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REVIEW ARTICLE

The myriad essential roles of microRNAs in cardiovascular homeostasis and disease

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Abstract According to the World Health Organization, cardiovascular disease accounts for approximately 30% of all deaths in the United States, and is the worldwide leading cause of morbidity and mortality. Over the last several years, microRNAs have emerged as critical regulators of physiological homeostasis in multiple organ systems, including the cardiovascular system. The focus of this review is to provide an overview of the current state of knowledge of the molecular mechanisms contributing to the multiple causes of cardiovascular disease with respect to regulation by microRNAs. A major challenge in understanding the roles of microRNAs in the pathophysiology of cardiovascular disease is that cardiovascular disease may arise from perturbations in intracellular signaling in multiple cell types including vascular smooth muscle and endothelial cells, cardiac myocytes and fibroblasts, as well as hepatocytes, pancreatic β-cells, and others. Additionally, perturbations in intracellular signaling cascades may also have profound effects on heterocellular communication via secreted cytokines and growth factors. There has been much progress in recent years to identify the microRNAs that are both dysregulated under pathological conditions, as well as the signaling pathway(s) regulated by an individual microRNA. The goal of this review is to summarize what is currently known about the mechanisms whereby microRNAs maintain cardiovascular homeostasis and to attempt to identify some key unresolved questions that require further study.

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Introduction

Cardiovascular Disease (CVD) is the leading cause of morbidity and mortality worldwide. Broadly defined, CVD includes primary diseases of the heart (i.e. myocardial infarction, valvular stenosis or insufficiency, congenital cardiac defects, hypertrophic and dilated cardiomyopathy, and pericarditis) as well as diseases of the circulatory system (i.e. ischemia and atheroslcerosis, vascular stenosis, pulmonary hypertension, subarachnoid and intracerebral hemorrhage, and arterial aneurysm and dissection). According to disease classifications outlined in the International Statistical Classification of Diseases and Related Health Problems by the World Health Organization, when adjusted for age, CVD accounts for approximately 30% of all deaths[1,2] in the United States alone. Hypertension affects ~65 million Americans; it increases the risk of stroke, coronary artery disease, congestive heart failure and mortality, yet approximately 43% of those undergoing pharmaceutical treatment fail to respond.[3] In addition to the human cost, spending on CVD between 2010 and 2030 is projected to increase from $272.5 billion to $818.1 billion in total direct medical costs.[4] It is anticipated that elucidating the molecular mechanisms underlying the etiology of CVD will lead to improved therapeutic outcomes.

With the discovery of RNAi[5–7] and a new class of small RNA molecules that inhibit translation of mRNAs,[8–10] siRNA and microRNA (miRNA) directed RNAi have become both a widely used technique, and an important biological process. MiRNAs are short (~22 nt) RNA molecules known to be involved in embryogenesis and development,[11–13] physiological homeostasis[14,15] and the etiology of disease.[16–18] With the recent discovery of miRNAs in blood,[19–21] it is anticipated that miRNAs may become supplemental prognostic and/or diagnostic biomarkers of CVD.

In this review, we will identify 1) clinical correlations between the progression of disease and the dysregulation of microRNAs, and 2) the causal relations between miRNA mediated RNAi and the observed pathology. As such, we will not discuss the mechanisms of miRNA biogenesis, but rather invite readers to some elegantly written reviews on the topic (see Refs. 22–28). Instead, this review will focus on the current state of our knowledge regarding individual miRNAs and their molecular role in maintaining physiological homeostasis in the cardiovascular system. Further, we will discuss novel non-canonical functions and signaling of miRNAs, as well as key unresolved questions that warrant future study. Herein we will give high priority to clinical studies identifying correlations between miRNA expression and diagnosis/prognosis, as well as *in vivo* mechanistic studies indentifying their molecular target(s). Finally, we would like to sincerely apologize to our many outstanding colleagues, whose work may have been inadvertently overlooked or not thoroughly discussed due to space constraints.

Myocardial infarction

Myocardial Infarction (MI) afflicts 7.6 million Americans each year, increases their susceptibility of a subsequent MI, and often precedes cardiac remodeling, cardiomyopathy, and in some cases congestive heart failure.[3] An acute MI (AMI) results from the sudden impairment or cessation of blood flow through one of the coronary arteries when an atherosclerotic plaque (see Atherosclerosis) becomes unstable and ruptures, leading to the rapid formation of thrombosis. Clinically, ST-segment Elevation Myocardial Infarction (STEMI) involves the full thickness of the myocardial wall and may also be caused by pericarditis, whereas Non-ST-segment Elevation Myocardial Infarction (NSTEMI) does not involve the entire ventricular wall thickness. In a 2048 patient cohort, the odds ratio of fatality by day 28 was 2.23 for STEMI patients compared to NSTEMI patients, whereas the adjusted 7-year mortality for 28-day survivors was higher in NSTEMI than in STEMI patients.[29] Rapid diagnosis of AMI is a critical determinant for therapeutic intervention and survival prognosis as the duration of impairment or cessation of blood flow to the working myocardium greatly influences the severity of cellular damage from ischemia to apoptosis and necrosis. In addition to the initial cellular damage, cardiac remodeling occurs in the infarct region which can lead to cardiomyopathies and Heart Failure (HF) (see Cardiomyopathy and heart failure).

MicroRNAs have recently been identified as potential prognostic indicators of HF following AMI. MiR-208b (Table 1) was found to be increased ~1600-fold in the plasma of AMI patients when compared with patients presenting with atypical chest pain without AMI.[30] Alternatively miR-208a, which was undetectable in non-AMI patients with atypical chest pain, was found to be readily detectable in ~90% of patients having been diagnosed with AMI.[31] However, other groups found that miR-208a and miR-208b are expressed at very low levels in the human heart[32] and in only ~30% of AMI patients.[33] Additionally, miR-499-5p was detectable in all control patients, elevated in all AMI patients and AMI animal models, and found to track cTnI in blood.[32,33] Perhaps more importantly, circulating miR-499-5p levels were associated with 12-month mortality in elderly NSTEMI subjects,[34] indicating its potential use as a prognostic indicator. However, it should be noted that miRNA-208a is specifically expressed in the heart, whereas miR-208b and miR-499-5p are also expressed in skeletal muscle,[35] thereby confounding the interpretation of circulating miR-208b and miR-499-5p for cardiac diagnostic and prognostic purposes.

Recent advances in diagnostic and therapeutic intervention have reduced in hospital mortality rates of patients with AMI, but have lead to increased mortality rates in patients who survive the initial AMI only to develop in-hospital or post-discharge Heart Failure (HF).[36,37] Data from a subset of ~200 patients in the original Framingham cohort showed a 14% incidence of HF 5-years post-MI.[38] In a more recent population-based cohort of 7733 patients, 71% of AMI survivors developed HF within 5 years, 64% of which were diagnosed within their first year post-MI.[37] While not all patients surviving AMI go on to develop HF (see Cardiomyopathy and heart failure), predicting those more likely to develop HF may increase their long term prognostic outcome and quality of life. Matsumoto et al[39] demonstrated that in Osaka Acute Coronary Insufficiency Study (OACIS) patients surviving AMI, p53-responsive miRNAs -192, -194, and -34a were significantly upregulated in sera of

Table 1 Validated targets of miRNAs implicated in the pathophysiology of cardiovascular disease. What is evident from this table is that multiple gene targets are targeted by multiple miRNAs under a variety of pathological conditions.

