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MicroRNAs in Cardiac Disease

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Abstract

MicroRNAs (miRs) are transcriptionally-regulated single-strand RNAs that depress protein expression through post-transcriptional mRNA silencing. A host of recent studies have established essential roles for miRs in cardiac development and cardiac health. Regulated myocardial miR expression is seen in a variety of cardiac syndromes, and serum miR levels are being evaluated as disease biomarkers. Manipulation of miR levels in mouse hearts using genetic techniques or engineered miR mimetics and antagonists is elucidating the roles of specific cardiac miRs in cardiac development, the cardiac response to injury or stress, and heart disease. Targeting of multiple factors within a single biological response pathway by a given miR has prompted development of small miR-targeting molecules that can be readily delivered and have sustained in vivo effects. These advances establish a foundation for novel diagnostics and new therapeutic approaches for myocardial infarction, cardiac hypertrophy, and heart failure.

Introduction: miRs and their mRNA targets

Transcriptional mechanisms regulating gene expression during cardiac development and in the adult heart after physiological stress or injury are well established ^{1, 2}. Less progress has been made elucidating post-transcriptional control mechanisms, such as differential mRNA splicing and translational suppression. The concept that protein expression can be modulated at the stage where protein is translated from mRNA derives in part from early work in *Drosophila* showing regulation of heat shock protein Hsp70 by targeted deadenylation and destabilization of its mRNA transcript ³. A decade ago, studies in *Drosophila* and mammalian cells showed that introduction of small RNA duplexes (RNAi) could induce post-transcriptional gene silencing via these same mechanisms ^{4, 5}, and other studies identified endogenous microRNAs (miRs) performing similar functions in yeast and *Drosophila* ^{6–8}. Shortly thereafter it was determined that miRs must be incorporated into Argonaute protein-containing RNA silencing complexes to target mRNAs for translational suppression ^{9, 10}.

Now, miRs are a major investigative focus in cardiovascular research. In the 5 years since Srivastava and colleagues began to describe roles for specific miRs in cardiac development ¹¹ and the 4 years since van Rooij, Olson, and co-workers published two seminal papers describing pathological roles for variably expressed miRs in cardiac hypertrophy and failure ^{12, 13}, there have been over 350 published articles examining miRs in the heart; >120 of

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these during calendar year 2010 alone. Such excitement is engendered in part by the realization that miRs comprise a different type of biological control mechanism with unique pathological effects and therapeutic implications. Improved understanding of miR biology is prompting a re-evaluation of older concepts that protein content is largely determined by transcriptional mechanisms that regulate RNA production from DNA templates (DNA>RNA>protein). While it is true that tissue- and context-specific protein activators and suppressors of transcription (transcription factors) regulate gene expression (and therefore protein make-up) in health and disease, we now know that these mechanisms are further modified by miRs that modulate protein translation from messenger RNAs. Since miRs are themselves transcribed from DNA, and their expression is subject to transcriptional control, miR effects on translation comprise a means of fine-tuning protein expression via complex feed-back, feed-forward, and cross-talk regulatory pathways. A metaphor for the different roles of transcriptional and miR regulation is the modern propeller-driven airplane. The engine (like transcriptional control) is the overall determinant of plane function; idle when parked, full throttle for take-off, and cruise for normal flight. Varying the pitch of the propeller blades (like miR-regulation of translation) is then used to provide fine control over airspeed while the engine continues to operate at its most efficient cruising RPM.

Here, major findings describing miR expression and function in adult cardiac disease are reviewed, emphasizing new data, existing challenges, and translational applications. For aspects of miR biology primarily related to cardiac development, the interested reader is referred to Liu and Olson's recent excellent review ¹⁴.

