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CLINICAL REVIEW

Basic science for the clinician

microRNAs in heart disease: putative novel therapeutic targets?

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microRNAs (miRs) are short, approximately 22-nucleotide-long non-coding RNAs involved in the control of gene expression. They guide ribonucleoprotein complexes that effect translational repression or messenger RNA degradation to targeted messenger RNAs. miRs were initially thought to be peculiar to the developmental regulation of the nematode worm, in which they were first described in 1993. Since then, hundreds of different miRs have been reported in diverse organisms, and many have been implicated in the regulation of physiological processes of adult animals. Of importance, misexpression of miRs has been uncovered as a pathogenic mechanism in several diseases. Here, we first outline the biogenesis and mechanism of action of miRs, and then discuss their relevance to heart biology, pathology, and medicine.

Keywords microRNA • Heart

Introduction

The genomes of eukaryotes intricately encode a wide range of RNA species. The functions of most of these RNAs have been only partially elucidated or are still unknown. Alongside more common-or-garden types such as messenger (m)—or protein-coding—RNA and those with infrastructural roles—such as transfer and ribosomal RNAs—two varieties of non-protein coding RNA, broadly defined as large RNA and small RNA, have emerged over the last few decades (for review see[1]). Of these, much interest has been garnered by a group of small RNAs, called microRNA (miR), because of their role in regulating post-transcriptional gene expression via the repression of targeted mRNAs. In fact, miRs, which are approximately 22 nucleotides long, modulate protein expression by binding to complementary sequences on mRNAs and, in doing so, target them for translational inhibition and/or degradation. Since 1993, when they were described for first in the nemotode worm,[2,3] there has been an acceleration in the publication of reports concerning miRs. These have documented the presence of miRs in a variety of animals, plants, and viruses, their role in developmental biology and their involvement in regulating many physiological processes in the adult organism.[4] Moreover, much research is shedding light on how the dysregulation of miRs is implicated in pathogenesis; miRs are, therefore, potentially important clinically and might one day be used for diagnosis, prognosis, and/or therapy. To date, over 700 miR entries have been registered for humans in the central online database of the Wellcome Trust Sanger Institute.[5]

In the present review, we will first outline the biogenesis and mechanism of action of miRs, and then discuss their relevance to heart biology, pathology, and medicine.

The biology of microRNA

Biogenesis

Two converging pathways have been discovered for the biogenesis of miRs in animals (*Figure 1*). In the first—the canonical pathway—transcription of miR genes yields transcripts, termed primary miRs (pri-miRs), that are up to several thousands of bases long.[6] pri-miRs have a characteristic hairpin morphology, comprising a loop and an imperfectly paired stem incorporating the mature miR sequence on one of the strands near the loop. Transcription of miR genes is polymerase II-dependent[6] (some miRs that are interspersed among repetitive DNA elements, however, are polymerase III dependent)[7] and is regulated by transcription factors (for review see[8]). Many miR genes are polycistronic in that they

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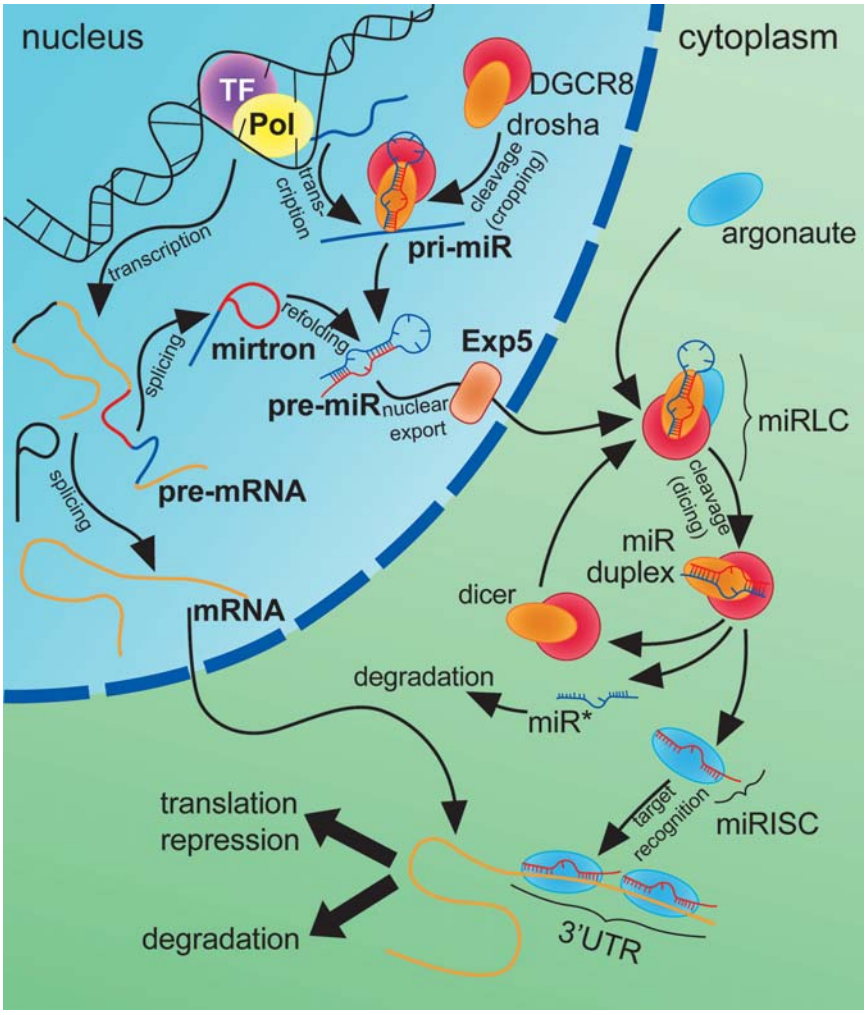