miRNA	Gene targets	Pathophysiology	Ref(s).
Let7	Nf2	INFR, Cancer	250
1	RhoA, Cdc42, Nelf-A/WHSC2, Kcnj2, Gja1, Ppp2r5a, Vegfa	CH, ARR, ANG	81,82,106,151
10	MAP3K7, βTRC, Flt1	ATH, ANG	122,154
15	Vegfa	ANG	149
16	Kdr	ANG	155
20	Vegfa, Hif1a	ANG	150
21	Pten, Pdcd4, Ppara, Mpv17l, Sorbs2, Pdlim5	Cancer, ISC, INFR, CH	64,65,123,165,166,253
23	Xiap, MuRF1	CI, CH	95,261
25	Serca2, Ip3r1	ARR, HF	83
26	Kcnj2	ARR	90
29	Adamts7, Col1a1, Col1a2, Col3a1, Fibrillin	ATH, ARR, CR	56,57,169
30	CTGF, MTP	CR, ATH, DLD	54,182
33	CROT, CPT1a, HADHB, AMPKa, IRS2, ABCA1, Npc1	ATH, DLD	179,180,183
34	Ppp1r10, vinculin, Sema4b, Pofut1, Bcl6	MI, CH	52,53
93	Vegfa	MetS	153
122	Slc7a1, Agpat1, Alpl, Cs, Klf6, Prom1, Sox4	Cancer, DLD	185,186,217
125	Grin2a	NSP	273
126	Spred1, Vcam1, Rgs16, Foxo3, Bcl2, Irs1, Tsc1	ANG, ATH, INFR	130,133–135, 157,159
130	Pparg	MetS	291
132	FoxO3, p300, p120RasGAP, MeCP2	CH, INFR, ANG, Rett Syndrome	102,137–139
133	CTGF, RhoA, Cdc42, Nelf-A/WHSC2	CH, CR	54,106
143	Klf5, Ssh2, Mrtfb, Orp8	ATH, T2DM	128,302
144	ABCA1	DLD	181
145	Klf4, Klf5, Srgap1/2, Ssh2, Ace	ATH	127–129
146	Tlr4, Myd88, Irak1, Traf6	INFR	251
155	Jarid2, Bcl6, eNOS, Socs1, c-MAF, Tnf	CH, ATH, HT, INFR	96,99–101,160,211
181	Cyld, Gria2	Cancer, NSP	253,274
192	Sip1	CR	51
199	Dyrk1a	CH	93
200	Vegfa	Diabetic Retinopathy	152
204	Mafa	T1DM	299
208	Med13	CH, MetS	67,68,287
206	Vegfa	ANG	151
210	Gpd1l, Efna3, Ptp1b, Rad52, E2f3, Casp8ap2, Iscu	HYP, cMet	230–236
212	FoxO3	CH	102
214	Slc8a1, Bcl2l11, Ppif	CR	80
221/222	p27kip1	ATH	168
223	Icam1, Gria2, Grin2b, Pknox1	ATH, CI, MetS	176,275,290
296	Hgs	ANG	156
301	Pias3	INFR	252
320	Aqp1, Aqp4, Hsp6b, Ets2, Mmp9, Emilin2, Nrp1, Pfkm	CI, MI/ISC, Cancer, ANG, cMet	265,266,268–270
328	Pim1, Cacna1c, Cacnb1, Igf1r	Cancer, ARR, HYP	84,239,249
342	Akt1, Bmpr2	ATH	161
375	Cadm1, Gphn, Cav1, Id3, Smarca2, Rasd1, Aifm1, Mtpn, Vti1a	T1DM	294,298
378	Med13, Crat	DLD, MetS	286
380	Tp53	Cancer	78

Table 1 (*continued*)

miRNA	Gene targets	Pathophysiology	Ref(s).
424	Cul2	HYP, ANG	228
425	Nppa	HT	222
484	Fis1	HYP, cMet	77
486	Pten, Foxo1a	CH	158
499	Calcineurin, Drp1, Sox6, Purβ, Sp3, Hp-1β, Med13, Mstn, Mapk6	CH, Striated Muscle Performance	35,73,74
637	ATP6VOA1	HT	218

Arrhythmia — ARR, Atherosclerosis — ATH, Angiogenesis — ANG, Cardiac Hypertrophy — CH, Cerebral Infarct — CI, Cardiac Remodeling — CR, Cellular Metabolism — cMet, Dyslipidemia — DLD, Heart Failure — HF, Hypertension — HT, Hypoxia — HYP, Inflammation and Immune Response — INFR, Ischemia — ISC, Metabolic Syndrome — MetS, Myocardial Infarction — MI, Neuronal and Synaptic Plasticity — NSP, Type 1 Diabetes — T1DM, Type 2 Diabetes — T2DM.

those who developed *de-novo* HF within 1 year. In a subset of the OACIS cohort who died within a year of discharge due to cardiac cause, sera expression of miR-155 and miR-380* were found to be increased ~3—4-fold in the convalescent stage when compared with those who returned for their 1 year follow-up.[40] Though this would suggest the possibility that miR-155 and miR-380* may be potential prognostic indicators for HF, the causal roles these miRNAs play in the progression of HF remain uncertain.

Cardiomyopathy and heart failure

Cardiac remodeling is a complex biological process involving deleterious perturbations in extracellular matrix (ECM) deposition and composition, morphological and phenotypic changes in the resident cardiac fibroblast population, and under extreme conditions, heart failure. While there are numerous pathophysiological causes of the various clinical subtypes of the cardiomyopathies, miRNAs are now being identified as crucial mechanistic players in the progression from normal to deleterious cardiac function under multiple pathophysiological stimuli. For the purposes of this review we will consider cardiomyopathy in general rather than each specific subset and attempt to clarify the newfound role of miRNAs in the progression of these diseases.

Fibrotic extracellular remodeling of the cardiac ECM is a deleterious consequence of ischemia, cardiac cell death and ventricular pressure overload. Cardiomyocytes reside in an ECM consisting of collagen fibrils that are composed mainly of collagen type I (~80%) and type III (~10%), with smaller contributions to the matrix coming from collagen types IV, V, and VI, in addition to the matrix contributions of laminin, elastin, glycosaminoglycans and others.[41,42] In clinical studies and in animal models of MI and cardiomyopathy, it has been well established that transient and prolonged ischemia as well as left ventricular pressure overload results in fibrotic scar formation in which resident myofibroblasts secrete excessive extracellular matrix components including collagens 1a1 (*Col1a1*), 1a2 (*Col1a2*), 3a1 (*Col3a1*) and matrix metalloproteinases which ultimately lead to impaired left ventricular function.[43—45] Similar ECM remodeling of the right ventricle occurs clinically and in animal models of pulmonary hypertension, chronic hypoxia, and AMI.[46—49]

Cardiomyocyte specific postnatal deletion of *Dicer* induced rapid death in juvenile and extensive cardiac remodeling in adult mice,[50] indicating the critical role of miRNAs in maintaining cardiac homeostasis. Specifically, miR-192 (Table 1) was found to inhibit Smad-interacting protein 1 (SIP1), an E-box repressor similar to δEF1, known to repress TGF-β dependent transcription of *Col1a2* expression.[51] In addition, Bernardo et al demonstrated that inhibition of the miR-34 family, but not miR-34a alone, attenuates morphological changes and pathological left ventricular remodeling in both pressure overload and MI models.[52] Mechanistically it was determined that the miR-34 family targets vinculin, Sema4b, Pofut1 and Bcl6. Inhibition of the miR-34 family resulted in significant increases in post-TAC αMHC/βMHC and phospho-AKT/total-AKT ratios as well as capillary density. Interestingly, repression of miR-34a prevented both age-associated and post-AMI cardiomyocyte apoptosis and interstitial fibrosis by maintaining/increasing PNUTS (PPP1R10) expression,[53] suggesting that the miR-34 family regulates signal transduction pathways necessary for cellular adhesion and cytoskeletal rearrangements. Further, miR-133b and miR-30c were found to repress CTGF expression thereby reducing *Col1a1* and *Col3a1* levels,[54] while miR-133a transgenic mice exhibited significantly reduced interstitial fibrosis and cardiomyocyte apoptosis in a mouse model of pressure overload CVD,[55] suggesting a role for miR-133 in repressing ECM remodeling and cardiomyocyte loss. Further, inhibition of miR-29b alone was sufficient to significantly increase *Col1a1*, *Col1a2*, and *Col3a1* expression *in vivo*.[56,57] In addition to miR-29, miR-21 expression was found to be increased >2-fold in intermediate and late-stage heart failure in mice.[58] Mechanistically, it has been suggested that miR-21 contributes to the extent of interstitial fibrosis and progressive heart failure and by selectively promoting fibroblast survival through targeting of *i*) Spry1 and thereby augmenting ERK-MAP kinase activity,[58,59] *ii*) PDCD4 thereby inhibiting apoptosis,[59,60] *iii*) PTEN thereby enhancing Akt signaling[61—63] and *iv*) PPARα thereby altering lipid metabolism.[64] Interestingly, myofibroblast derived exosomes, enriched in miR-21*, were found to induce cardiomyocyte hypertrophy by targeting SORBS2 and PDLIM5.[65] However, genetic ablation of miR-21 fails to inhibit cardiac hypertrophy and pathological cardiac remodeling in multiple models of CVD,[66] suggesting that the concerted signaling

of miR-21, miR-21*, and other miRNAs are necessary for pathological remodeling within the heart.