A brief overview of miR biology

MicroRNAs (miRs) are small (~18-25 nucleotide) non-coding single-stranded RNAs that regulate protein expression of target mRNAs having complementary sequences, typically in their 3' untranslated regions. Analysis of sequences within the human genome predicts over 1,000 different miR genes, located either in the introns of protein-coding genes (so-called "miRtrons") or as independent entities in the spaces between genes. The location of miR genes is an important determinant of their expression and regulation: When they are located within a parent gene, primary miR biogenesis is controlled by the same transcriptional mechanisms as the parent gene mRNA (although miR and parent mRNA splicing and stability may differ). In contrast, an independent miR gene will have its own transcriptional controls. In either case, the long primary nuclear miR transcript ("pri-miR") undergoes splicing by the RNase-III Drosha/Dgcr8 enzyme complex, generating a characteristically hair-pin folded precursor miR ("pre-miR") that is exported by exportin-5 from the nucleus (Figure 1a) ¹⁵. Cytoplasmic pre-miRs are cleaved by a Dicer-containing complex ¹⁶ to generate a double-stranded miR-miR* duplex containing the mature miR on one strand and a complementary non-functional miR* on the other. In some instances, the miR duplex contains two complementary functional miRs, designated 3p- and 5p- based on their positions within the pre-miR. In either case, the mature single-strand miR is loaded by an as-yet unidentified protein homologous to *Drosophila* R2D2 and *Caenorhabditis* RDE-4 ¹⁷ into an Argonaute protein-containing RNA-induced silencing complex (RISC), the site of translational repression, where it can be presented to target mRNAs having complementary sequences (Figure 1b).

Because Drosha and Dicer nucleases specifically impact miR processing, some early studies examining the impact of miRs on the heart used the approach of ablating these miR-processing enzymes, thus generally diminishing all miR production. Interrupting miR formation at its proximal step by striated muscle-specific ablation of DiGeorge syndrome critical region 8 (Dgcr8), which participates with Drosha in intra-nuclear miR processing, induced left ventricular remodeling that progressed to lethal heart failure and suggesting a

broad requirement for miRs in cardiac development and homeostasis¹⁸. The first studies to interrupt miR processing at its penultimate step through *Dicer* gene ablation were germ-line knockouts, decreasing miR production in the entire organism and throughout development. The resulting embryonic lethality¹⁹ proved that one or more miRs are necessary for normal fetal development, but revealed little about miR functioning specific to the heart. To better address this issue, mice were created in which *Dicer* was ablated only from cardiac myocytes, either in the early embryonic heart (directed by *Nkx2.5-Cre*), the fully developed heart within days of birth (directed by *MYH6-Cre*), or conditionally in juvenile and adult mouse hearts (directed by *MYH6*-driven tamoxifen activated MER-Cre-MER)^{20 21, 22 23}. Each approach of interrupting cardiac myocyte miR processing generated a cardiomyopathy: *Dicer* deletion early in cardiac development produced lethal intrauterine cardiac hypoplasia whereas postnatal and adult cardiac *Dicer* ablation produced lethal cardiomyopathies exhibiting pathological cardiac gene expression, abnormal sarcomeric structure, and cardiomyocyte hypertrophy and/or apoptosis. These results support an important role for miRs in cardiac development and health, but do not define specific critical events that are controlled by miRs, or establish the important targets of cardiac-expressed miRs.

As noted above, the effect of a miR is translational suppression or degradation of its target mRNA(s). miRs located within RISCs recognize and attract target mRNAs by binding, through Watson-Crick pairing, to the mRNA 3' untranslated regions. Bioinformatics searches for mRNA sequences complementary to those of predicted human and animal miRs suggest that one third of all transcripts may be miR targets²⁴. Binding of miR "seed sequences" (nt 2-7) to their complementary mRNA sequence appears to be the major determinant of miR-mRNA pairing, with extra-seed sequence pairing providing fine discrimination between multiple miR targets. Recent work suggests that there are at least four distinct types of extra-seed sequence miR-mRNA pairings, with different functional connotations: 3'-supplementary and complementary seed sites (nt 13-16) enhance mRNA target recognition^{25, 26}, central sites in the middle of miRs (nt 9-12)²⁷, and cleavage sites that occur at miR positions 10 and 11 when miRNAs have extensive 5' and 3' sequence complementarity.

