Targeting MicroRNA Targets

Paula A. Da Costa Martins, Leon J. De Windt

The intriguing biology of microRNAs, small regulatory noncoding RNA molecules encoded by the genome that coordinately regulate gene expression by targeting messenger RNAs, has opened new territories for our understanding of gene regulatory circuits in cardiac homeostasis and disease. It is now estimated that the human genome may encode more than 1000 miRNAs,[1,2] which may regulate up to 60% of mammalian genes, and certain miRNA species are remarkably abundant in mammalian cell types.[3] On average, a single miRNA has approximately 100 target sites.[4]

Article, see p 521

The miRNAs perform their gene regulatory function by guiding the RNAi-induced silencing complex (RISC) to partially complementary regions in the 3′UTR region of protein coding messenger RNAs (mRNAs). Within the RISC complex, miRNAs display imperfect Watson-Crick base pairing with mRNAs, ultimately leading to suppression of gene expression primarily by mRNA decay and, to a lesser extent, translational repression.[5] The most commonly accepted mechanism of miRNA targeting in metazoans involves an interaction between the 5′-end bases 2 to 7 of the miRNA, designated the miRNA "seed region," and the 3′ untranslated region (3′-UTR) of the target mRNA.[6] Whereas, occasionally, additional "compensatory" sites more toward the 3′-end of the miRNA compensate for "seed" mismatches and, more rarely, more "centered" sites are also involved in base-pairing between miRNA and mRNAs,[7] these different options do occur for validated targets for one single miRNA.[4]

Over the past decade, deciphering the roles of individual miRNAs has relied heavily on the identification of their targets to provide mechanistic context to their cellular function. As Watson-Crick base-pairing underlies miRNA–mRNA interactions and multiple metazoan genomes are now readily available, prediction of miRNA seeds throughout the genome is a seemingly straightforward task that can be tackled by the field of bioinformatics. Parallel to the discovery and annotation of the metazoan miRNOME, a plethora of online, user-friendly target prediction algorithms have surged, based around such a model. Small differences remain between databases in which relative preference is given to miRNA "seed" regions or inclusion of additional "compensatory" sites. Other sites focus on manually curated resources of experimentally validated targets, harbor bibliographic searches, include miRNA expression patterns and miRNA–disease relationships, or are focused on listed polymorphisms in miRNA sequences and targets. An updated, comprehensive overview of currently available databases is available at https://sites.google.com/site/mirnatools/mirna-databases.

Although computational methods for miRNA target prediction are the prevailing means to analyze their function, experimental evidence indicates that all databases still miss a large fraction of the targeted genes and, more worrisome, also predict a large number of false-positives. Failure to accurately predict miRNA targets in silico is likely attributable to a combination of factors, including the finding that targeting also can be mediated through sites other than the 3′-UTR and that seed region base-pairing is not always required. Finally, in metazoans, each cell type likely has its unique miRNA and mRNA profiles, and because the vastly different cellular transcriptomes (both under homeostasis and disease states) are still not incorporated in the predictive databases, the potential for false-positive predicted targets is greatly increased. In conclusion, the power and validity of in silico data are hindered by the simplified rules used to represent targeting interactions, and experimentation remains an essential element to identify genuine miRNA targets.

Several approaches for the experimental validation of miRNA–mRNA interactions are available. In the most simplified approach, a reporter system is used in which binding of a given miRNA to its specific mRNA target site will repress reporter protein production, thereby reducing activity/expression that can be measured and compared with a control. Commonly, the 3′-UTR of the target gene of interest is fused immediately downstream of a luciferase reporter open reading frame. Another simplified approach encompasses the modulation of miRNA concentration by inducing its transient overexpression in a cell type known to express the putative target protein and subsequent quantitative reverse-transcriptase polymerase chain reaction and Western analysis to assess expression levels of the target gene at the mRNA and protein levels, respectively. Identification of individual miRNA–mRNA interactions does not account for the fact that miRNAs are able to regulate complex gene networks, which requires large-scale and unbiased methods of miRNA target identification. The majority of large-scale miRNA target validation approaches involve differential expression of a single miRNA, followed by downstream genome-wide gene expression or proteomic analysis. Despite widespread use, miRNA modulation experiments induce supraphysiological miRNA levels after transient transfection, baring the risk to saturate RISC complexes, displace other endogenous miRNAs,[8] and cause low-affinity target sites to appear functionally important.[9]