We, and others, first demonstrated the causative role of miR-208a in the pathological remodeling of the heart in a mouse model of pressure overload cardiac hypertrophy.[67,68] Mechanistically, it was demonstrated that miR-208a represses THRAP1/MED13 to inhibit T3 signaling, thereby increasing βHMC and miR-208b expression, as well as other fetal genes characteristic of cardiac remodeling. Of therapeutic interest, inhibition of miR-208a led to a dose dependent prevention of βHMC and ECM remodeling while simultaneously improving cardiac function in a rat model of hypertension (see Hypertension) induced heart failure.[69] Further, T3 signaling induces miR-208a-dependent cardiac expression of miR-499 to control skeletal muscle fiber type by targeting Sox6.[35] MiR-499 was found to be increased in cardiac Gαq transgenic mice,[70] which stimulates cardiomyocyte hypertrophy, apoptosis, and heart failure *in vivo*.[71,72] Previously demonstrated to target Med13/Thrap1, myostatin (Mstn), Mapk6, and Sp3,[73] cardiomyocyte specific ectopic expression of miR-499 in mice lead to progressive cardiac enlargement, contractile dysfunction, and overt dilated cardiomyopathy by 20 weeks of age,[70] thus indicating a causal relation between deleterious miR-499 expression and heart failure. Paradoxically, miR-499 transgenic mice ~8–10 weeks of age were found to be resistant to left ventricular remodeling after ischemia–reperfusion injury through repression of calcineurin and Drp1,[74] proteins which play key roles in hypertrophic signaling, and mitochondrial fission and energetics. However, these disparate observations may be reconciled by the ages of the mice in question. Given these observations, it seems likely that miR-499 plays a short-term protective role under ischemia–reperfusion injury by regulating Drp1 levels, and thus mitochondrial energy production, whereas long-term expression of miR-499 is deleterious by promoting hypertrophic signaling through its repression of myostatin.

A metabolic shift from fatty acids in the healthy myocardium to that of increased glucose metabolism is known to accompany heart failure.[75,76] Interestingly, miR-484 was found to target *Fis1* thereby preventing mitochondrial fission and apoptosis in anoxic cardiomyocytes,[77] whereas miR-380-5p was found to directly target P53, thereby inhibiting apoptosis.[78] In dilated cardiomyopathy (DCM) patients, and in a mouse model of DCM, the expression of *Dnm3os* encoded miRs-199a and -214 were found to be dramatically increased.[79] Interestingly, it was observed that inhibition of both miR-199a and miR-214, but not individual inhibition, attenuated both pathological remodeling and the metabolic switch from fatty acids to glucose by preserving the expression of PPARδ. MiR-214$^{-/-}$ mice in which miR-199a expression is preserved, exhibited decreased survival following MI and intensified ischemia–reperfusion induced cardiomyocyte apoptosis and ECM remodeling.[80] Mechanistically, it was determined that miR-214 directly targets multiple genes involved in metabolism and apoptosis including the sodium/calcium exchanger Ncx1 (*Slc8a1*), pro-apoptotic Bcl2 family member Bcl2-like 11 (*Bcl2l11*), and *Ppif* (cyclophillin D), a mediator of Ca^{2+} and oxidative damage-induced cell death independent of the Bcl2 pathway.

Interestingly, miR-208$^{-/-}$ mice demonstrate a marked absence of P-waves on ECGs,[68] indicating a potential role of miR-208 in controlling atrial depolarization. MiR-1 expression was found to be significantly increased in humans with coronary artery disease as compared with those without coronary artery disease, and to precede Phase II arrhythmia following AMI in a rat model.[81] Repression of miR-1 was found to inhibit arrhythmogenesis in the ischemic heart by derepressing connexin-43 (Gja1) and K^+ channel subunit Kir2.1 (Kcnj2) expression.[81] Further, the arrhythmogenic activity of miR-1 was found to be through selective repression of the protein phosphatase PP2A regulatory subunit B56α, thereby resulting in enhanced RyR2 phosphorylation at S2814, elevated diastolic SR Ca^{2+} leak, and arrhythmogenic myocyte Ca^{2+} cycling.[82] Interestingly, *in vivo* inhibition of miR-25 was found to sustain the expression of Serca2a and IP3R1,[83] thereby maintaining normal Ca^{2+} dynamics during excitation–contraction coupling and halting the progression of heart failure. MiR-328 expression was found to be increased >2-fold in atrial samples from atrial fibrillation (AF) patients, and demonstrated to repress atrial expression of the cardiac L-type Ca^{2+} channel α1c and β1 subunits,[84] thereby contributing to further atrial remodeling and the characteristic defects in L-type Ca^{2+} current densities associated with AF.[85–88] Expression of miR-26a and miR-26b is reduced in a mouse model of pressure overload hypertrophy[89] and in human AF patients.[90] Interestingly, miR-26a/b were found to be transcriptional targets of NFAT and to directly repress Kir2.1/KCNJ2 protein levels and I_{K1} density,[90] indicating a mechanistic role of reduced miR-26a/b expression in cardiac hypertrophy and the progression of AF. Collectively, these studies indicate that miRNAs are key regulators of physiologically relevant ion currents necessary for cellular homeostasis, excitation–contraction coupling, and cardiac function.

NFAT transcription factors, both necessary and sufficient to mediate cardiac hypertrophy, are antagonized by a variety of kinases including GSK3β, p38 and JNK thereby inhibiting their nuclear accumulation and diminishing hypertrophic gene expression. Calcineurin (Ppp3ca) is a calcium/calmodulin-dependent serine/threonine protein phosphatase that dephosphorylates NFAT thereby initiating hypertrophic gene expression.[91,92] Recent evidence suggests that miRNAs play both synergistic and antagonistic roles in calcineurin/NFAT signaling. MiR-199b was identified as a direct transcriptional target of calcineurin/NFAT to repress the NFAT inhibitory nuclear kinase Dyrk1a in a feed forward mechanism to enhance calcineruin-dependent gene expression.[93] Increased calcineurin/NFAT signaling in conjunction with decreased miR-25 signaling converged to deleteriously re-express Hand2 in order to initiate cardiac remodeling.[94] In addition, miR-23a has been shown to be necessary for the induction of hypertrophy *in vivo* by directly inhibiting MuRF1 in response to calcineurin/NFATc3 signaling.[95] Mir-155 has also been demonstrated to play a critical role in the pathological hypertrophic response as genetic ablation of miR-155 was shown to inhibit cardiomyocyte hypertrophy and dramatically increase the long term survival of calcineurin transgenic mice,[96] suggesting that miR-155 targets genes involved in anti-hypertrophic signaling. Mechanistically, it was found that miR-155

directly inhibits the expression of Jarid2, a known regulator of PRC2 activity,[97,98] suggesting that miR-155 plays a role in regulating epigenetic gene silencing. Interestingly, transplantation of bone marrow from miR-155$^{-/-}$ to WT mice was sufficient to inhibit hypertension induced cardiomyocyte hypertrophic growth and the concomitant deleterious loss of cardiac function by maintaining macrophage expression of suppressor of cytokine signaling 1 (SOCS1).[99] This is consistent with prior reports indicating a crucial role of miR-155 in regulating immune responses and cytokine signaling by targeting c-Maf[100] and TNF.[101] Collectively, these results demonstrate the necessity of miR-155-dependent paracrine signaling from bone marrow-derived cells in pathological cardiac remodeling and hypertrophy.

Further, elevated expression of miR-212 and miR-132 (the miR-212/132 family is separated by less than 500 nt within the genome) is necessary and sufficient contributors to pathological cardiac hypertrophy by targeting FoxO3 expression[102]; acting largely to activate the expression of Atrogin-1 (Fbxo32), an E3-ubiquitin ligase that initiates the degradation of calcineurin in cardiomyocytes.[103,104] Polycistronic miR-1 and miR-133, first identified as factors necessary for normal skeletal muscle myogenesis,[105] were found to be dramatically downregulated in mouse model of pressure overload cardiac hypertrophy.[106] Adeno-associated viral 9 (AAV9) cardiac delivery of miR-1 improved cardiac function in a rat model of pressure overload CVD,[107] while overexpression of miR-1 or miR-133, and *in vivo* inhibition of miR-133, reduced and enhanced, respectively, the severity of pressure overload cardiac hypertrophy by maintaining the normal physiological levels of RhoA, Cdc42 and Nelf-A/WHSC2.[106] RhoA and Cdc42 are known to be associated with cytoskeletal and myofibrillar rearrangements,[108,109] key hallmarks of cardiomyocyte hypertrophy. Collectively, these data indicate the myriad signaling and biological processes by which miRNAs regulate cardiac function, homeostasis, and the progression of cardiac remodeling and hypertrophy.

Atherosclerosis

Atherosclerosis, characterized by a progressive narrowing of the lumen of blood vessels, is a complex disease involving vascular endothelial cells, vascular smooth muscle cells, macrophages and their monocyte progenitors, neutrophils, and others,[110,111] and a primary risk factor for Myocardial Infarction (see Myocardial infarction) and stroke (see Subarachoid and intercerebral haemorrhage and infarction).[1,3] One of the primary factors in the etiology of atherosclerosis is the accumulation of oxidized phospholipids in atherogenic regions of the arterial tree with subsequent infiltration by inflammatory cells and activated endothelial cells, forming a fibrous plaque.[112–114] As the plaque expands, inducing neointimal formation and vascular remodeling, the luminal cross-sectional area decreases resulting in a progressive loss of volumetric blood flow and the onset of ischemia.