Figure 1 Schematic of microRNA biogenesis and action. See text for explanation. The mature microRNA sequence is given in red. TF, transcription factor; Pol, RNA polymerase II or III; Exp5, exportin 5.

encode two or more stem-loops that can each be processed into distinct mature miRs.[4] As the pri-miR is transcribed, a nuclear enzyme called Drosha—bound to a cofactor, the DiGeorge syndrome critical region 8 (DGCR8)—processes the pri-miRNA by cropping the distal stem portion. Important for their recognition in later processing, cleavage by Drosha introduces staggered cuts on each side of the RNA stem, resulting in a 5′ phosphate and a two-nucleotide overhang at the 3′ end. This produces a shorter hairpin, called precursor miR (pre-miR).[9] The pre-miRs can then be transported to the cytoplasmic compartment of the cell by exportin-5.[10] Final processing is carried out by the miR-induced silencing complex (miRISC)-loading complex (miRLC).[11] The miRLC is an agglomeration of proteins that removes the loop portion of the pre-miR—by an enzyme called Dicer[12]—to form a double-stranded miR duplex, strips away what is called the passenger (or miRNA*) strand from the duplex to leave a mature miR, and transfers the mature miR from Dicer to another protein of the miRLC, called Argonaute (Ago).[13] The effector of miRNA-mediated RNA silencing is the miRISC, composed of the mature miR attached to an Ago protein and a GW182 protein (for a review see[14]).

In 2007, a second pathway was identified in which the miRs—termed mirtrons—derive from introns that are the correct size to form pre-miRs directly.[15,16] The mirtrons are spliced out of their host gene to form looped intermediates (or lariats) that are then debranched and refolded into the usual stem-loop structure of pre-miRs; mirtrons, therefore, bypass the Drosha processing step. From here, mirtrons access the canonical biogenesis pathway described above. To date, only a small number of mirtrons have been found in primates.[17] However, some mammalian mirtrons might have a longer 5′-tail (tailed mirtrons), so the introns that potentially contain this type of miR might be more numerous than first thought.[18] (For an extensive review on animal miR biogenesis, see ref. 19.)

Mechanisms of action

miR-induced silencing complexes act by first binding to sites that seem to be predominantly present on the 3′ untranslated region

(UTR) of mRNAs. The function of the miR is to serve as the target-recognition component of the miRISC[20] because it can bind to a complementary sequence when these are accessible. Binding is thought to be initiated at nucleotides 2–7/8 of the miR, the so-called seed region; the rest of the miR binds imperfectly, creating bulges and mismatches in the miR:mRNA heteroduplex (for a reviews on target recognition, see[21,22]). The great advantage of this system is that a given binding-site sequence can be present on any number of mRNAs, so a single miR sequence is sufficient to target (and regulate) hundreds of mRNAs—which can be part of related processes or pathways—contemporaneously; moreover, in order to target a different set of mRNAs, only the miR needs to be changed while the protein components of the miRISC can remain the same. The efficacy of the system can be regulated through the presence of multiple target sites on a given mRNA's 3′UTR, enabling a number of miRs to bind cooperatively and, thus, effect a stronger action.

Once locked on an mRNA, the proteins making up the miRISC promote downregulation of the protein the mRNA encodes (for extensive reviews, read[23,24]). A number of animal miRs have been reported to promote gene silencing through mRNA degradation.[23,24] However, it has been speculated that translational repression is the default mechanism of miR-mediated repression of gene expression:[25] whether this occurs at the initiation or a post-initiation step is still being debated. In addition, a few reports have described activation of translation by miRs.[26–29] Further studies are needed to ascertain whether the various findings are due to distinct mechanisms, diverse moments of an initial miR-mediated event or experimental artefacts.

Targets of microRNA

Many programs are available online for the prediction of individual targets of miRs (for reviews, see[30,31]). However, the identification of bona fide targets in animals remains problematic because animal miRs bind to mRNA with imperfect complementarity and we still have an only partial understanding of how binding sites are recognized. Thus, some bioinformatically predicted targets turn out to be false and others are overlooked altogether. Experimental validation of targets is, therefore, an important step in defining the functions of individual miRs (for review, see[32]). Unfortunately, the identification of targets within a living system is not without its own problems too, and the data obtained need to be evaluated with care. For example, the use of an artificial reporter constructed with a large number of binding sites and without the other features that probably affect target recognition (such as tertiary mRNA structure and binding sites for other miRs and proteins)[4] can produce an exaggerated, not necessarily physiologic, outcome (e.g. $<$30-fold change), whereas the effect of the cognate miR on the expression of a given native protein may in reality be relatively quite small (e.g. less than four-fold change).[33] Also, because a given miR might target multiple mRNAs to produce a discernible phenotype, an effect studied on any single protein might be relatively unimportant on its own.[33,34] Moreover, targets validated through the overexpression of ectopically introduced miRs are not necessarily authentic biological targets because of, for example, the presence of differentially expressed UTR-binding proteins that block the formation of the heteroduplex in another cell type.[34]

microRNA and the heart

Cardiac-expressed microRNAs

Of the thousands of miRs described to date in humans and other animals, many exhibit tissue-specific patterns of expression.[35] Because of their ability to target classes of mRNAs that direct cell proliferation, differentiation, and programmed death,[36] tissue-specific miR expression gave rise to the idea that they would play a major role in tissue differentiation and organ development. Although this was shown to be correct in specific instances, i.e. in muscle, miR-1 and miR-133a are co-expressed in response to several myocyte differentiation factors,[37,38] it has not held up as a general rule. Instead, there appear to be ~150–200 cardiac-expressed miRs, many of which are dynamically regulated in response to acute cardiac stress, and in some instances during the long-term compensatory response of the heart to chronic injury or haemodynamic overload. Thus, there is increasing evidence that modulated miR expression is an important part of the acute stress-response mechanism of the heart, and that this additional level of regulatory complexity contributes both to cardiac homeostasis in health, and to myocardial pathology in disease.

