum microRNAs are closely associated with the presence of microvascular complications in type 2 diabetes mellitus. Sci Report. 2016;6:20032. doi:10.1038/srep20032.

Wang H, Ach RA, Curry B. Direct and sensitive miRNA profiling from low-input total RNA. RNA. 2007;13:151–9.

Wang K, Zhang S, Marzolf B, et al. Circulating microRNAs, potential biomarkers for drug-induced liver injury. Proc Natl Acad Sci U S A. 2009;106:4402–7. doi:10.1073/pnas.0813371106.

Wang K, Zhang S, Weber J, et al. Export of microRNAs and microRNA-protective protein by mammalian cells. Nucleic Acids Res. 2010;38:7248–59. doi:10.1093/nar/gkq601.

Wang X, Huang W, Liu G, et al. Cardiomyocytes mediate anti-angiogenesis in type 2 diabetic rats through the exosomal transfer of miR-320 into endothelial cells. J Mol Cell Cardiol. 2014;74:139–50. doi:10.1016/j.yjmcc.2014.05.001.

Widera C, Gupta SK, Lorenzen JM, et al. Diagnostic and prognostic impact of six circulating microRNAs in acute coronary syndrome. J Mol Cell Cardiol. 2011;51:872–5. doi:10.1016/j.yjmcc.2011.07.011.

Willeit P, Zampetaki A, Dudek K, et al. Circulating microRNAs as novel biomarkers for platelet activation. Circ Res. 2013;112:595–600. doi:10.1161/CIRCRESAHA.111.300539.

Willems M, Moshage H, Nevens F, et al. Plasma collected from heparinized blood is not suitable for HCV-RNA detection by conventional RT-PCR assay. J Virol Methods. 1993;42:127–30.

Yokota M, Tatsumi N, Nathalang O, et al. Effects of heparin on polymerase chain reaction for blood white cells. J Clin Lab Anal. 1999;13:133–40.

Zampetaki A, Kiechl S, Drozdov I, et al. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. Circ Res. 2010;107:810–7. doi:10.1161/CIRCRESAHA.110.226357.

Zampetaki A, Mayr M. MicroRNAs in vascular and metabolic disease. Circ Res. 2012;110:508–22. doi:10.1161/CIRCRESAHA.111.247445.

Zampetaki A, Willeit P, Burr S, et al. Angiogenic microRNAs linked to incidence and progression of diabetic retinopathy in type 1 diabetes. Diabetes. 2016;65:216–27. doi:10.2337/db15-0389. Epub 2015 Sep 22

Zampetaki A, Willeit P, Drozdov I, et al. Profiling of circulating microRNAs: from single biomarkers to re-wired networks. Cardiovasc Res. 2011;93:555–62. doi:10.1093/cvr/cvr266.

Zampetaki A, Willeit P, Tilling L, et al. Prospective study on circulating MicroRNAs and risk of myocardial infarction. J Am Coll Cardiol. 2012;60:290–9. doi:10.1016/j.jacc.2012.03.056.

Zardo G, Ciolfi A, Vian L, et al. Polycombs and microRNA-223 regulate human granulopoiesis by transcriptional control of target gene expression. Blood. 2012;119:4034–46. doi:10.1182/blood-2011-08-371344.

Zernecke A, Bidzhekov K, Noels H, et al. Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. Sci Signal. 2009;2:ra81. doi:10.1126/scisignal.2000610.

Zhang Y, Liu D, Chen X, et al. Secreted monocytic miR-150 enhances targeted endothelial cell migration. Mol Cell. 2010;39:133–44. doi:10.1016/j.molcel.2010.06.010.

Zisoulis DG, Kai ZS, Chang RK, et al. Autoregulation of microRNA biogenesis by let-7 and Argonaute. Nature. 2012;486:541–4. doi:10.1038/nature11134.

Zook JM, Samarov D, Mcdaniel J, et al. Synthetic spike-in standards improve run-specific systematic error analysis for DNA and RNA sequencing. PLoS One. 2012;7:e41356. doi:10.1371/journal.pone.0041356.

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