Previously thought of as a lipid storage disease, atheroprotective and athero-genic endothelial inflammatory signaling has emerged as a critical mechanistic focal point in the etiology of atherosclerosis.[115,116] Pro-inflammatory effector molecules potentiate plaque formation and many of the immune cells enriched in the nascent plaque are activated, actively producing inflammatory cytokines.[117–120] Nascent atherosclerotic plaque formation was found to be significantly attenuated in Vcam-1$^{-/-}$ mice,[121] indicating a critical role of this adhesion molecule in the etiology of atherosclerosis. In primary human endothelial cells, miR-10a (Table 1) was found to inhibit Vcam-1 expression by directly targeting MAP3K7 and βTRC,[122] upstream activators of NF-KB signaling known to induce Vcam-1 expression. More interestingly, miR-10a was found to be expressed at significantly lower levels in the atherosclerosis prone aortic arch (as compared with the descending thoracic aorta) and the caudal and cranial walls of the renal artery (adjacent to the descending abdominal aorta, as compared with the distal renal artery). Though, this would suggest a causal relationship between miR-10a expression and Vcam-1 dependent atherogenic signaling, further *in vivo* studies are needed.

Non-laminar/low-sheer chaotic flow, such as occurs at atherosclerosis prone bifurcations and curves within the vascular tree, has been shown to act in a positive feedback mechanism to induce and maintain the expression of miR-21 and the endothelial pro-inflammatory/pro-atherogenic genes Vcam-1 and Mcp-1. Mechanistically, it was determined that miR-21 directly targets PPARα, which is an inhibitor of AP-1 dependent Vcam-1, Mcp-1, and miR-21 expression.[123] However, atheroprotective pulsatile laminar flow, such as occurs during straight vascular segments, has been shown to increase KLF2[124] and the miR-23b cluster (miR-23b and miR-27b)[125] to maintain endothelial homeostasis. Interestingly, atheroprotective flow actively maintains endothelial homeostasis, by increasing KLF2 expression in part by suppressing the expression of miR-92a,[124] while simultaneously increasing the expression of miR-23b in order to repress E2F1 expression and Rb phosphorylation,[125] thus maintaining endothelial quiescence and physiological homeostasis. In addition, endothelial KLF2 induces miR-143/145 expression, which is then transported by non-apoptotic microvesicles to smooth muscle cells (SMCs) to promote an atheroprotective phenotype.[126] Consistent with this observation, miR-143/145 null mice are hypotensive, exhibit profound thinning of the tunica media in large conduit vessels, and have a decreased ratio of quiescent contractile to synthetic SMCs.[127,128] Interestingly, miR-143 and miR-145 are necessary for the acquisition and maintenance of the quiescent contractile SMC phenotype by targeting *Ace*,[127] whereas loss of miR-143, miR-145, or both, significantly reduces neointimal formation following carotid artery ligation[128,129] through repression of multiple genes involved in SMC migration and hypertrophic growth. Collectively these data indicate that precise temporal control of miRs-143/145 expression is a primary determinant in neointimal formation, and suggest that transient inhibition of miR-143 and/or miR-145 in the region of vascular injury (i.e. vascular stent or balloon angiography) would provide therapeutic benefit.

Alternatively, endothelial derived apoptotic (vesicular) bodies (AB) enriched in miR-126 are found to deliver their miRNA-cargo to recipient cells in order to repress SPRED1

and RGS16, thereby leading to the increased secretion of the pro-angiogenic chemokine CXCL12 in Apoe$^{-/-}$ mice. Repetitive injections of miR-126 containing ABs significantly reduced carotid artery atherosclerotic plaque area, as well as its macrophage and SMC content, by increasing the number of circulating Sca1^{+} lineage^{-} progenitors.[130] Plasma levels of CXCL12 and its receptor CXCR4 were significantly decreased in patients with unstable angina, as compared with patients with angina pectoris,[131] whereas CXCR4^{+} bone marrow-derived mononuclear cells (BMCs) were found to be enriched in pro-angiogenic cytokines HGF and PDGF-BB, improving neovascularization in a mouse hind limb ischemia model.[132] Additionally, miR-126 is necessary for normal angiogenesis and vascular integrity in both the mouse and zebrafish model systems.[133,134] Though an integral component of normal vascular development, miR-126 null mice show inhibited neointimal formation through decreased SMC proliferation and apoptosis in a carotid artery ligation model,[135] suggesting that miR-126 and other pro-angiogenic miRNAs may be of therapeutic interest.

Interestingly, post-AMI transplantation of saphenous vein-derived pericyte progenitor cells (SVPs) resulted in dramatic improvement of myocardial blood flow and neovascularization through the secretion of VEGFA, angiopoietin-1, angiogenic cytokines, and miR-132,[136] thereby enhancing paracrine signaling to further induce angiogenic and survival responses within the myocardium. Mir-132 is known to target MeCP2,[137] p300,[138] and the anti-angiogenic Ras inhibitor p120RasGAP.[139,140] While angiopoietins-1 and -2 are both ligands for the receptor tyrosine kinase TIE2,[141] angiopoietin-2, sensitizes endothelial cells to TNFα induced expression of pro-inflammatory adhesion molecules Icam-1 and Vcam-1,[142] and antagonizes the pro-angiogenic signaling of angiopoietin-1.[141,143] VEGFA was originally identified as a heparin-binding protein present in serum-free tumor cell culture medium that induced normal guinea pig skin microvasculature to become hyperpermeable, and referred to as vascular permeability factor (VPF).[144,145] Interestingly, VEGFA/VPF is also a potent pro-inflammatory cytokine. $VEGF_{165}$ transgenic mice have an asthma-like phenotype characterized by mucus metaplasia, smooth muscle hyperplasia, vascular remodeling, inflammation, edema and angiogenesis.[146] Further, reduction in VEGFA levels (VEGF $Trap_{R1R2}$) significantly reduced inflammatory cell recruitment *in vivo* in a mouse model of corneal neovascularization,[147] while angiopoietin-1 can repress the VEGFA induced increase in Vcam-1 and Icam-1 mRNA expression and leukocyte adhesion in HUVECs.[148] Pro-angiogenic VEGF signaling is necessary to induce the quiescent to proliferative phenotypic switch necessary for both the repair the vascular endothelium following injury, and for angiogenesis and neovascularization. VEGFA is known to be directly targeted by multiple miRNAs including miR-15a,[149] miR-20b,[150] miR-1 and miR-206,[151] miR-200b,[152] and miR-93.[153] In addition, intracellular VEGF signaling is similarly regulated by multiple miRNAs, including miR-10 targeting of the VEGF receptor *Flt1*,[154] miR-16 and miR-424 targeting of the VEGF receptor *Kdr*,[155] miR-296 mediated repression of PDGF receptor-β and *Kdr* through direct targeting of *Hgs*,[156] miR-126 targeting of the Ras inhibitor *Spred1*,[133,134,157] miR-486 targeting of the PI3K/Akt inhibitors PTEN and *Foxo1a*,[158] and miR-126 targeting of the mTor inhibitor *Tsc1*.[159] Collectively, these studies indicate that miRNA-dependent regulation of VEGFA/angiopoietin signaling plays a crucial role in maintaining the delicate balance between pro/anti-angiogenic and pro/anti-inflammatory signaling within the endothelium.

In ApoE deficient mice, leukocyte-specific loss of miR-155 was sufficient to reduce plaque size and the number of plaque associated macrophages after partial carotid ligation. In addition, miR-155 null macrophages stimulated with oxidized phospholipids expressed markedly lower levels of the monocyte recruiting chemokine CCL2. Mechanistically, miR-155 was found to directly target the NF-κB inhibitor Bcl6.[160] Interestingly, miR-342-5p may be an upstream activator of miR-155-dependent inflammatory signaling, as its inhibition increased Akt1, Bmpr2 and anti-inflammatory signaling while repressing miR-155 expression and atherosclerotic plaque formation.[161] Further, inhibition of miR-342-5p reduced the number of macrophages and smooth muscle cells in neointimas. Together these data indicate causal roles for miR-155 and miR-342-5p in progression of vascular inflammation, neointima formation and atherosclerosis. In addition, TLR4 (an upstream regulator of miR-146a/b, miR-132, and miR-155[162]) has been found to be preferentially expressed by macrophages enriched in atherosclerotic plaques.[163] Interestingly, miR-21 was shown to be necessary to inhibit pro-inflammatory PDCD4-dependent TLR4 signaling and NF-κB activation, thus negating the toxic effects of repressed anti-inflammatory signaling in response to lipopolysaccaride .[164] MiR-21 has been previously shown to repress PDCD4 expression in cancer cells,[165,166] resulting in increased tumor cell proliferation, invasion and metastasis. In addition, elevated miR-21 expression is observed in carotid artery neointimal lesions following balloon angioplasty, whereas inhibition of miR-21 dramatically reduces neointimal formation.[167] Together these results strongly suggest a critical role of miR-21 in atherogenic inflammatory signaling, which may be due, in part, to is pro-proliferative effects regardless of cell type. Expression of miR-221/222 was found to be significantly and transiently increased following balloon-injury in adult male rats. Consistent with its pro-proliferative effects by repressing p27Kip1, inhibition of miR-221 significantly represses neointimal formation following balloon injury.[168] Further, miR-29 was shown to play a crucial role in repressing ADAMTS-7 ,[169] previously identified in GWAS of patients with coronary artery disease[170] and coronary artery calcification.[171] Silencing of ADAMTS-7 *in vivo* substantially inhibited neointimal formation and SMC migration in response to pro-inflammatory cytokines,[172] thus implicating miRNAs as multifactorial mediators of vascular inflammation, atherosclerotic plaque formation and subsequent ECM remodeling and neointima formation.