Of the miRs expressed in the heart, some are either enriched in or specific to this organ. miR-1, miR-133, miR-206, and miR-208 have been found to be particularly important for muscle. The miR-1 family comprises the miR-1 subfamily and miR-206, the latter expressed in skeletal but not cardiac muscle. The miR-1 subfamily consists of two identical transcripts, miR-1-1 and miR-1-2, which are differentiated by the addition of the numerical suffix because they are encoded on different chromosomes (ch2 and ch18, respectively). The miR-1 members are expressed from bicistronic units together with members of the miR-133 family, which comprises miR-133a-1, miR-133a-2 and miR-133b (the lettered suffix denotes that the miRs differ at only one or two positions). Only one miR, miR-208, is acknowledged as being cardiac-specific. It is encoded in an intron of the *alpha myosin heavy chain* gene. Other miRs that might be potential cardiac-enriched miRs include miR-128, miR-302, miR-367, and miR-499.[39] The most recent data on the miR profile of adult mouse heart, determined by sophisticated and highly sensitive RNA resequencing, is reported by Rao *et al.*[40]

Experimental validation of cardiovascular-related miR targets has only recently got under way, and only a few targets have been described as pertinent to the cardiomyocyte to date: these have been reported to be involved in the regulation of various aspects such as heart development,[41] hypertrophic cardiac growth,[42–47] electrophysiology,[48–50] apoptosis,[51–54] and metabolism[55] (*Table 1*). Moreover, miRs and relative targets are being uncovered for the other cells making up the cardiovascular system, such as fibroblasts,[56–59] endothelial cells,[60] and smooth muscle cells,[61–65] which cannot be overlooked when studying the physiology of the cardiovascular system or its response to stress.

The importance of microRNA biogenesis to the developing and adult heart

Dicer splicing of mature miRs from pre-miRs is the penultimate step of miR biogenesis, and is common to all miRs. For this

Table 1 **Cardiomyocyte-expressed microRNAs with experimentally verified targets**

microRNA	Setting (model[a])	Dysregulation	Target(s)	Effect of miR on
miR-1[42]	Hypertrophy (TAC); iCMP (h)	Down	*Cdk9, Rheb, RasGAP, Fibronectin*	Hypertrophy (−)
miR-1[47]	Hypertrophy (TAC); acromegaly (h)	Down	*IGF1*	Hypertrophy (−)
miR-1[51]	Oxidative stress (H9c2)	Up	*HSP60, HSP70*	Apoptosis (+)
miR-1[54]	Oxidative stress (H9c2)	Up	*Bcl-2*	Apoptosis (+)
miR-1[48]	Various	—	Sarcolemmal channel protein mRNAs	Electrophysiology
miR-1[50]	miR overexpression (ARVC)	Up	*B56α*	Sarcoplasmic reticulum Ca^{2+} release (+)
miR-1-2[37]	miR deletion (miR-1-2 KO)	Down	*Irx*	Repolarization (I_{to})
miR-21[52]	Oxidative stress (NRVC)	Up	*PDCD4*	Apoptosis (−)
miR-23a[44]	Hypertrophy (NRVC)	Up	*MuRF1*	Hypertrophy (+)
miR-30c[56]	Hypertrophy (Ren2); AS (h)	Down	*CTGF*	Fibrosis (−)
miR-133[45]	Hypertrophy (TAC) (training) (Akt-Tg); iCMP (h)	Down	*Rho-A, NELF, Cdc42*	Hypertrophy (−)
miR-133[51]	miR Overexpression (H9c2)	Up	*Caspase9*	Apoptosis (−)
miR-133[48]	Various	—	Sarcolemmal channel protein mRNAs	Electrophysiology
miR-133[56]	Hypertrophy (Ren2); AS (h)	Down	*CTGF*	Fibrosis (−)
miR-133[55]	miR downexpression (ARVC); hypertension (Dahl rats)	Down	*KLF15*	Glucose uptake (+)
miR-208[46]	Hypertrophy (miR-208 KO)	Down	*THRAP-1*	Contraction (+)
miR-208[49]	miR overexpression (Tg)	Up	*THRAP-1; Myostatin*	Hypertrophy (+)
miR-320[53]	Ischemia/reperfusion (rat)	Down	*HSP20*	Apoptosis (+)

TAC, transverse aortic constriction; iCMP, idiopathic cardiomyopathy; h, human; CaN calcineurin; Tg, transgenic; H9c2, rat embryonic ventricle-cell line; A/NRVC, adult/neonatal rat ventricular cardiomyocytes; KO, knockout; Ren2, homozygous rat model of hypertension-induced heart failure; AS, aortic stenosis; (−), anti; (+), pro.
[a]Mouse if not otherwise stated.

reason, experimental manipulation of Dicer expression has afforded an opportunity to examine the consequences of global miR perturbations on cardiac formation and function. The results provide insight into the distinct roles of regulated miR expression during embryonic development and in the physiologically stressed adult heart.