Importantly, a single mRNA may be simultaneously targeted by one or more different miR-containing RISC complexes that cooperate synergistically to repress the mRNA, and a single miR is likely to have multiple mRNA targets^{28, 29}. Perhaps due to evolutionary pressures³⁰, a miR, miR cluster, or members of a miR family tend to target multiple mRNA transcripts within common cellular response pathways, e.g. proliferation, apoptosis, angiogenesis, or fibrosis. As a consequence, miRs can coordinately regulate multiple determinants of a given cellular response. The multiplicity of miRs targeting different mRNAs within a given functional pathway, especially in the context of regulated expression of both miRs and their mRNA targets, provides for remarkable richness and complexity in adaptive regulatory control. However, defining miR-mRNA interactions in their proper biological context and determining the functional consequences of these miR-mRNA pairing events remains a major challenge.

Because miR targeting of mRNAs for cleavage or deadenylation and translational suppression is directly linked to RNA-RNA binding events, it was expected that bioinformatic analysis of miR-mRNA sequence complementarity would accurately predict mRNA targeting by specific miRs. However, practical applications of bioinformatic algorithms are limited because miR-mRNA pairing has multiple determinants, whereas prediction programs tend to rank interaction based on a single feature, such seed sequence complementarity, cross-species conservation, or structural determinants of miR-mRNA binding thermodynamics. Furthermore, bioinformatic tools do not incorporate critical information on miR and mRNA expression levels that inevitably vary between tissues, and

in health and disease. Consequently, when we asked four different bioinformatic algorithms to predict which of ~10,000 known cardiac-expressed mRNAs are likely targets of 139 cardiac-expressed miRs³¹, the results showed poor agreement between different programs³². Likewise, comparative miR and mRNA expression profiling to identify reciprocally-regulated miR-mRNA pairs has met with limited success compared to luciferase reporter studies of the same pairs³³. This disparity is likely due to other mechanisms of mRNA regulation (synthesis and degradation), to indirect mRNA regulation by other miR targets, and to translational inhibition without mRNA destabilization (Figure 1c)³⁴. Finally, luciferase reporter studies show that specific miR-mRNA pairings can occur and have functional consequences, but do not tell you if the interactions have relevance to specific in vivo contexts under which miR and mRNA levels themselves are regulated.

Recently, our laboratory modified previously described cardiac RNA sequencing techniques³⁵ to profile both the population of miR-targeted mRNAs in the heart, and to identify the specific cardiac mRNA targets of particular miRs. The procedure, which we term *RISC-Seq*³⁶, addresses some of the limitations of bioinformatics, comparative profiling, and luciferase studies. We took advantage of miR-mRNA pairing at RISC complexes that are the exclusive domain of Argonaute proteins to develop immunoprecipitation methods for mouse heart RISCs. We then coupled RISC immunoprecipitation (anti-argonaute 2) with procedures to isolate and sequence, without prior amplification, the RISC-associated mRNA. By RNA-sequencing total cardiac mRNA (profiling the transcriptome) and RISC-associated mRNA (profiling the RISCome) from the same hearts, we identified mRNAs that are specifically enriched at cardiac RISCs. Performing this procedure in normal and surgically modeled or genetically modified hearts can provide insight into how miR-mediated post-transcriptional controls modify cardiac disease.

To define the cardiac mRNAs that are targets of a particular miR, we used cardiac transgenesis to achieve what has been called “RISC programming”³⁷, and then performed RISC-Seq. RNA sequencing was used to profile RISC-associated mRNAs in mouse hearts that transgenically express miR-133a or miR-499 (both muscle-specific miRs) in cardiac myocytes, and compared to the RISComes of non-programmed nontransgenic mouse hearts to identify mRNAs specifically targeted to the RISCs by those miRs. This technique successfully catalogued mRNAs differentially recognized by the two programming mRNAs, but is limited by relative insensitivity to detect mRNA targets of the programming miR if that mRNA is highly enriched in the normal cardiac RISC, either because the programming miR is highly expressed in normal hearts or because the mRNA is a target of multiple miRs. It is likely that the coming years will see use of RISC-Seq in combination with complementary techniques that delineate miR-mRNA interaction sites by RNA footprinting³⁸ and that assess the impact of miRs on the proteome³⁹ to better and more comprehensively define relevant miR-mRNA interactions in normal and diseased hearts.