While the causal relation between lipid accumulation and atherosclerosis has been well documented for years, the cellular and molecular causes of lipid accumulation and subsequent atheroslcerosis in atheroprone regions of the vasculature are becoming increasingly clear. Increasing the circulating levels of HDL were found to induce regression of atherosclerosis in ApoE deficient mice [173] and in humans ,[174]

suggesting the therapeutic benefit of increasing circulating HDL levels. Interestingly, the anti-atherosclerotic effects of HDL may be due, in part, to HDL-delivered miRNAs whose expression signature changes under disease conditions.[175] Endothelial Icam-1 was found to be directly targeted by HDL-delivered, rather than endothelial transcribed, miR-223 in order to repress inflammatory gene expression,[176] indicating heterocellular communication through HDL-delivered miRNAs *in vivo*. Northern blot analysis indicates that miR-223 is highly expressed in bone marrow, lung, and spleen with detectable expression in liver and thymus,[177] suggesting that the liver is the likely source of HDL-delivered miR-223 to the vascular endothelium. Interestingly, 4 weeks after inhibition of miR-33, LDL receptor null mice were found to have significant reductions in previously established atherosclerotic plaques and elevated circulating HDL levels,[178] through direct targeting of the adenosine triphosphate-binding cassette transporter 1 (ABCA1) and the endolysosomal transport protein NPC1.[179] Further, inhibition of miR-33a and miR-33b increased hepatic expression of cholesterol transporter ABCA1 leading to increased circulating HDL levels and a marked repression of VLDL associated triglycerides in non-human primates.[180] MiR-144 is found to be upregulated in conditions of hyperlipidemia and hypercholesterolemia by LXR signaling in the liver, and to directly repress ABCA1 expression,[181] while miR-30c directly represses MTP expression with corresponding reductions in APOB secretion, total cholesterol and triglycerids, and atherosclerotic plaques.[182] MiR-33a is also known to directly target CROT, CPT1a, HADHB, AMPKα and IRS2,[183] key regulators of cholesterol homeostasis, fatty acid metabolism and insulin signaling. Finally, inhibition of miR-122 was found to significantly reduce total serum cholesterol and liver steatosis in mice fed a high-fat diet for 19 weeks,[184] thus indicating potential therapeutic benefits of miR-122 inhibition in patients with dyslipidemia. Mechanistically, miR-122 was found to target Agpat1,[185] an enzyme critical for triglyceride synthesis, and multiple genes (see Table 1) involved in hepatic differentiation, fibrosis, and homeostasis.[186] Together these data implicate multiple miRNAs as key regulators of circulating HDL levels, thereby modulating circulating LDLs, triglycerides and other lipids involved in the pro-atherosclerotic vascular inflammatory response.

Hypertension

Essential hypertension effects ~65 million Americans,[3] however, the prevalence and hospitalization rates of HF patients with prediagnosed hypertension has continued to increase. In addition, the rates of end-stage renal disease (ESRD) have been increasing yearly since the early 1990s, with hypertension second only to diabetes as its primary precursor.[187] Although the precise pathogenesis of essential hypertension remains elusive, it has been suggested that defective sodium handling and regulation of fluid volumes by the kidney are a primary cause.[188] Further, virtually all the known Mendelian disorders impacting blood pressure are caused by dysregulation of salt and water reabsorption in the distal nephron.[189] Alternatively, other studies have identified primary vascular defects impacting peripheral resistance without direct involvement of the renin-angiotensis-system (RAS).[190–194] Current oral antihypertensive treatment options include diuretics, beta-blockers, aldosterone receptor blockers, angiotensin-converting enzyme inhibitors, calcium channel blockers, α1 blockers and direct vasodilators.[187] Given their effectiveness, ACE inhibitors and Angiotensin receptor blockers, remain a mainstay in the treatment of diabetes, heart failure, atherosclerosis and hypertension.[195]

Terminal pulmonary arterial hypertension (PAH) is characterized by lesions composed of hyperproliferative PA endothelial cells and smooth muscle cells.[196,197] Although the intrinsic cellular and molecular mechanisms contributing to EC and VSM proliferation and phenotypic switching have been studied for years,[110,198–201] recent evidence strongly suggests that crosstalk between these two cellular populations is dramatically altered, thereby inducing perturbations in molecular signaling and contributing to the loss of vascular homeostasis.[197,202–204] Of particular interest in PAH, Apelin (APLN) is known to be highly expressed in the endothelium of the pulmonary and systemic vasculature,[205] and to decrease blood pressure in both normal and hypertensive mice[206] likely through activation of eNOS.[207] Recently, perturbations in the apelin (APLN) — apelin receptor (APLNR, also referred to as APJ or AGTRL1) signaling axis have been observed in PA hypertensive patients.[208,209] Mechanistically, it was reported that impaired APLN signaling leads to increased FGF2, FGFR1, PASMC proliferation and vascular tone by a reduction in APLN-mediated expression of miR-424 and miR-503.[210] In addition, miR-155 (Table 1) was found to repress eNOS expression, and endothelium dependent vasorelaxation, in a TNFα model of endothelial dysfunction.[211]

Angiotensin II regulates a variety of non-hemodynamic physiological processes including fluid homeostasis, renal function, aldosterone production, induction of reactive oxygen species, apoptosis and hypertrophy, in addition to its role as a vasoconstrictor. Crowley et al[212] demonstrated that renal angiotensin type-1 receptors (AT_1R) are required for the development of Ang II-dependent hypertension and cardiac hypertrophy. Interestingly, a high correlation has been identified in hypertensive patients between Ang II sensitivity and the presence of the +1166A/C single nucleotide polymorphism (SNP) in the *AT_1R* gene.[213] The presence of the +1166A/C SNP has been associated with increased Ang II-dependent vasoconstriction in isolated human arteries.[214] Although consistent with *in silico* analyses suggesting that the +1166A/C SNP would inhibit miR-155 mediated repression of AT_1R, this has yet to be validated *in vivo*. Additionally, decreased expression of the cationic amino acid transporter encoded by *Slc7a1* in hypertensive patients is associated with a +2157C/T SNP located within its 3′UTR.[215] The cationic amino acid transporter encoded by *Slc7a1* (CAT-1) is the principal arginine transporter in endothelial cells; physiologically, arginine is an essential metabolite in the production of the potent vasodilator nitric oxide. Interestingly, an inverse relation between miR-122 and CAT-1 protein expression is observed in the developing liver, with miR-122 expression increasing dramatically from ED12.5 to P21.[216] In addition, miR-122 has been shown to directly inhibit translation of *Slc7a1* in a hepatocarcinoma cell line.[217] However, the mechanism

whereby the *Slc7a1* SNP promotes decreased protein expression levels remains to be determined. A 3′UTR polymorphism at T+3246C (rs938671) in the vacuolar H^+-ATPase subunit ATP6VOA1 is found to create a miR-637 binding motif, thereby inducing changes in vacuolar pH and secretory protein processing of Chromogranin A (CHGA) into Catestatin.[218] CHGA is the precursor to several bioactive peptides including catecholamine release-inhibitory Catestatin (human $CHGA_{352-372}$), the vasoconstrictor inhibitors Vasostatin I (human $CHGA_{1-76}$) and Vasostatin II (human $CHGA_{1-115}$),[219,220] and is linked to familial hypertension along with ATP6VOA1.[221] Patients with the rs5068 SNP (AG vs. AA, located in the 3′UTR) of the *NPPA* gene, were found to have significantly higher blood pressure, and up to 50% higher ANP levels in AG individuals than in AA individuals.[222] Mechanistically it was determined that the AG allele inhibited the ability of miR-425 to repress plasma ANP levels. Using a bioinformatics approach, Nossent et al identified SNPs associated with hypertension in the 3′UTR of the arginine vasopressin 1A receptor (AVPR1A, rs11174811), and hypotension in the 3′UTRs of bradykinin 2 receptor (BDKRB2, rs5225 and rs2069591), and the thromboxane A2 receptor (TBXA2R, rs13306046).[223] Thromboxane A2 is an important platelet derived[224] modulator of vascular tone in the pulmonary arterial[225] and cerebral arterial[226] vasculature (see Subarachnoid and intercerebral haemorrhage and infarction). Together, this data strongly suggests that many of the genetic variations associated with hypertension may be due to deleterious gain-of-function and loss-of-function mutations to enhance or repress miRNA-directed post-transcriptional gene expression (Fig. 1).