Sophisticated molecular techniques have been employed to ablate the *Dicer* gene from mouse hearts at various times during the life of the organism. Cardiac-specific *Dicer* ablation is necessary because somatic *Dicer* ablation produces early embryonic lethality due to an apparent defect in gastrulation.[66] Accordingly, mice carrying floxed *Dicer* genes have been bred to three different cardiac-specific Cre mice to specifically ablate *Dicer* from cardiomyocytes at different times or under different experimental conditions: early in the developing heart (Nkx-2.5 Cre)[37]; shortly after birth (αMHC-Cre)[67]; and either in 3-week-old or adult mice (tamoxifen-inducible αMHC Cre).[68] Specific details of the resulting phenotypes can be found in the respective reports and have recently been summarized.[69] Because Dicer deletion may have effects that are not specifically a consequence of interfering with miR biogenesis, the parallel approach of conditionally ablating *DGCR8*, also important for miR production, was recently employed.[40] In this case, the use of a muscle-specific Cre driver that is most active after birth to ablate *DGCR-8* again resulted in progressive cardiomyopathy. The importance of these collective findings is that interference with miR biogenesis at any time from very early in heart development to the fully developed adult heart results in catastrophic cardiac failure, with induction of pathological cardiac genes, loss of normal sarcomeric organization, and cardiomyocyte hypertrophy and/or apoptosis. These results support a critical role for miRs not only in growing or developing hearts but also in maintaining normal cardiac homeostasis. Thus, it has been important to examine the pattern of miR expression and regulation in adult heart disease, as described in what follows.

microRNA and heart pathology

microRNA signatures in experimental heart disease

Most of the existing *in vivo* basic research describing patterns of cardiac miR expression, and elucidating their individual effects, has been performed using mice. Mice are excellent models because their genes (and miRs) are readily manipulated, the anatomy of the cardiovascular system is similar to that of humans, routine physiological analysis is possible using echocardiography, magnetic resonance imaging, and invasive catheterization-based haemodynamic measurements, and the time course of cardiac disease progression is compressed in comparison with larger animal models.

There are two broad classes of models of experimental murine heart disease: genetic and physiological. In genetic models, a critical causal factor is overexpressed under control of the promoter from a cardiac-specific gene, typically the αMHC promoter. Because expression of the transgenic factor is driven in a cardiomyocyte-specific manner, the molecular phenotype (including mRNA and miR expression profiles) is generally assumed to be the direct consequence of the transgene rather than of a collateral systemic event. Thus, *genetic mouse models have the advantage of revealing cardiomyocyte autonomous changes*. And as with conventional hypertrophy and heart failure, different genetic models produce different 'flavours' of disease. In miR profiling studies, there are substantial data from two genetic models: Akt transgenic mice that produce 'physiological hypertrophy', similar to a trained athlete's heart that does not progress to failure;[45] and calcineurin (CaN) transgenic mice, which produce 'pathological hypertrophy' that more resembles pressure overload hypertrophy and that can progress to heart failure.[70] There are also data from pressure overload hypertrophy that occurs in response to surgical banding of the aorta,[42,71,72] and myocardial infarction produced by surgical ligation of the left anterior descending coronary artery.[57] These models have the advantage of being more relevant to human pressure overload and myocardial infarction, but the data interpretation can be confounded by non-cardiac effects of the physiological manipulations.

miRs reported to be up- or down-regulated in the various mouse models described earlier are listed in *Table 2*. There are a few notes of caution before broad generalizations can be derived from these cumulative expression profiling data. First, the data need to be interpreted in the context of evolving platforms for assaying miR levels, and the rapid expansion of recognized miRs. Older studies assayed a limited number of miRs compared with more recent studies. Second, a cardiac-expressed miR is not necessarily a cardiomyocyte-expressed miR. Interventions, such as pressure overloading and myocardial infarction that affect the entire heart including interstitial cells, the coronary macro- and micro-vasculature and resident or migratory inflammatory cells, will not produce the same RNA expression signatures as genetic manipulations that directly affect only cardiomyocytes. Finally, genetic models are artificial and even experimental pressure overloading and myocardial infarction are acute interventions that do not precisely mimic the chronically progressive conditions seen in human diseases with the same names. Thus, the miR expression profiling data from mouse models identify a large number of miRs that are expressed in the heart at relatively high levels, and a subset of these that are subject to dynamic regulation under conditions of myocardial stress or injury. If associations between these experimentally regulated miRs and their human counterparts were also observed in clinical heart disease, the implication would be that cross-species conservation of regulation would more strongly suggest biological relevance.

microRNA signatures in clinical heart failure

As with mouse cardiac models, microarrays have been used by a number of groups to profile miR expression signatures in human heart disease, most commonly dilated or ischaemic cardiomyopathies. The initial report of miR profiling in human heart disease was by Thum *et al.*, who compared mRNA and miR expression signatures from four non-failing and six failing hearts.[73] The Ambion miR microarrays contained probe sets for 384 miRs. The authors noted significant (defined as $P < 0.05$, >1.5-fold increase or decrease) upregulation of 67 miRs, with downregulation of 43 miRs, in the failing vs. control hearts (*Table 3*). The miRNA expression signature was similar to that of ($n = 6$) fetal hearts, suggesting that both mRNA and miR expression in heart failure partially recapitulates that of the embryonic heart.

Table 2 microRNA expression and regulation in experimental models of heart failure