Regulated microRNA expression in cardiac disease

The effects of miR-mediated post-transcriptional regulation of cellular protein production are enhanced by dynamic regulation of miRs and their mRNA targets under conditions of physiological stress and disease. Myocardial miR profiling using microarrays, quantitative PCR, and Northern blotting has identified ~200 miRs that are consistently expressed in hearts, many of which are regulated in cardiac hypertrophy, ischemia, and/or heart failure. Van Rooij, et al were the first to describe regulated miRs in heart disease by microarray profiling miR expression in mouse models of cardiac hypertrophy/failure; these findings for selected miRs were then extended in human cardiomyopathy¹². A number of subsequent studies have also taken advantage of murine models to explore the physiological determinants of miR expression, including a recent interesting report indicating that miR

expression differs in experimental pressure and volume overload⁴⁰. Likewise, there are several reports of comprehensive miR profiling in diseased human myocardium: Thum, et al⁴¹ compared mRNA and miR expression profiles from four normal and six failing human hearts and identified a group of 67 upregulated and 43 downregulated miRs in failing hearts that recapitulated the miR expression signature observed in six fetal hearts. In a larger study of fifty-seven diseased human hearts (twenty-five dilated cardiomyopathy, nineteen ischemic cardiomyopathy, and thirteen aortic stenosis) and ten normal hearts, Ikeda et al identified 87 cardiac-expressed miRs, 43 of which were regulated in at least one of the disease groups⁴². The authors concluded that miR expression profiling provides a diagnostic accuracy of approximately 70%, suggesting for the first time that myocardial miR signatures could have diagnostic utility. This notion has received additional support in a smaller study from Sucharov, et al⁴³ and a larger study from Naga Prasad, et al, who used a custom microarray to identify eight upregulated miRs in fifty heart failure cardiac samples⁴⁴. The latter study is the first to validate clinical associations of miR expression levels in an independent case-control cohort (twenty dilated cardiomyopathy and ten nonfailing). Seven of the eight regulated miRs had previously been identified as regulated in either human or mouse heart failure, further validating these findings.

Our laboratory used miR profiling as a means to functionally stratify human heart failure on the basis of a “recovery” phenotype manifested by end-stage hearts placed on mechanical support. We compared mRNA and miR expression profiles in the same myocardial samples obtained from heart failure with and without mechanical left ventricular assist device support (LVAD) to determine if miR expression would better track functional improvement than mRNA profiles⁴⁶. Microarrays were used to interrogate expression of 467 human miRs and the whole human transcriptome (mRNAs). miR and mRNA expression signatures were generated from seventeen cardiomyopathic hearts not treated with LVAD (“heart failure”), ten hearts treated with LVADs (“failure recovery”), and eleven non-failing control hearts. Consistent with previous findings⁴⁵, mRNA signatures varied little between heart failure and failure recovery groups. In contrast, of twenty-eight miRs that were significantly upregulated in heart failure, twenty were fully normalized in the LVAD failure recovery group and the remaining 8 miRs exhibited strong trends toward normalization. This clinical study uncovered the remarkable sensitivity of miR expression profiling to clinical status in late heart failure, which is not seen in mRNA profiles. The results are consistent with the notion that transcriptional regulation of mRNAs is a major response to disease, whereas regulated miR expression is applied for finer adjustments. The corollary to this schema is that miR expression levels may more accurately reflect the acute functional status of the heart.

microRNAs as circulating biomarkers of cardiac disease

No matter how potentially useful, miR expression profiling is unlikely to achieve widespread use as a clinical diagnostic tool if an invasive procedure (cardiac biopsy) is required to obtain myocardium samples. Fortunately, recent studies suggest that circulating miR levels can reflect their tissue expression. Mitchell et al established the concept of assaying circulating miRs to detect cancer⁴⁷, demonstrating that tissue-specific circulating miRs (for prostate) could be reliably detected in serum, and showing that miR profiles in serum reflected regulated changes in miR expression induced by the cancerous prostate tissue. Importantly, their findings in experimental models and human subjects revealed that endogenous circulating miRs are remarkably stable, persisting for days in serum samples, whereas exogenous miRs added to serum are rapidly degraded. The unexpected stability of endogenous circulating miRs is attributed to their packaging and secretion into the blood within small cell-derived microvesicles, or “exosomes”⁴⁸.