Chronic hypoxia is the most common cause of pulmonary hypertension. Hypoxic conditions induce transcriptional activation of multiple genes involved in angiogenesis and metabolic adaptation. The central mediator in this signaling cascade is the hypoxia-inducible factor (HIF-1), which is composed of HIF-1α and HIF-1β subunits. Cytosolic HIF-1α is maintained at low levels under normoxia by proteasomal degradation following prolyl-hydroxylation.[227] Interestingly, miR-424, previously identified as a downstream contributor of APLN signaling,[210] was demonstrated to target the ubiquitin ligase scaffold *Cul2*, thereby stabilizing HIF-1α isoforms,[228] and thus contributing to a feed-forward signaling pathway to potentiate HIF-1 dependent gene expression. However, this data also suggests that many of the phenotypic observations associated with miR-424 are due to its mechanistic effects on repressing E3-ubiquitin ligase assembly and proteosome mediated proteolysis. Alternatively, HIF-1 dependent miR-210 was found to induce plieotropic effects in multiple cell lines.[229] MiR-210 was found to act in a positive feedback loop to promote HIF-1α stability and nuclear translocation by repressing prolyl-hydroxylase activity by targeting GPD1L.[230] In addition, receptor tyrosine kinase ligand ephrin A3,[231,232] homology-dependent DNA repair pathway member RAD52,[233] transcription factor E2f3,[234] and caspase-8 associated protein 2[235] have all been examined as miR-210 targets in hypoxia.

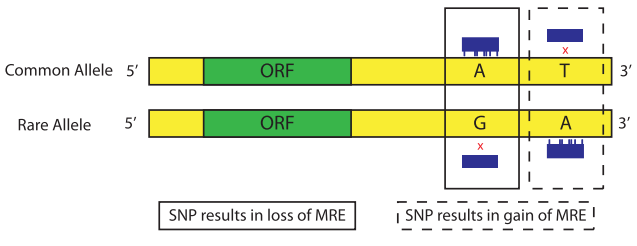

Figure 1 Schematic representation of the effect of SNPs on miRNA target sites. SNPs in the 3′UTR of protein coding genes may result in the loss or gain of a miRNA response element (MRE), thereby deleteriously altering post-transcriptional gene regulation.

Perhaps most interestingly, miR-210 was shown to directly repress ISCU1/2, thereby reducing iron-sulfur cluster formation and mitochondrial function via Complex I and aconitase.[236] In addition, HIF-1 signaling is known to *i*) induce multiple genes involved in glucose transport and glycolysis, *ii*) enhance the expression of enzymes responsible for the shunting of pyruvate from the TCA cycle to anaerobic metabolism, and *iii*) mediate a subunit switch in cytochrome c oxidase thereby enhancing electron transport chain (ETC) efficiency under hypoxic conditions.[237,238] Inhibition of ISCU1/2 expression and thereby TCA cycle (aconitase) and ETC (Complex I) activities by miR-210, further enhances the ability of HIF-1 to control the hypoxia-induced metabolic switch to ensure precise control over energy production and cell survival. In addition, ectopic expression of miR-328 decreased the deleterious increase in hypoxia-dependent right ventricular pressure and pulmonary artery (PA) remodeling.[239] Mechanistically, it was determined that miR-328 inhibits the expression of the L-type calcium channel-α1c (CaV1.2) and IGF-1R which are essential for normal smooth muscle and cardiac excitation—contraction coupling[240–242] and postnatal hypertrophic growth.[243] Interestingly, hypoxia induced prolyl-hydroxylation of Ago2 was found to be a critical determinant in its association with Hsp90, translocation to stress granules, and miRNA-dependent endonuclease activity,[244] suggesting that perturbations in the regulation of miRNA directed Ago2/RISC activity, in addition to deleterious changes in miRNA expression, may contribute to molecular etiology of disease.

Emerging evidence suggests that inappropriate activation of the STAT3/NFATc2/Pim1 axis has a causal role in human pulmonary arterial hypertension.[245–247] Mesenchymal stromal cell derived exosomes (MEX) were found to inhibit hypoxia induced lung vascular remodeling and inflammation, right ventricular hypertrophy, and PAH in mice.[248] Mechanistically, it was determined that MEX, enriched in miR-16, miR-21, and let7b precursor, inhibit hypoxic induction of the miR-17 superfamily, thereby increasing the levels of antiproliferative miR-204 expression to block the STAT3-miR-204-STAT3 feed-forward loop in distal pulmonary vessels. In addition, miR-328 was found to directly target *Pim1* and to act as a 'decoy' sequestering hnRNP E2 in order to rescue CEBPA mRNA translation.[249] Let-7a was found to repress STAT3 activation by targeting NF2,[250] and miR-146b was found to repress STAT3 signaling by targeting its upstream activators TLR4, MyD88, IRAK-1, and TRAF6.[251] In addition, miR-301a was found to enhance STAT3 signaling by targeting the STAT3 inhibitor Pias3,[252] while STAT3 dependent miRNA-21 and miR-181b-1

were found to be part of a positive feedback look enhancing STAT3 expression and cellular transformation by targeting *Pten* and *Cyld* respectively .[253]

Subarachnoid and intercerebral haemorrhage and infarction

Cerebrovascular diseases including atherothrombotic or embolic infarction (stroke), as well as subarachnoid and intercerebral hemorrhage was the number 4 cause of death in the United States from 2009 to 2010, causing approximately 5.2% of all deaths.[1] Of all strokes, 87% are ischemic, whereas 10% are classified as intercerebral hemorrhage and ~3% are of subarachnoid hemorrhage classification. Among ischemic stroke survivors ≥65 years of age from the Framingham Heart Study, ~50% experienced hemiparesis, ~46% had cognitive defects, ~19% had aphasia and ~26% were either dependent in daily living activities or institutionalized in a nursing home.[3] Rapid diagnosis of stroke and treatment with antithrombotic or thrombolytic is crucial to patient survival and subsequent morbidity,[254] by minimizing and preventing neuronal cell death. Post stroke neuronal cell death is due to cytotoxic edema and/or increased intracranial cerebral pressure resulting from *i*) anoxic membrane depolarization which leads to increased intracellular Na^+, water influx and cellular swelling, and *ii*) breakdown of the blood–brain-barrier leading to indiscriminate accessibility of intravascular proteins and ions to the brain parenchyma.[255,256]

In a rat model of transient focal cerebral ischemia followed by reperfusion for 24 or 48 h, the expression level of multiple miRNAs circulating in the blood and isolated from the brain were found to be differentially altered,[257] suggesting a role for miRNAs in the maintenance of cerebral homeostasis. Interestingly, many of the dysregulated miRNAs are not known to be expressed within the brain or blood, suggesting that circulating miRNAs may act as messengers to distal organs/cells following cerebral ischemia–reperfusion injury. Although women have a lower age-adjusted stroke incidence than men, women have a longer lifetime risk of stroke,[3] likely due to increased lifespan. Interestingly, several experimental studies have suggested that estrogens have neuroprotective effects following stroke.[258–260] In an experimental model of stroke, gender differences in infarct size have been correlated with miR-23a (Table 1) expression.[261] Post stroke decreases (male) and increases (female) in miR-23a expression were found to be linked to infarct size and neuronal apoptosis by targeting X-linked inhibitor of apoptosis (*Xiap)*, in which the increased XIAP protein expression (male) inhibits Caspase-3 mediated apoptosis.