From the Department of Cardiology (P.A.D.C.M., L.J.D.W.), CARIM School for Cardiovascular Diseases, Faculty of Health, Medicine and Life Sciences, Maastricht University, Maastricht, the Netherlands.

Correspondence to Leon J. De Windt, Department of Cardiology, CARIM School for Cardiovascular Diseases, Maastricht University, Maastricht, the Netherlands. E-mail l.dewindt@maastrichtuniversity.nl

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Finally, biochemical approaches are increasingly used to identify miRNA–mRNA pairs by immunoprecipitation and purification of RISC components such as Argonaute[10] or TNRC6,[11] followed by identification of bound targets by microarray or deep sequencing. Apart from the useful elucidation of genome-wide miRNA target gene networks, these studies also revealed biologically intriguing data in which approximately half of miRNA binding sites were mapped to 3′-UTRs and a remarkably similar percentage also were mapped to mRNA coding regions, and approximately 5% even bound 5′-UTRs.[11,12]

The cardiac sarcomere is primarily composed of transcripts from three myosin heavy chain genes, *Myh6*, the fast twitch α-myosin heavy chain, *Myh7*, which encodes the slow twitch β-isoform, and low amounts of *Myh7b*, another fast twitch isoform.[13] The relative composition of the sarcomere for these proteins determines contractile properties and energy consumption of the heart.[14] A miRNA family designated "MyoMirs" are encoded by introns of the separate myosin heavy chain genes, in which miR-208a, miR-208b, and miR-499 are located within the *Myh6*, *Myh7*, and *Myh7b* genes, respectively. MyoMirs play a crucial role in the regulation of myosin gene expression and the cardiac stress response in rodent species.[13] The MyoMirs miR-499 is an evolutionary conserved muscle-specific miRNA and likely plays a role in myosin gene regulation.[13,15] In addition, miR-499 has been implicated in suppression of apoptosis in myocardial infarction and after ischemia/reperfusion.[16] In contrast, Shieh et al[17] generated transgenic mice with increased expression levels of miR-499 under control of the cardiac *myh6* promoter, in which elevated levels of miR-499 were sufficient to provoke spontaneous enlarged hearts, contractile dysfunction, and downregulation of immediate early response genes known to be relevant for the cardiac stress response.[18] Being a member of the MyoMirs family, miR-499 expression is expected to increase in pressure overload-induced hypertrophy. Clinical data, however, have shown variable expression levels of miR-499 in the human heart. However, experimental overexpression strategies have revealed inconsistency with either protective or deteriorating cardiac effects. These discrepancies keep the exact function of miR-499 in the heart unclear. As with many of the reported functional miRNA studies relevant for cardiac remodeling and heart failure, in which single miRNAs and their single targets were often the center of attention,[19–22] this strategy inadvertently could contribute to discrepant findings and ambiguity of cardiac miRNA function.

In this issue of *Circulation Research*, Matkovich et al[23] exhaustively studied miR-499 contribution to heart disease. The authors applied a comprehensive approach including determination of miR-499 expression patterns in clinical hypertrophy and failure and genome-wide RISC and RNA sequencing after targeted overexpression of miR-499, yielding crucial new insights into this MyoMirs family member. First, comparison of normal, failing, and nonfailing hypertrophied human hearts not only confirmed but also correlated increased miR-499 expression levels in heart failure and hypertrophy with repression of 98 cardiac-expressed mRNA targets predicted by in silico bioinformatics tools. Similarly, miRNA microarrays identified miR-499 as firmly upregulated in the Gqα-overexpression mouse model, which recapitulates essential aspects of pressure overload-induced cardiomyopathy,[24] and in which 13 of the predicted target mRNAs were downregulated.

