Decoding the Cardiac Message
The 2011 Thomas W. Smith Memorial Lecture

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Abstract: This review reflects and expands on the contents of my presentation at the Thomas W. Smith Memorial Lecture at American Heart Association Scientific Sessions, 2011. “Decoding the cardiac message” refers to accumulating results from ongoing microRNA research that is altering longstanding concepts of the mechanisms for, and consequences of, messenger RNA (mRNA) regulation in the heart. First, I provide a brief historical perspective of the field of molecular genetics, touching on seminal research that paved the way for modern molecular cardiovascular research and helped establish the foundation for current concepts of mRNA regulation in the heart. I follow with some interesting details about the specific research that led to the discovery and appreciation of microRNAs as highly conserved pivotal regulators of RNA expression and translation. Finally, I provide a personal viewpoint as to how agnostic genome-wide techniques for measuring microRNAs, their mRNA targets, and their protein products can be applied in an integrated multisystems approach to uncover direct and indirect effects of microRNAs. Experimental designs integrating next-generation sequencing and global proteomics have the potential to address unanswered questions regarding microRNA–mRNA interactions in cardiac disease, how disease alters mRNA targeting by specific microRNAs, and how mutational and polymorphic nucleotide variation in microRNAs can affect end-organ function and stress response. (Circ Res. 2012;110:755-763.)

Key Words: RNA silencing ■ transcriptional regulation ■ genetic mutation ■ microRNA

We are members of a biomedical research community whose world-view has been completely altered over the past 150 years, first by the discovery of the principles of genetics, then by the development of the techniques of molecular biology, and most recently by the application of computerized/roboticized “omics” approaches to broad biological questions. The scientific topic of this session, microRNAs in the cardiovascular system, is an example of an area of research that did not exist even a decade ago. Yet the ongoing explosion of information on microRNAs has provided insight into integration and regulation of biological systems. We must therefore react by adjusting our concepts and experimental approaches.

Because “a dwarf on a giant’s shoulders sees farther of the two,”1 I offer here some perspective on the scientific road that has brought molecular genetics to its current state. I then propose a conceptual paradigm for investigating microRNA–mRNA–protein regulation that extends the typical reductionist experimental approach in favor of global analyses that lend themselves to integrative interpretation of biological pathways in terms of functional modules. Finally, I discuss future developments that have the potential to integrate basic molecular, proteomic, and physiological research with clinical genetics of microRNAs.

The Genesis of Molecular Biology

The underlying principle of molecular biology can be summarized as follows: genes are vertically transmitted packets of biological information that determine phenotype, and DNA is the stuff of which genes are made. The experimental basis for this paradigm, now accepted as natural law, was developed by 2 19th-century Europeans who were contemporaries, and yet apparently unaware of each other’s work. Fr. Gregor Mendel is familiar to every middle school student. During the period immediately before and during the American Civil War, his meticulous observation and quantitative documentation of patterns for vertical phenotype transmission of specific traits in *Pisum sativum* (peas) identified dominant and recessive genetic characteristics and enabled him to derive the “laws of inheritance.”2 Mendel’s original work was misunderstood and largely ignored until it was independently reproduced and acknowledged over 30 years later by Hugo de Vries and Carl Correns.3,4

Contemporaneous with Mendel’s botanical studies, the young physician Friedrich Miescher was embarking on what would now be considered a postdoctoral research fellowship studying the chemistry of blood cells. Although his project in Felix Hoppe-Seyler’s program was to have focused on

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lymphocytes, they were difficult to obtain. By contrast, leukocytes were abundant in the purulent suppurations of bandages obtained from the local surgical clinic. From leukocyte nuclei, Miescher isolated an acid-insoluble, sodium hydroxide- and disodium phosphate-soluble nonproteinaceous substance he designated “nuclein,” later termed nucleic acid, or DNA. Publication of Miescher’s results in his mentor’s journal was delayed for 2 years while Hoppe-Seyler reproduced them, extended them to yeast, and published his own data.6

The work of Mendel and Miescher and their successors established the foundations of molecular biology by defining genetic principles and discovering DNA. However, the revelation that genes are made of DNA required 70 additional years, until World War II. As Professor Emeritus at Rockefeller University, 66-year-old Oswald Avery, MD, and his colleagues reported that DNA was the substance that induced virulent transformation of nonpathological pneumococcus.7

The identity of DNA as the “transforming principle” of genes was confirmed by tracing bacteriophage32P-DNA in studies performed by Alfred Hershey and Martha Chase.8 Together, the Avery-MacLeod-McCarty and Hershey-Chase experiments established the molecular basis for heredity and helped to focus attention on the mechanism by which DNA encodes genetic principles and discovering DNA. However, the revelation that genes are made of DNA required 70 additional years, until World War II. As Professor Emeritus at Rockefeller University, 66-year-old Oswald Avery, MD, and his colleagues reported that DNA was the substance that induced virulent transformation of nonpathological pneumococcus.7

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From DNA to RNA

Understanding DNA was the key that unlocked the door of molecular genetics. A timeline of Nobel Prizes given for DNA research since World War II reveals a burst of DNA research that characterized the second half of the 20th century (Figure 1, top). Discovery of the enzymes responsible for DNA replication and RNA transcription, elucidation of the mechanism of bacterial transduction, and development of the technique for saturation mutagenesis were acknowledged in the late 1950s, followed by recognition of Watson and Crick’s elucidation of the 3-dimensional structure for DNA in 1962. Over the next 15 years the attention of the Nobel committee was elsewhere, but in the 15 years that followed (ie, 1978–1993), 4 Nobel prizes were awarded for DNA research, 3 related to development of DNA manipulation or diagnostic techniques (restriction endonucleases in 1978, rDNA expression and DNA sequencing in 1980, and polymerase chain reaction and site-directed mutagenesis in 1993).