Upregulated microRNAs			
miR-10a[57]	miR-92b[57]	miR-199a[42,70]	miR-352[57]
miR-10b[57,70]	miR-103[42,57]	miR-199a*[42,70]	miR-365[57]
miR-15b[42,57]	miR-106a[72]	miR-199a-3p[57]	miR-379[57]
miR-16[57]	miR-107[42,57]	miR-199a-5p[57]	miR-483[57]
miR-17-5p[72]	miR-125b[42,70,72]	miR-199b[42]	miR-497[57]
miR-18b[72]	miR-126[70]	miR-199b*[57]	miR-574-5p[57]
miR-19a[67]	miR-127[42,57]	miR-200a[72]	miR-638[57]
miR-19b[72]	miR-132[57]	miR-208[72]	miR-705[57]
miR-20b[72]	miR-140[42,72]	miR-210[70,72]	miR-711[57]
miR-21[42,57,70,72]	miR-140*[42,57]	miR-211[70]	miR-739[57]
miR-23a[42,70]	miR-142-3p[72]	miR-214[42,57,70]	miR-762[57]
miR-23b[42,70]	miR-146a[57]	miR-217[70]	miR-923[57]
miR-24[42,70]	miR-146b[57]	miR-218[57,70]	let-7b[42,57]
miR-25[70]	miR-153[72]	miR-221[42,57,72]	let-7c[42]
miR-26b[57]	miR-154[70]	mR-222[42,57,72]	let-7d[57]
miR-27a[42,70]	miR-155[57]	miR-223[57]	let-7e[57]
miR-27b[42,70]	miR-184[72]	miR-330[70]	let-7g[57]
miR-31[42]	miR-195[42,70]	miR-335-5p[57]	let-7h[57]
miR-34a[57]	miR-199*[57]	miR-351[42,57,70]	let-7j[57]
Downregulated microRNAs			
miR-1[42]	miR-30a-3p[42]	miR-93[70]	miR-155[42]
miR-10a[42]	miR-30a-5p[42]	miR-133a[70]	miR-181b[70]
miR-10b[42]	miR-30b[42,72]	miR-133b[70]	miR-185[42]
miR-26a[42]	miR-30c[42,72]	miR-139[42]	miR-194[42]
miR-26b[42]	miR-30d[42]	miR-149[42]	miR-218[42]
miR-29a[42]	miR-30e[42,70]	mR-150[42,70,72]	miR-378[42]
miR-29c[42,70]	miR-30e*[42]	miR-151[42]	let-7d*[42]
Non-regulated microRNAs			
miR-15a[71]	miR-125a[71]	miR-148a[71]	miR-451[71]
miR-22[71]	miR-126-3p[71]	miR-152[71]	miR-486[71]
miR-29b[71]	miR-126-5p[71]	miR-181a[71]	let-7a[71]
miR-98[71]	miR-143[71]	miR-191[71]	let-7f[71]
miR-99a[71]	miR-145[71]	miR-341[71]	let-7i[71]

In a substantially larger clinical study published just months later, Ikeda *et al.*[74] described the miR profiles of human dilated cardiomyopathy ($n = 25$), ischaemic cardiomyopathy ($n = 19$), and pressure overload hypertrophy (aortic stenosis, $n = 13$), compared with that of 10 normal hearts. Their assay measured levels of 428 individual miRs using a high-throughput bead-based platform[75] that detected 87 cardiac-expressed miRs, 43 of which were regulated in at least one of the disease groups ($P < 0.05$, FDR $< 5\%$) (*Table 3*). Importantly, the miR expression profile appeared to be distinguishable between disease groups, and within the primary data set the miR signature was able to predict the diagnosis with an accuracy rate that approached 70%.

A similarly designed study by Sucharov *et al.*[76] compared miR expression in five ischaemic and five non-ischaemic cardiomyopathic hearts, compared with six non-failing hearts. The assay used a microarray containing probes for 470 miRs. Thirty-three miRs were reported as regulated in either ischaemic and/or non-ischaemic cardiomyopathic hearts ($P < 0.1$) (*Table 3*), several of which were shown to have measurable effects in cultured neonatal rat ventricular cardiomyocytes. Naga Prasad *et al.*[77] used a custom microarray to identify eight miRs (seven of which had been previously identified in human or mouse heart failure) that were upregulated in 50 heart failure cardiac samples, compared with twenty non-failing specimens, and independently validated the associations in 20 dilated cardiomyopathy and 10 non-failing samples. Taken together, these studies support the idea that miR regulation may be sufficiently distinct in different forms of cardiac injury to be able to discriminate between heart failure of ischaemic vs. non-ischaemic aetiology.

In a recent study, Matkovich *et al.*[78] examined whether miRs that were regulated in heart failure would be normalized by left-ventricular assist device (LVAD) therapy, i.e. they measured the sensitivity of miR dynamism to functional changes in the failing heart. miR and mRNA expression profiles were generated for 17 cardiomyopathic hearts not treated with LVADs, 10 hearts on treatment with LVADs and 11 non-failing control hearts. The Invitrogen miR array contained probe sets for 467 miRs, of which 81 were confidently expressed in hearts. Of these, 28 were upregulated in cardiomyopathic hearts ($P < 0.001$, greater than two-fold increase), with three others showing strong trends ($P < 0.01$) (*Table 3*). The most interesting finding was that 20 of the 28 upregulated miRs were fully normalized in LVAD-supported hearts, and the other eight decreased towards normal. A limited qPCR study of four miRs also found that LVAD treatment normalized abnormal expression, but suggested that this effect was more pronounced in ischaemic cardiomyopathy.[79] The remarkable sensitivity of the miR signature to biomechanical support, which is known to favourably effect both cardiac performance and remodelling, parallels regulated expression of Dicer, the critical enzyme for processing to mature miRs (see above). Dicer levels are decreased in human heart failure, but normalized by LVAD treatment.[67] It is possible that acute regulation of Dicer expression in human heart failure plays a role in the dynamic expression of members of the heart failure miR program, but mechanistic linkage between the two processes has not yet been established.

microRNAs in myocardial ischaemia and vascular diseases

miR plays an important role in regulating endothelial homeostasis, and angiogenesis *in vivo*. For example, targeted deletion of the endothelial cell-restricted miR miR-126 causes leaking of vessels, haemorrhaging and partial embryonic lethality, due to a loss of vascular integrity and defects in endothelial cell proliferation, migration, and angiogenesis. Mutant surviving mice display defective cardiac neovascularization following myocardial infarction. A link was demonstrated between miR-126 and VEGF and FGF in that it increases the pro-angiogenic effects of these two cytokines.[80,81] Evidence of the potentially relevant role of miRs in vascular diseases was also provided by the miR-17approximately92 cluster, which is highly expressed in endothelial cells; miR-92a, a component of this cluster, controls the growth of new blood vessels.[82] Forced overexpression of miR-92a in endothelial cells blocked angiogenesis *in vitro* and *in vivo*. In mouse models of limb