Cytotoxic cerebral edema is a major contributor to post-stroke infarct volume and apoptosis. Aquaporins (MW ~30 kDa) are small integral membrane proteins that are critical for water transport and are highly expressed in may cell types of the kidney, lung, and brain that are involved in fluid transport. Aquaporin 4 (*Aqp4*) is found to be highly enriched in perivascular glial processes, the opposite cellular pole of glial high affinity glutamate transporter GLAST (*Slc1a3*), and in subpopulations of ependymal cells lining the ventricles.[262,263] Counter-intuitively, genetic ablation of *Aqp4* was found to reduce cerebral edema in a mouse model of human ischemic hemispheric stroke (middle cerebral artery occlusion),[264] leading to increased survival. Contrary to this observation, intracerebral injection of anti-miR-320a was found to increase AQP1 and AQP4 expression, and decrease infarct volume in a mouse model of human ischemic hemispheric stroke.[265] In light of the observations in $Aqp4^{-/-}$ mice, it is appears unlikely that the antagonistic effects of miR-320a on AQP1 and AQP4 expression are solely responsible for the reduction in infarct volume, as miR-320 is also known to directly target the cardioprotective and neural expressed Hsp20 (*Hspb6*),[266,267] metastasis associated *Ets2*, *Mmp9* and *Emilin2*,[268] pro-angiogenic Nrp1[269] and gycolytic *Pfkm*.[270] In addition, miR-320 was found to recruit Ago1 and the Polycomb group component Ezh2 to the Polr3d genetic locus in order to enhance H3K27me3 thereby repressing Polr3d transcription.[271]

Glutamate excitotoxicity resulting from increased glutamate receptor activation is an additional causative mechanism leading to neuronal cell death. Excessive calcium influx through NMDA receptors requires membrane depolarization induced by sodium influx through AMPA receptors.[272] Neuronal miR-125b targets NR2A (*Grin2a*, ionotropic glutamate receptor, NMDA receptor subunit epsilon 1) to regulate synapse structure and function,[273] while miR-181a is known to target GluA2/GluR2 (Gria2, ionotropic glutamate receptor, AMPA receptor subunit alpha 2).[274] Interestingly, the bone marrow and spleen enriched miR-223[177] was found to directly target NR2B (Grin2b, ionotropic glutamate receptor, NMDA receptor subunit epsilon 2) and GluA2/GluR2 to protect neurons from cell death following transient global cerebral ischemia.[275] Although below the Northern blot detection threshold,[177] quantitative miRNA RT-PCR analysis indicates that miR-223 is differentially expressed within the cerebrum, with its highest expression in the cerebral cortex.[275] Interestingly, DAVID (v6.7) and TargetScan (release 5.1) analyses indicate that the majority of computationally predicted miR-223 targets are brain specific, thus raising speculation that the cerebral effects of miR-223 are from non-neuronal derived miR-223 via heterocellular signaling.

Risk factors for cardiovascular disease

Broadly defined, Metabolic Syndrome (MetS) consists of multiple risk factors for CVD and type-2 diabetes mellitus (T2DM) and involves various combinations of dyslipidemia, hypertension, impaired fasting glucose, insulin resistance, and increased body mass index (BMI) or waist-to-hip ratio.[276] During 2009 and 2010, the age-adjusted prevalence of MetS was 22.9% of the U.S. population.[277] According to the National Health and Nutrition Examination Survey (NHANES) from 2003 to 2006, approximately 34% of adults met the criteria for MetS.[278] Similar to other complex disorders, multiple genes in various cell types are implicated in the etiology of MetS. Genetic markers identified by GWAS, genomic SNP analysis, copy-number variants and compared to clinical risk scores derived from extended models containing multiple genetic markers for T2DM and CVD identify *i*) PPARG, KCNJ11, IGF2BP2, KCNQ1 and SNPs associated with SLC2A2 and IGF1 correlate highly with

T2DM, and *ii*) PPARG, IRS1, ADAMTS9, DUSP9, GCK and GCKR correlate highly with obesity and insulin resistance.[279] Similar to protein coding genes, correlations between miRNA expression and MetS are observed. Serum miRNA analysis, genome wide gene expression, and serum NMR metabolomics analyzed from 71 participants of the Young Finns Study found that *i*) miR-144-5p concentration correlated with fasting glucose levels, *ii*) miR-1207-5p with glycosylated hemoglobin, *iii*) miR-484 with metabolites associated with insulin resistance, *iv*) miR-625-3p with total and esterified cholesterol, IDL lipids and phospholipids, and *v*) miRs-1288-3p, -129-1-3p, -129-2-3p with glycerol.[280] In a 3 month study of 21 morbidly obese patients and 14 lean controls, it was found that the circulating monocyte levels of miR-181a/b was increased to that of the lean controls in obese patients following bariatric surgery induced weight loss.[281] Interestingly, the same study identified that miR-181a levels in monocytes were similarly decreased in a 125 patient cohort diagnosed with MetS and who underwent coronary angioplasty. MiR-181b (Table 1) is known to regulate CYLD,[253] a deubiquitinating enzyme that negatively regulates NF-κB activity.[282,283] Interestingly, miR-181 expression is highest in thymus, brain, lung with noticeable expression in bone marrow and spleen.[177] Together, these data indicate that plasma or leukocyte-derived miRNAs contribute to the multitude of causes and consequences of MetS and CVD; the mechanisms of which are only now coming into focus.

The peroxisome proliferator-activated receptor γ coactivator 1 (PGC-1) family of transcriptional cofactors regulate crucial aspects of energy metabolism including adaptive thermogenesis in brown adipose tissue, adipocyte cell fate determination, mitochondrial biogenesis, glucose uptake and gluconeogenesis.[284,285] Encoded within PGC1α, miRs-378 and -378* were found to counter the metabolic actions of PGC1α through repression of carnitine *O*-acetyltransferase (CRAT) and MED13.[286] CRAT encodes a mitochondrial enzyme involved in fatty acid metabolism and MED13 is a component of the Mediator complex controlling nuclear hormone receptor activity. Interestingly, pharmacological inhibition of miR-208a and cardiac specific MED13 transgenic mice displayed similar resistance to obesity and glucose intolerance after 6 weeks of a high fat diet.[287] In addition, cardiac specific deletion of MED13 enhanced susceptibility to obesity following 6 weeks of a high fat diet. Cardiac specific miR-208a is known to target MED13/THRAP1/TRAP240.[67,68] Together these data indicate a critical role of cardiac miR-208a and MED13 in the regulation of systemic energy homeostasis and whole body metabolism; however, the heterocellular signaling mechanism linking cardiac miR-208a/MED13 to white adipose tissue accumulation and glucose tolerance remains to be identified. MiR-21 knockout mice and inhibition of miR-21 in WT mice have reduced kidney fibrosis and epithelial injury in unilateral ureteral obstruction and unilateral ischemia reperfusion injury models of kidney injury.[64] Mechanistically, it was determined that miR-21 targets peroxisome proliferator-activated receptor α (Ppara), a transcription factor that regulates multiple metabolic and lipid oxidation pathways, and Mpv17l (also known as M-LP), an inner mitochondrial membrane protein which inhibits mitochondrial oxidative stress.[288,289]

A causal link between insulin resistance and macrophage activation (see Atherosclerosis) has been elucidated. Genetic ablation of the bone marrow macrophage enriched miR-223 was found to exacerbate high-fat diet induced insulin resistance macrophage activation/polarization through repression of Pknox1.[290] Importantly, a bone marrow transplant from miR-223$^{-/-}$ mice into WT mice demonstrated that myeloid cell-specific loss of miR-223 was sufficient to exacerbate systemic insulin resistance and adipose tissue inflammation. Significantly repressed in adipocytes of obese patients, miR-130a/b was found to repress adipocyte differentiation by targeting peroxisome proliferator-activated receptor γ (Pparg).[291] Interestingly, the induction of miR-130a/b expression in differentiated adipocytes in response to TNFα stimulation is partially due to NF-κB enhanced transcription,[292] further suggesting cross-talk between pro-inflammatory signaling and insulin resistance. In addition, silencing of miR-103/107 (miRs-103 and -107 differ by one nucleotide at position 21) alleviates hyperglycemia in *ob*/*ob* and diet-induced obese mice.[293] Of potential therapeutic interest, it was further demonstrated that hepatic silencing of miR-103/107 expression is *i*) sufficient to reverse metabolic abnormalities in obese and insulin-resistant states, and *ii*) directly leads to increased Cav1 levels and subsequent improvements in insulin signaling by increased adipocyte insulin receptor β-subunit (IRβ) expression and increased insulin dependent phosphorylation of Akt1 and IRβ in adipose tissue and the liver.