The results of circulating miR profiling in heart disease are also beginning to appear in the literature. Tijssen et al described increased circulating miR-423-5p in heart failure⁴⁹, but without comparison to conventional heart failure biomarkers such as BNP⁵⁰. Other surveys likewise describe regulation of different miRs in chronic heart failure and/or stable coronary artery disease⁵¹⁻⁵³. A recent report suggests that serum levels of cardiac-expressed miRs react to cardiac injury in a manner similar to cardiac enzymes: Corsten et al describe strikingly increased (>1,000 fold) plasma levels of miRs-208b and -499 (two related members of the myomiR family; see below) after myocardial infarction that mirrored levels of troponin T. Likewise, viral myocarditis was associated with smaller (6-30 fold) increases of these miRs in plasma, a myomiR “leak” if you will, and acute heart failure showed an even smaller increase (2-fold) of plasma miR-499 only. In contrast, the plasma miR profile was not changed in patients with diastolic dysfunction. This important study indicates that circulating cardiac miRs are released as a specific response to myocardial injury, a notion supported by results of other circulating miR profiling surveys in myocardial infarction⁵⁴⁻⁵⁹. It is interesting to speculate how serum or plasma miR profiling may be able to refine current diagnostics. For example, if the miR expression profile is different in virally-infected myocardium than infarcted/ischemic myocardium, then circulating miR signatures could provide greater etiologic specificity than cardiac enzyme determinations.

Specific miR functions in heart disease

MyomiRs and myofiber identity

The myomiRs are a family of three highly expressed muscle-specific miRs, miRs-208a, -208b, and -499, that the Olson group has shown control myosin heavy chain isoform expression⁶⁰ (this elegant story is fully detailed in their recent review;¹⁴), and that more recent work has also implicated in cardiac differentiation of embryonic stem cells⁶¹. MyomiR regulation of myosin heavy chain (MHC) protein isoforms is a major mechanism directing the α to β myosin isoform shift⁶² that occurs in pathological hypertrophy, heart failure, and thyroid disease.

The human genome has 11 myosin heavy chain *MYH* genes, all likely derived from a single ancestral *MYH* gene. Only two *MYH* genes are expressed to a significant extent in myocardium, *MYH6* encoding α MHC and *MYH7* encoding β MHC. A third *MYH* gene, *MYH7b*, is closely related to the two cardiac myosin heavy chain genes, but its expression is very low in the heart⁶³. The human *MYH6* and *MYH7* genes occur in tandem on chromosome 14q12, as the consequence of a gene duplication event⁶⁴. The same tandem genetic positioning and complicated intron/exon structures are seen for these two genes in the mouse genome. However, β MHC is the dominant cardiac isoform in large mammals and humans (over 90% of total ventricular MHC;⁶⁵), whereas β MHC in the fetal mouse ventricle is almost entirely replaced by α MHC after birth through the influence of thyroid hormone. The relative proportions of α MHC and β MHC are inverted in human and mouse hearts, but their reciprocal regulation in heart disease or by thyroid hormone is similar, reflecting common transcriptional and post transcriptional mechanisms⁶⁶. Thyroid hormone (T3) shifts *MYH* gene expression in favor of α MHC, whereas hypothyroidism induced by propyl-thiouracil (PTU), pressure overload hypertrophy, and heart failure favor β MHC⁶⁶.

Within each of the three *MYH* genes are intronic miRs that have also been duplicated, miR-208a in α MHC/*MYH6*, miR-208b in β MHC/*MYH7*, and miR-499 in *MYH7b*. Although intronic sequences differ substantially between the three genes, the myomiR sequences encoded within their miRtrons have been almost completely conserved, suggesting evolutionary pressure to maintain these miRs and their functions. Each of the myomiRs is transcribed into the same precursor mRNAs as its parent *MYH* gene, but because of feedback regulation between *MYH* genes^{13, 60} and differences in stability between miR-499

and the parent *MYH7b* mRNA⁶⁷, their expression does not precisely parallel that of the parent mRNA: Like its parent mRNA (*MYH6*/αMHC), miR-208a is potently induced by T3 and suppressed by PTU. However, because miRs are generally more stable than mRNAs, αMHC mRNA declines in response to PTU, whereas miR208a is not rapidly degraded and its levels are sustained¹³. Residual miR-208a can therefore suppresses the translation of transcriptional repressors of *MYH7* and *MYH7b* transcription, de-repressing βMHC expression (and that of its daughter, miR-208). Collectively, these findings describe regulation and counter-regulation by the myomiRs of their family members' parent genes, driving the α/βMHC “switch”.