Just 5 years ago the Nobel Prize in Physiology or Medicine was awarded for targeted gene manipulation in mice. Thus, by the time I graduated from Medical School over 30 years ago, we had a detailed understanding of the fundamental mechanisms of DNA replication and transcription. An appreciation of the impact of DNA mutations provided a mechanism for “Mendelian” human disease, including the first-recognized example, alkaptonuria, the heritable features of which had originally been described in 1902.13

Our understanding of the roles for genetic mutation and regulated gene transcription in heart disease is built on these fundamental observations of the middle and late 20th century. Using a combination of genetic techniques and functional genomics, Christine and Jon Seidman first described in 1990 a molecular basis for the most common Mendelian cardiac disease, familial hypertrophic cardiomyopathy.14,15

This seminal pair of publications inaugurated an era of molecular genetics in cardiology that has, to date, uncovered
over 1000 mutations of more than 30 different genes causing “monogenic” or Mendelian heritable hypertrophic and dilated cardiomyopathies. (For a fascinating personal perspective of the Seidmans’ body of work and the overall field, the interested reader is referred to Seidman & Seidman.16) Likewise, the pathways by which molecular regulation of muscle gene expression determines cardiac development, and the contribution of dysregulated cardiac gene expression to heart disease, were initially uncovered by Eric Olson and his colleagues (reviewed in Olson,17 Potthoff & Olson,18 Hill & Olson,19 and Haberland et al20). The general paradigm established by these and other researchers engendered detailed investigations of genetic reprogramming in heart disease, provided a foundation for molecular diagnostics in human and experimental heart failure, and suggested novel therapeutic avenues aimed at normalizing the pathological effects of disease-regulated cardiac genes.21–23

Observations that there were distinctive gene expression patterns within disease was one of many factors prompting mechanistic investigations that moved beyond DNA to examine its messenger RNA gene products. The evolutionary advantage of DNA as the archive for biological design lies in its chemical stability, ability to undergo repair, and high fidelity of replication permitting faithful vertical transmission of encoded phenotypes. In other words, DNA is comparatively static and immutable. For these reasons, results from the Human Genome Project have provided not only a blueprint of our species’ molecular architecture in comparison with that of other species,24,25 but also revealed to what extent past Homo sapiens genetic admixture with other ancient hominids impacts modern humans.26 Metaphorically, however, when purchasing a car, one does not just compare parts lists and schematic diagrams of various models; one takes the car for a test drive. By the same logic, in moving beyond the genetic blueprint it was necessary to determine how mRNA and protein gene products are regulated and interact to direct biological processes.

In comparison with DNA, mRNA is evanescent and susceptible to enzymatic and physical degradation. Thus, the major insights into RNA biology followed and built on those of DNA. Returning to the Nobel prize timeline (Figure 1, bottom), the first RNA Nobel prize was awarded in 1968 for deciphering the mechanisms of translation, followed by a 20-year hiatus that was brought to an end by recognition of RNA splicing produces protein diversity (1993). By 2006, RNA science had clearly arrived when the Nobel prizes for both Chemistry and Physiology or Medicine were awarded for RNA biology, the former for deciphering the mechanisms of transcription and the latter for the discovery of modulated gene expression by double-stranded RNAs, ie, RNA interference. This brings us to the modern era and the overall topic of this scientific session, microRNAs.

**RNA Interference and MicroRNAs**

The first microRNA, lin-4, was described in *Caenorhabditis elegans* in 1993 by Victor Ambros and colleagues.27 At the time, lin-4 was largely considered to be a biological curiosity limited to nematodes: it was widely accepted that an antisense RNA complementary to a given mRNA could suppress that mRNA, but it was not appreciated how endogenous (or exogenous) double-stranded small RNAs could function as modulators of biological function. This apparent contradiction was addressed by Andrew Fire and Craig Mello’s subsequent discovery that RNA interference by double-stranded RNA was more efficient than that by single-stranded antisense RNA28 (for which they received the 2006 Nobel Prize in Physiology or Medicine). This observation revealed the presence of previously unknown mechanisms for processing double-stranded small RNAs and presenting the active single strand to its mRNA target. These revelations were rapidly followed by identification and characterization of the second microRNA, let-7, by Gary Ruvkun,29,30 setting the stage for 3 concurrent 2001 reports in *Science* that demonstrated unexpected abundance and evolutionary conservation of microRNAs, and suggested their broad impact on biological function.31–33

These 3 papers inaugurated the burst of microRNA research that continues to the present; they also received the AAAS Newcomb Cleveland Prize for the most significant papers published in *Science* during that year.