Table 3 microRNA expression and regulation in human heart disease

Upregulated miRNAs			
miR-1[77]	miR-125b[74,77]	hsa-miR-29a[73]	hsa-miR-525-AS[73]
miR-15a[77]	miR-126[77]	hsa-miR-32[73]	let-7b[74]
miR-15b[74]	miR-130a[77]	hsa-miR-34b[73]	let-7c[74]
miR-16[77]	miR-133a[77]	hsa-miR-100[76]	let-7e[74]
miR-21[77]	miR-133b[77]	hsa-miR-125a[73]	let-7f[77]
miR-22[77]	miR-140*[74]	hsa-miR-125b[76]	let-7g[77]
miR-23a[74,77]	miR-143[77]	hsa-miR-126-AS[73]	let-7i[77]
miR-24[74,77]	miR-145[74]	hsa-miR-130a[73]	hsa-let-7a[73]
miR-26a[77]	miR-181a[74]	hsa-miR-132[73]	hsa-let-7c[73]
miR-26b[76,77]	miR-191[74]	hsa-miR-181b[76]	hsa-let-7d[73]
miR-27a[74,77]	miR-195[74,77]	hsa-miR-195[76]	hsa-let-7e[73]
miR-27b[74,77]	miR-199a*[74]	hsa-miR-199[76]	mmu-miR-17-3p[73]
miR-28[76]	miR-199a-3p[77]	hsa-miR-212[73]	mmu-miR-215[73]
miR-29a[77]	miR-214[74]	hsa-miR-213[73]	mmu-miR-292-3p[73]
miR-29b[77]	miR-320[74]	hsa-miR-302a[73]	mmu-miR-295[73]
miR-30a-5p[77]	mR-342[74,76]	hsa-miR-320[73]	mmu-miR-297[73]
miR-30b[77]	miR-378[77]	hsa-miR-365[73]	mmu-miR-322[73]
miR-30c[77]	miR-423*[74]	hsa-miR-372[73]	mmu-miR-330[73]
miR-30d[77]	miR-499[77]	hsa-miR-373[73]	mo-miR-297[73]
miR-93[74]	miR-638[77]	hsa-miR-382[73]	mo-miR-333[73]
miR-99b[74]	hsa-miR-1[73]	hsa-miR-423[73]	
miR-100[74]	hsa-miR-21[73]	hsa-miR-424[73]	
mR-103[74,77]	hsa-miR-23a[76]	hsa-miR-429[73]	
Downregulated miRNAs			
miR-1[74,76]	miR-126[74]	hsa-miR-30c[76]	hsa-miR-486[73]
miR-10a[74]	miR-126*[74]	hsa-miR-92[76]	hsa-miR-494[73]
miR-17-5p[74]	miR-222[74,76]	hsa-miR-133a[76]	hsa-miR-515-5p[73]
miR-19a[74]	miR-224[76]	hsa-miR-133b[76]	hsa-miR-520d-AS[73]
miR-19b[74]	miR-374[74]	hsa-miR-139[76]	hsa-miR-594[76]
miR-20a[74]	miR-451[74]	hsa-miR-150[76]	let-7a[76]
miR-20b[74]	miR-484[76]	hsa-miR-182[73]	let-7c[76]
miR-26b[74]	miR-499[74]	hsa-miR-197[76]	let-7d[76]
miR-28[74]	hsa-miR-10b[76]	hsa-miR-221[76]	let-7f[76]
miR-30e-5p[74]	hsa-miR-20a[76]	hsa-miR-422b[74,76]	
miR-101[74]	hsa-miR-22[76]	hsa-miR-452-AS[73]	
miR-106a[74]	hsa-miR-30a-5p[73]	hsa-miR-483[76]	
Non-regulated miRNAs			
miR-23b[77]	miR-148a[77]	miR-208[77]	miR-452[77]
miR-29c[77]	miR-151[77]	miR-221[77]	miR-487b[77]
miR-30a-3p[77]	miR-152[77]	miR-223[77]	miR-520h[77]
miR-98[77]	miR-185[77]	miR-324-5p[77]	miR-652[77]
miR-99a[77]	miR-188[77]	miR-337[77]	miR-663[77]
miR-107[77]	miR-198[77]	miR-361[77]	
miR-125a[77]	miR-199a-5p[77]	miR-377[77]	
miR-146a[77]	miR-199b[77]	miR-422a[77]	

ischaemia and myocardial infarction, administration of an antagomir designed to inhibit miR-92a led to enhanced blood vessel growth and functional recovery of damaged tissue. miR-92a appears to target mRNAs corresponding to several pro-angiogenic proteins, including the integrin subunit alpha5. Thus, miR-92a may serve as a valuable therapeutic target in the setting of ischaemic disease.[83]

The role of miRs in cardiovascular diseases has been recently further confirmed by studying their expression in the smooth muscle cell (SMC) compartment. For example, the miR-143/145

cluster has been demonstrated to be specifically expressed in SMCs;[61–65] their expression is controlled by SRF and is decreased during acute (re-stenosis) or chronic (atherosclerosis) stress. Knockout of miR-143/145 induces defects in SMC terminal differentiation which is reflected by a decreased capacity for vasoconstriction after vasopressor challenge.[61,63,64] The cytoskeletal apparatus is particularly affected by the knockout of miR-143/145. Smooth muscle cell proliferation and migration seem also to be regulated by the miR-143/145 cluster.[61–65]