The mechanism by which myomiRs regulate myosin isoforms is made even more intricate and elegant by recently described alternate splicing of cardiac *MYH7b* transcripts. As previously noted, MYH7b protein is not measurable in myocardium. Despite the absence of its parent mRNA and protein, miR-499 is one of the most highly expressed miRs in the heart, and is released in measurable amounts into plasma and serum after myocardial injury⁶⁸. An explanation for this apparent discrepancy is preferential alternate splicing of *MYH7b* exon 7 in the heart, as recently described by Leslie Leinwand's group⁶⁷. Cardiac *MYH7B* transcripts lacking exon 7 are degraded through the mechanism of nonsense-mediated mRNA decay. Because nonsense mRNA decay occurs at the ribosome after nuclear export⁶⁹, intra-nuclear processing of the primary miR-499 transcript is unaffected by exon 7 splicing. Thus, alternate mRNA splicing in the heart uncouples production of mature miR-499 from expression of the parent *MYH7b* mRNA. The fascinating implication of these findings is that the human *MYH7b* gene has evolved as a non-functioning host for its incorporated daughter miR (miR-499), which itself is essential for normal expression of the cardiac myosin heavy chain proteins encoded by a different MYH gene, *MYH7*/βMHC.

miR1/133 regulation of cardiac hypertrophy and remodeling

It has long been recognized that gene expression in reactive cardiac hypertrophy recapitulates that in the growing embryonic heart⁷⁰. This makes intuitive sense as programs for cardiac growth are potently induced in both embryonic heart development and the hypertrophic response to hemodynamic overload. Recent studies have extended the concept of parallel cardiac developmental and stress responses to miRs⁴¹, especially the miR1/133 cluster. A miR cluster consists of two or more miRs that are linked genetically and transcribed as a single polycistronic primary transcript (see Figure 1a). Clusters may account for up to half of all mammalian miRs, and evidence is accumulating that miRs within a cluster cooperate in their functional effects, despite having very different sequences (and, by implication, mRNA targets). In the heart, the best understood example of a miR cluster includes miR-1 (or related miR-206) and miR-133, which exist as miR-1/miR-133 pairs at three different genomic loci, each of which is transcribed as a bi-cistronic transcript. miR-1 helps to direct cardiomyocyte differentiation from embryonic stem cells in vitro⁷¹ and cardiac development in vivo^{20, 11}. A recent report has also implicated miR-1 in cardiomyocyte hypertrophy⁷². Likewise, miR-133a can affect both cardiac development and pathological hypertrophy: Combined ablation of miR-133a-1 and miR-133a-2 was lethal, and produced ventricular septal defects and dilated cardiomyopathy in the few mice that survived⁷³. In adult hearts, inhibition of miR-133a using an engineered synthetic oligonucleotide miR antagonist (a class of miR silencing molecules called “antagomirs”⁷⁴) induced hypertrophy⁷⁵, whereas forced miR-133a expression in the embryonic heart potently suppressed cardiomyocyte proliferation and produced lethal ventricular hypoplasia⁷³. However, expression of miR-133a in the postnatal heart did not alter normal cardiac growth or the hypertrophic response to sustained pressure overload⁷⁶. Rather, the miR-133a transgenic heart exhibited diminished myocardial fibrosis and improved diastolic function after surgical aortic coarctation, consistent with regulation of myocardial fibrosis in adult

hearts ⁷⁷. Since fetal cardiac growth is mediated by cardiomyocyte proliferation, but cardiomyocytes exit the cell cycle shortly after birth and reactive hypertrophy in adult hearts is accomplished by cardiomyocyte enlargement without proliferation, it is interesting to postulate that miR-133a directs cardiomyocyte cell cycling rather than cardiomyocyte growth in general.