Three transgenic mouse lines engineered to overexpress miR-499 in a heart-restricted manner were generated, two lines expressing miR-499 to similar levels as observed in human heart failure and one line expressing miR-499 at approximately three-fold higher levels. Transgenic mice overexpressing miR-499 displayed pathological cardiac remodeling reflected by hypertrophic growth accompanied by cardiac dysfunction. The ensuing adverse remodeling events were clearly aggravated when transgenic animals were subjected to transverse aortic constriction. All three lines had development of a similar cardiac phenotype as a consequence of the induction of genetic reprogramming in which 136 mRNAs were regulated in 4-week-old and 8-week-old miR-499 transgenic hearts, with 31 of which identified as potential miR-499 target genes by bioinformatics tools. Interestingly, microRNA profiling revealed an overlap in microRNA expression patterns between miR-499 transgenic animals and pressure-overloaded nontransgenic animals, suggesting that similar gene reprogramming occurred in these two animal models of heart failure.

Because a single microRNA exerts its function by affecting a complete network of genes either directly or indirectly, analyzing targets beyond the immediate direct targets will further aid in our understanding of microRNA function and the cardiac phenotypes it may provoke. In this regard, and for the first time to our knowledge in our field, Matkovich et al have addressed this aspect elegantly by identifying the direct and indirect targets of miR-499 by a combination of whole-genome RISC sequencing and analyzing their function in the context of heart failure. This method allowed the identification of a set of mRNAs that were significantly enriched in miR-499 transgenic hearts; some of them previously validated targets, including Sox6. Although most of the target mRNAs showed decreased expression levels, a small number of mRNAs were increased, reflecting indirect targeting or an, as of yet, incompletely understood miRNA–mRNA interaction. Gene ontology and pathway analysis, incorporating all direct and indirect mRNA targets for miR-499, disclosed major regulation of different kinases, particularly members of the mitogen-activated protein kinase cascade. Also, several phosphatases within the prosurvival Akt signaling pathway such as PTEN and Phlpp1 were regulated by miR-499. Interestingly, integration of this transcriptome analysis with proteomic data deciphered a function for miR-499 in regulating signaling pathways that coordinate posttranslational protein modification.

Potential technical limitations of this new technology were either properly acknowledged or controlled for in the study. First, RISC programming by transgenic miRNA overexpression may bare the risk for uncontrolled overexpression of both the mature miRNA and the "passenger" strand (miRNA*). Inappropriate miRNA* levels may recruit its targets to the RISC, which will be detected by RISC sequencing. In their studies,[23] the miR-499/miR-499* ratio was

measured and remained the same as in a wild-type situation. A second, more theoretical, consideration concerns the overexpression of miRNAs that are already expressed at high levels, such as MyoMirs, and bares the potential of saturating miRNA–mRNA incorporation into the RISC, which may explain the nonlinear relationship between miR-499 expression and its phenotypic effects in low versus high overexpressors. Finally, an as-of-yet experimentally uncontrollable risk is associated with the consequence that expression levels of other miRNAs may become altered by genetic miRNA overexpression strategies, changing the RISCome and baring the risk of identifying RISC-enriched mRNAs as direct targets of the RISC-programming miRNA.

Notwithstanding these uncontrollable experimental caveats, this is the first study in our field that fully supports the premise that miR-499 is enriched in human heart disease, that forced cardiomyocyte miR-499 expression suffices to induce progressive experimental heart failure, and that elevated miR-499 expression is deleterious in the context of pressure overload. Moreover, the effects of miR-499 on gene regulatory networks, protein contents, and posttranslational protein modifications are remarkably complex, but the technological strategies used in this study may well prove to become exemplary to decipher and target single microRNA targets in the cardiovascular context.

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Disclosures

None.

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