The tenuous relationship between mRNA and microRNA signatures

Given that miRs exert their effects by directing specific target mRNAs to miRISCs for degradation, it has been widely assumed that comparative miR and mRNA profiling informed by bioinformatics identification of consensus miR binding sequences would link regulated miRs with their relevant mRNA targets. However, this has not generally been the case. The two human studies in which comprehensive mRNA and miR signatures were obtained in the same clinical cardiac samples failed to reveal clear reciprocal relationships between upregulated miRs and downregulation of their putative mRNA targets.[73,77] Nevertheless, miRs have important pathophysiological effects on cardiomyocytes, as shown by *in vitro* and *in vivo* manipulation.[45,70] We believe that the answer is suggested by accumulating data suggesting the major effect of miRs in mammalian systems might not be mRNA destabilization, but rather translational suppression.[33] If this is the case, mRNA profiling can identify only those mRNA targets of miRs that are, directly or indirectly, destabilized, and will miss the majority of bona fide targets that undergo translational inhibition without altering mRNA levels. This situation requires a different approach to connect specific miRs and their mRNA targets, such as profiling mRNAs from the RISCs of cells or model organisms in which specific miRs are overexpressed.[84]

Implications of microRNA for clinical practice

From mRNA to microRNA expression profiling in cardiac disease

A mechanistic role for altered mRNA expression levels in heart disease has been recognized for many years.[85] A few hallmark genes are regulated in virtually every clinical and experimental model of cardiac hypertrophy and/or heart failure. The most sensitive transcriptional marker for heart failure is increased cardiomyocyte expression of mRNAs for the atrial and brain natriuretic peptides, ANF and BNP. On the other hand, cardiomyocyte hypertrophy is indicated by a redistribution of myosin heavy chain isoform mRNA from alpha (α-MHC) to beta (β-MHC). Transcriptional upregulation of natriuretic peptides and β-MHC is observed in cultured cardiac myocytes induced to undergo hypertrophy, in genetic rodent models of cardiac hypertrophy and cardiomyopathy, in rabbit, dog, and porcine experimental models of surgically induced cardiac disorders, and in the analogous human diseases. Thus, a conserved transcriptional signature for heart disease appears to be a nearly universal response, and in many instances the individual regulated transcripts have been mechanistically connected to specific pathological features of hypertrophied and failing myocardium.[86] However, because end-stage cardiomyopathy combines features of heart failure with cardiomyocyte hypertrophy, ANP/BNP and β-MHC are typically increased together with many other members of the so-called 'fetal gene program' in fully developed adult cardiomyopathies. Combinatorial regulation of many mRNAs in cardiac disease diminishes the specificity of the response for a particular condition. For example upregulation of ANP along with β-MHC in a cardiomyopathic heart does not provide data as to whether cardiomyocyte hypertrophy led to heart failure, as in severe hypertension, or is part of a compensatory response to primary myocardial damage, as after myocardial infarction. Furthermore, since heart failure is the common terminal condition that results from irreparable myocardial damage of any cause, the transcriptional profile of late heart failure provides little insight into specific aetiology (ischaemic, viral, alcoholic) or information about likely prognosis.[87] For these reasons, mRNA profiling has not transitioned from the research laboratory to routine clinical practice.[88] There is, however, tremendous interest in determining whether dynamic regulation of cardiac-expressed miRs could prove more useful than mRNA profiling as a molecular signature for specific cardiac syndromes. With the development of faster and cheaper high-throughput technologies, this could be a particularly exciting prospect for diagnosis and prognostication in cardiology.

Modulation of dysregulated microRNAs

That the misexpression of an miR could be involved in a pathogenic mechanism was reported first for leukaemia[89] and then for many other pathologies.[36] This has spurred the setting up of biotech companies with the aim of developing miR-based drugs.[90] The therapeutic strategy would be directed at normalizing miR expression, silencing those that become inappropriately overexpressed or replacing those that become downregulated (*Figure 2A*).

Administration of a single-stranded oligonucleotide that is antisense to a disease-upregulated miR (anti-miR oligonucleotides or AMOs), for example, could act as a competitive inhibitor and determine the upregulation of sets of proteins. Chemical modification is necessary to improve the pharmacokinetic properties of oligonucleotide (for review see[91]). One modification involves conjugation with cholesterol, which enhances transport across cell membranes, to form so called antagomiRs:[92] these have been used successfully in *in vivo* models to inhibit miRs and produce a relevant pharmacological effect on the heart.[45,53,58] Two or more identical miR-binding sites can be synthesized in series in order to sequestrate a larger number of miRs (erasers[93] or sponges[94]). If these are constructed from partially complementary sequences harbouring a seed sequence, they can be used to sequester all the related miRs of an miR family. Moreover, sponges have been synthesized that target different miRs contemporaneously: these multiple-target AMOs (MTg-AMOs) were shown to be more effective than using mixtures of AMOs targeting individual miRs separately.[95] On the other hand, miRs that have been downregulated by disease might be pharmacologically