miRs, angiomiRs, and the cardiac response to ischemic injury

miRs are regulated in cardiac ischemia and may control the myocardial response to ischemic insults by modifying cell survival/death, tissue repair/fibrosis, or vascularization. The most compelling data to date describe roles for endothelial-specific miR-126 and cardiomyocyte miR-210 in the cardiac response to ischemia. Both of these miRs are regulated in ischemic myocardium, but their patterns of regulation and mechanisms of action differ.

miR-126 is downregulated in myocardial infarcts, but upregulated in border areas ⁷⁸. A critical role for miR-126 in development of the fetal vascular system (“angiomir” effect) was first described in companion *Developmental Cell* papers from the Olson and Srivastava laboratories: miR-126 gene ablation in mice or morpholino knockdown in zebrafish depressed vascular endothelial formation and angiogenesis ^{79, 80}. miR-126 deficiency in mice impairs vascularization after experimental myocardial infarction, suggesting that miR-126 directs both initial vascular development and revascularization ⁷⁹. A recent two-photon study of zebrafish indicates that blood flow activates the mechano-sensitive transcription factor, *klf2a*, which induces expression of miR-126 that enhances angiogenic VEGF signaling ⁸¹.

Like miR-126, miR-210 is upregulated in ischemic myocardium. Whereas miR-126 is expressed only in endothelial cells, miR-210 is expressed in cardiac myocytes, is induced by HIF1 α , and has been associated with increased survival/decreased apoptosis of ischemic cells ⁸²⁻⁸⁴. Recently, gene therapy using a minicircle DNA vector encoding the miR-210 precursor decreased ventricular remodeling, improved ventricular ejection performance, diminished cardiomyocyte apoptosis, and enhanced myocardial neovascularization in myocardial infarcted mice ⁸². Since the minicircle vector approach resulted in sustained cardiac expression of the miR, this study suggests both a therapeutic modality and a specific therapeutic target for miR-promoted myocardial angiogenesis.

In opposition to the angiomiRs are anti-angiogenic effects of endothelial cell-expressed miR-92a, which also modifies the response to myocardial ischemia. Bonauer, et al ⁸⁵ described increased miR-92a expression after experimental myocardial infarction, and observed that miR-92a inhibited neovascularization. Accordingly, they treated myocardial infarcted mice with a miR-92a antagomir and observed increased border zone vascularization, diminished infarct size, and enhanced post-infarction ventricular function.

Cardiomyocyte death after myocardial infarction is followed by myocardial fibrosis. Several miRs have been linked to cardiac fibrosis: miR-29 expression is greater in cardiac fibroblasts than in cardiac-myocytes, and miR-29 is downregulated in the border zone of infarcted hearts, but still increases myocardial fibrosis by de-repressing collagen and elastin translation ⁸⁶. In contrast, miR-21 expression is increased in the border zone of myocardial infarcts ^{78, 86} and in the fibroblasts of failing human hearts, where it enhances fibroblast survival/myocardial fibrosis by repressing programmed cell death 4 (PDCD4) and sprouty 1 (SPRY1), activating the ERK MAP kinases ^{78, 87}. Interestingly, cardiomyocyte (not fibroblast) miR-21 expression is downregulated by hypoxia, perhaps as part of the Akt survival pathway ⁸⁸. An initial report employed a cholesterol-modified miR-21 “antagomir” to reduced myocardial fibrosis and pathological remodeling in the mouse aortic banding pressure overload model, suggesting a therapeutic approach ⁸⁷. However, this story appears

more complex than first thought because a recent report indicates that neither genetic ablation of miR-21 nor treatment of mice with a locked nucleic acid modified miR-21 “antimiR” significantly affects cardiac hypertrophy, remodeling, or myocardial fibrosis in aortic banded mice ⁸⁹. It is possible that the miR-21 antagomir used in the original study has “off-target” effects. In any case, these studies suggest that miR gene ablation will continue to be a useful adjunct to pharmacological inhibition for dissecting the biological effects of individual miRs.