Perhaps most interestingly, miRNAs similarly play a regulatory role in the production of insulin in response to a variety of physiological stimuli. The pancreatic β-cell enriched miR-375 was found to target myotrophin (*Mtpn*) and vesicle transport through interaction with t-SNAREs 1A (*Vti1a*) and in order to repress glucose induced insulin secretion.[294] Myotrophin and VTI1A are known to be involved in cardiac hypertrophy[295,296] as well as neuronal vesicular transport and neurotransmitter release.[297] Importantly, miR-375 null mice are hyperglycemic with elevated gluconeogenesis and hepatic glucose output.[298] Interestingly, it was discovered that these mice have reduced β-cell mass due to impaired β-cell proliferation likely due to elevated expression of cell adhesion molecule 1 (*Cadm1*), gephyrin (*Gphn*), caveolin1 (*Cav1*), inhibitor of DNA binding 3 (*Id3*), *Smarca2*, Ras—dexamethasone-induced-1 (*Rasd1*), and mitochondrion-associated apoptosis-inducing factor 1 (*Aifm1*). Interestingly, β-cell thioredoxin-interacting protein (TXNIP) was found to induce the expression of miR-204, thereby repressing MAFA expression and insulin transcription.[299] MAFA is a basic leucine zipper transcription factor that is essential for glucose induced insulin secretion, but regulating the transcription of insulin.[300] Further, it was found that miR-143/145 null mice are protected from the development of T2DM, whereas transgenic overexpression of miR-143, but not miR-145, impaired insulin induced AKT activation and glucose homeostasis.[301] Mechanistically, it was found that the expression of miR-143/145 was dramatically elevated in the livers of obese mice lead to the miR-143 directed inhibition of oxysterol-binding-protein-related protein (ORP) 8.[302] Loss of pancreatic β-cell number is a critical factor in the etiology of diabetes, and a subject of targeted therapeutic research.[303,304] It is becoming evident that miRNAs

are key regulators of metabolic pathways contributing to CVD.

Conclusions and future directions

In this review we have attempted to summarize what is known regarding molecular mechanisms whereby miRNAs modulate normal physiological and pathophysiological signals within the cardiovascular system. A complex model has evolved in which miRNAs modulate key regulatory processes involved in fine-tuning signal transduction cascades, thereby maintaining cellular and physiological homeostasis (Fig. 2). This complexity is readily apparent from the multiple lines of evidence indicating the essential roles of miRNAs in the regulation of *i*) cytokine/paracrine/autocrine signaling, *ii*) lipid metabolism and the progression of atherosclerosis, *iii*) resting membrane potential and intracellular calcium handling, and *iv*) hematopoietic derived inflammatory cells in the progression of atherosclerosis and cardiac hypertrophy. In addition, from multiple genetic mouse models, we have learned of the critical roles that miRNAs play in normal development, physiological homeostasis, and cellular responses to pathological stimuli. Importantly, these data indicate the necessity of miRNAs in maintaining physiological homeostasis by directly modulating plasma HDL/LDL ratios, inflammatory cytokine production and signaling, systemic energy homeostasis and MetS, mitochondrial function, cellular proliferation, apoptosis, as well as ECM deposition and remodeling. However, confounding many of these results is the discovery that non-cell-autonomous miRNAs contribute to post-transcriptional gene regulation. While the relative contribution of non-cell-autonomous vs. cell-autonomous miRNAs remains to be determined, heterocellular communication via miRNAs is emerging as a physiologically relevant signaling paradigm.

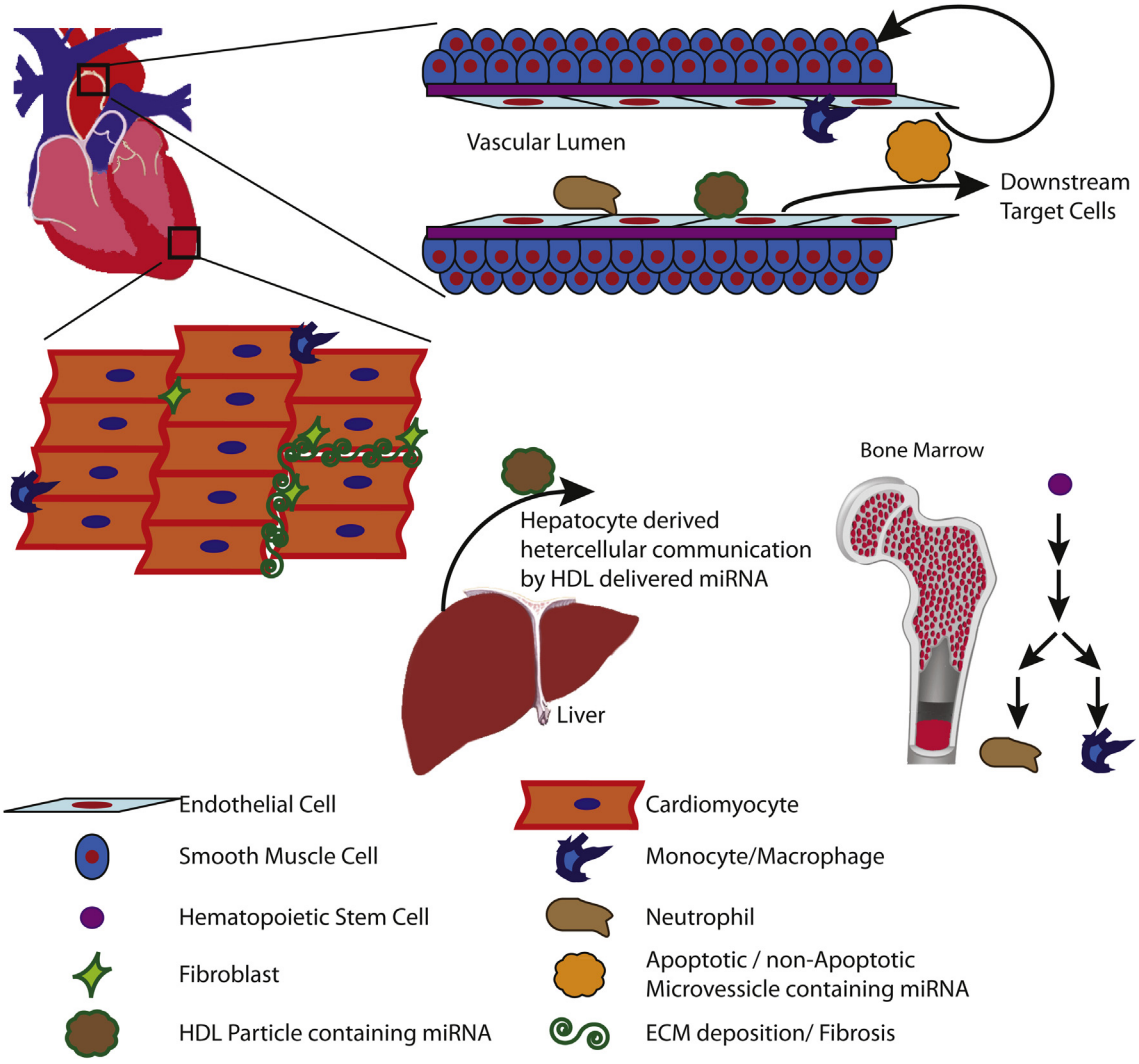

Figure 2 Known heterocellular signaling mechanisms involved in the progression of CVD. This figure summarizes what is currently known about multiple cell types within the cardiovascular system under normal physiological and pathophysiological conditions. The information presented above represents findings from multiple *in vivo* and *in vitro* experimental models in which genetic and disease models were utilized to determine the biological and physiological function of miRNAs.

Despite compelling evidence identifying post-transcriptional targets of individual miRNAs, and the pathophysiological effects of abrogating miRNA directed mRNA translational repression, we know relatively little about how multiple miRNAs work in concert, how polycistronic miRNAs are regulated, or if cells are capable of discriminating between cell-autonomous and non-cell-autonomous miRNAs. Key unresolved questions include the following: 1) Do non-cell-autonomous miRNAs supplant or complement cell-autonomous miRNAs? 2) Do multiple miRNA loaded RISCs compete or complement their biochemical activities with respect to a single mRNA? 3) Do non-cell-autonomous miRNA passenger strands act as a sponge for cell-autonomous derived miRNA target strands or do they participate in RISC loading and translational repression? 4) What are the environmental cues and extracellular stimuli that regulate the ratios of mature miRNAs derived from polycistrons? 5) What are the cellular origins of the miRNAs found to be circulating within blood? 6) How is the expression of these circulating miRNAs regulated under multifactorial diseases such as MetS, diabetes, hypertension, dyslipidemia and atherosclerosis?

Clearly, there has been much exciting progress in our understanding of signaling cascades regulated by miRNAs and their contribution to the etiology of CVD, but additional work is needed. Given that CVD is a multifactorial disease process involving not just the heart and vasculature, but also bone marrow derived cells, liver regulation of plasma lipids, and pancreatic insulin signaling among others, it is likely that major progress in this area is going to be dependent upon sophisticated cell-type specific loss of function approaches coupled with whole animal physiological studies and/or local inhibition of candidate regulatory factors. Finally, much additional work is also needed to identify dysregulated miRNAs, which are both causative and specific, for diagnosis/prognosis of end-stage clinical sequelae of hypertension, atherosclerosis, myocardial infarction and cardiac hypertrophy.

Conflict of interest

The authors declare no conflicts of interest.

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