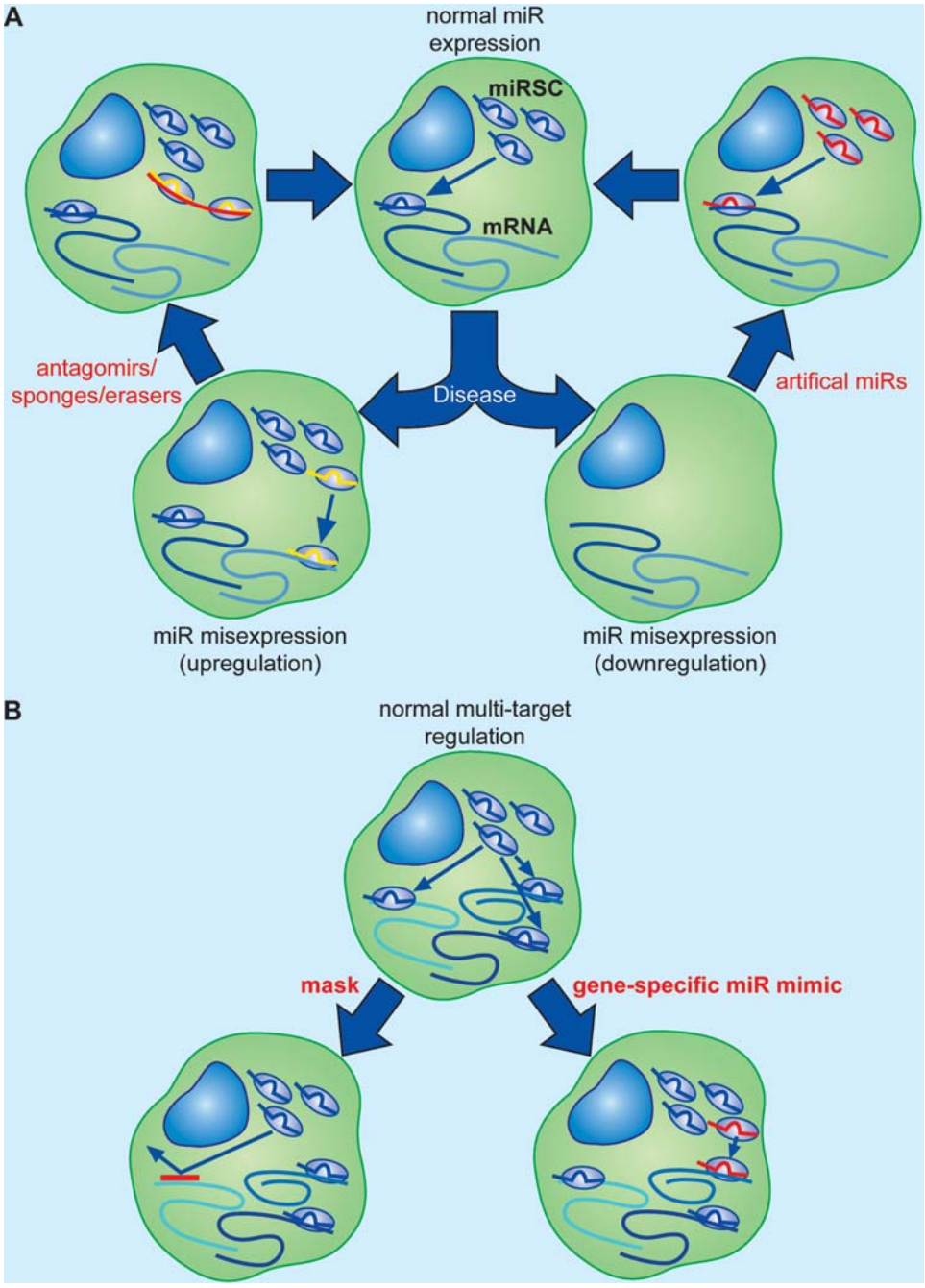

Figure 2 Schematic overview of strategies used to alter microRNA expression. (*A*) Cells express a microRNA profile that can become altered with disease. Antisense oligonucleotides, such as antagomirs, sponges, and erasers (in red) can capture microRNAs for knockdown or sequestrate inappropriately overexpressed microRNAs, whereas artificially introduced microRNAs (in red) can be used to overexpress microRNAs or, potentially, to replace expression of downregulated ones. These strategies have the potential to affect large numbers of different targets (for simplicity, only one target mRNA per microRNA is represented). (*B*) Masks and gene-specific microRNA mimics (in red) can be used to affect single targets specifically (mRNAs in different shades of blue represent a set affected by a given microRNA).

re-expressed via the introduction of precursors, or of their coding sequences, that co-opt the miR biosynthetic machinery.

The potential advantage of targeting, or artificially re-expressing, miRs directly lies in the potential to restore the expression of hundreds of dysregulated mRNAs to their pre-pathological level in one go and, in doing so, hopefully reverse disease. However, miR-based approaches might also be taken advantage of to affect only one target should this be deemed desirable (*Figure 2B*). By identifying an accessible and suitably long sequence on the 3′UTR that is unique to the gene of interest, an oligonucleotide precursor can

be synthesized to have a sequence that binds only to a given mRNA and to effect translation inhibition only of this specific mRNA (gene-specific miR mimics).[96] A sequence can also be generated in such a way as to occupy an miR-binding site of a single cognate mRNA without being first incorporated into an miRISC: these miR masks specifically impede the action of the miRISC on that specific mRNA without affecting any actions on other cognate mRNAs.[96]

Conclusions

Our understanding of miR has come a long way in a short period of time. miRs have emerged as important controllers of gene expression, as pathogenic triggers when misexpressed and as potential targets of therapeutic interventions. However, the biology of miR is complex and has not been fully clarified: it is probable that not all miRs have been discovered yet and, even if they have been, we are far from having a definite picture of the mRNAs they regulate; how miRs interact with transcription factors and how they themselves are regulated, modified, and degraded are still being defined. Moreover, miRs have been linked to gene silencing at the transcriptional level[97] and other, as yet unknown, functions may still be discovered.

Before miRs become useful clinically for heart disease diagnosis/prognosis, it will be necessary to obtain standardized and detailed expression profiles of healthy and diseased tissue. A parallel advance in procedures capable of analysing large numbers of miRs quickly and economically will be needed too. The use of miRs for therapy is also not still without problems. We have given only a brief outline of the techniques that can be used at the moment to modulate miR expression. Many of the above strategies have not been extensively tested yet in *in vivo* heart disease models, let alone in humans. They remain experimental techniques useful for the study of miRs. Moreover, the currently available antisense approaches—to transiently deliver preformed, short RNA sequences—or gene therapy technologies—to stably introduce sequences that encode miR-precursor transcripts—will need to be developed and adapted. Furthermore, because of the multiple miRs involved, cardiovascular diseases may turn out to be more problematical to treat than, for example, tumours that can arise from the misexpression of single oncogenic miRs. Therefore, hurdles, such as better understanding of the function of individual miRs and adequate, efficient, safe, and standardized delivery of oligonucleotide sequences to the heart parenchyma remain to be overcome. With these advances, miR-based diagnosis and therapy will, hopefully, become a reality for clinical cardiology.

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