The potential for miR-based therapeutics

miRs and the pathways they regulate are attractive therapeutic targets because of nodal control exerted by individual miRs or miR clusters over pathways that regulate cardiac hypertrophy, cardiomyocyte apoptosis, myocardial vascularization, and cardiac fibrosis. miRs are stable “small molecules” that can be measured in the circulation, inhibited through the use of antagomirs or antimiRs, and mimicked through exogenous expression, as with minicircle DNA vectors. Care et al first used an antagomir of miR-133a to induce cardiac hypertrophy and transcortical gene delivery of synthetic miR-133a to prevent hypertrophy in a genetic mouse model ⁷⁵. A miR-21 directed antagomir prevented myocardial fibrosis after pressure overloading, although it is not clear if this effect is mediated exclusively by inhibition of miR-21 ^{87,90}. miR-494 overexpression decreased myocardial infarct size and cardiomyocyte apoptosis while enhancing post-ischemic functional recovery in mice ⁹¹. The recent pre-clinical evaluation of minicircle miR-210 gene therapy in murine myocardial infarction further supports the concept of miR-based therapeutics ⁸². As suggested by the miR-21 studies described above however, a concern for developing miR-based therapeutics (as with any form of gene therapy) is defining unintended or “off-target” consequences before implementing such therapy in human disease. These are complex issues: Conceptually, how does the pharmacological term “off-target” apply to miRs, whose endogenous expression levels can be regulated over an order of magnitude, depending upon pathophysiological context? Because miRs target their mRNAs based not only on seed-sequence complementarity, but also on extra-seed sequence pairing, it seems likely that weaker miR binding partners that may be irrelevant under basal conditions can develop significant impact when the miR or mRNA target or both are upregulated in stress or disease. miR-mRNA pairing is an interactive continuum (strong to weak), not an “on/off” response. For this reason, therapeutic dosing of a miR has the potential to silence mRNAs that are not normally its targets. Furthermore, ectopic expression of a miR in a tissue or cell in which it is not normally present will have the effect of silencing mRNAs that are not normally regulated by this mechanism, representing a true promiscuous or “off-target” biological effect even though the miR-mRNA pairing itself is normal. Improved bioinformatics are needed to enhance prediction of miR targeting. Interpretation of animal studies will need to incorporate the consideration that, while miRs are highly conserved between mammalian species, miR binding sites in mRNAs are much more variable and miR targeting will differ accordingly. Notwithstanding these and other hurdles, the rapid technical advances we are currently witnessing and the insight that is being derived from application of these methods engenders enthusiasm that miRs will lead to “miRacles” rather than “miRage” ⁹².

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1. . Pathways for microRNA biogenesis, translational inhibition, and feedback regulation

A. miR biogenesis. Depicted are two miR genes forming a miR cluster located in the intron (miRtron) of a parent gene. Both parent and miR gene transcription is determined by protein transcriptional activators and repressors (transcription factors). The primary RNA product includes the pri-miR whose distinct secondary structure is recognized and cleaved by the Drosha/Dgcr8 RNA polymerases to form a pre-miR that is transported from the nucleus by exportin. **B. Translational inhibition by RISC-associated miRs.** After nuclear export, cytosolic pre-miRs are processed by Dicer into miR duplexes. Mature single stranded miRs are loaded onto the argonaute (Argo) protein-containing RNA induced silencing complex (RISC) for presentation to mRNAs. mRNAs with sequences in their 3'UTR complementary to the miR are bound and silenced or eliminated (indicated by the scissors). **C. Complex feedback regulation of mRNAs by miRs.** A mRNA recognized by two different miR-containing RISC complexes is shown at upper right. Blockade of ribosomal translation for the mRNA targets of these miRs depresses expression of the respective gene products. When these gene products are transcriptional repressors or activators, this produces secondary effects on expression of any genes and their mRNAs regulated by these factors. Likewise, expression of miRs encoded within these mRNAs or independently regulated by these transcription factors is affected, which can initiate a new regulatory cycle affecting translation and/or expression of mRNAs that are not direct targets of the original miRs (light grey).