Conceptually, what do microRNAs do? To quote a past student from my laboratory, “MicroRNAs make mRNAs not make protein.” Although there may be some exceptions, the general function of microRNAs is to prevent mRNA translation into protein. Two possible mechanisms have been identified, mRNA destabilization and translational suppression (although, again, this is an area of research that continues to evolve). As schematically depicted in Figure 2A, a fully processed single-stranded microRNA is incorporated into an Argonaute2-containing macromolecular complex, the RNA-induced silencing complex or RISC,44 to which target mRNAs are recruited via complementary Watson–Crick binding to the incorporated microRNA. mRNA destabilization takes place through nucleolytic cleavage and degradation of the complementary mRNA. Accordingly, steady state expression levels of mRNAs targeted by microRNAs may be decreased due to degradation. Alternatively, mRNAs incorporated into RISCs may undergo translational suppression via deadenylation, ie, removal of the polyA+ tail, which may or may not require participation in the RISC of poly(A) binding protein (PABP) and associated deadenylases35,36 in association with GW182 family proteins37 (Figure 2B). It is likely that translational suppression and mRNA degradation both occur, or that they take place sequentially.

My goal here is not to provide an encyclopedic review of cardiac microRNAs encompassing the explosion of data from the past few years. Others have contributed more to the field, and any such compendium would soon be made obsolete by ongoing progress and developments. Rather, I would like to offer a perspective on investigative and analytic approaches that may be useful for interrogating cardiac microRNAs in health and disease.

**An Unbiased and Genome-Wide Approach to MicroRNA Target Analysis**

Association of an mRNA with a microRNA–RISC complex is required for gene silencing. This association is reversible.
and therefore governed by the same general principles as noncovalent receptor–ligand binding: the microRNA–RISC is analogous to an unoccupied receptor, the mRNA is like its ligand, and the microRNA–mRNA duplex is like the active receptor–ligand complex. Thus, the steady state probability that a given microRNA–mRNA pair will form in the RISC is determined by the concentration of microRNA, the concentration of its mRNA target, and the binding affinity of the microRNA–mRNA duplex (Figure 2C). For RNA duplexes, binding affinity is more properly described by binding energy, which is a function of Watson–Crick complementarity and can be calculated using relatively straightforward methods. The formula for calculating the steady state interaction between a microRNA and a given mRNA target is simple, but each of the formulaic variables exhibits tremendous biological variability. The concentration (expression level) of ~200 cardiac microRNAs varies widely in human and experimental heart disease. Likewise, the concentration (expression level) of cardiac mRNAs varies independently due to transcriptional regulation and dependently due to destabilization by cardiac-expressed microRNAs. Finally, the binding energy for a given microRNA–mRNA complex will vary with the primary sequence complementarity and can be influenced by secondary mRNA structures that affect binding site accessibility to RISC-associated microRNAs. Figure 2C (right panel) provides an example of sequence variability within 5 bioinformatically predicted miR-499 binding sites in one of its thoroughly validated cardiac mRNA targets, Sox6.

One way to account for confounding biological variability is to quantify each of the variables simultaneously and in the proper clinical or experimental context. Multiple microRNAs and mRNAs are simultaneously regulated in cardiac disease (vide supra). Furthermore, a given microRNA has the potential to independently target multiple mRNAs, and a given mRNA is likely to be targeted by more than 1 microRNA. Thus, agnostic whole-genome approaches are essential to deconvoluting and understanding the pathophysiology of microRNA–mRNA interactions in heart disease. Toward this end we have used next-generation sequencing of cardiac mRNA (RNA-Seq) to digitally measure absolute mRNA expression levels (ie, the transcriptome) in normal and diseased hearts, and have adapted a similar approach to quantifying the mRNAs within cardiac RISC complexes (RISC-Seq). The latter utilizes Argonaute 2 immunoprecipitation combined with microextraction and next-generation sequencing of associated mRNAs to identify RISC-targeted mRNAs (the RISCome), ie, those mRNAs that are enriched in the RISC in comparison with the transcriptome due to the actions of endogenous microRNAs. It is therefore also essential to quantify all the endogenously expressed microRNAs, for which next-generation sequencing protocols are available. By comparing the results of microRNA sequencing, RNA-Seq, and RISC-Seq performed under different sets of pathophysiological conditions (Figure 3A), it is possible to obtain an overview of how different diseases alter the cardiac microRNA–mRNA interactome. To date, this type of comprehensive and unbiased genome-wide approach to cataloguing disease-induced microRNA–mRNA interactions has not been performed for the heart.

The combination of RISC-Seq and RNA-Seq will identify microRNA targets, but does not indicate which of the expressed microRNAs is directly targeting which of the RISC-associated mRNAs. Available bioinformatics platforms can help refine the universe of potential microRNA–mRNA pairs, but their results can be inconsistent and are heavily biased toward near-perfect seed-sequence (microRNA nucleotides 2–8) complementarity. The procedure for “RISC programming” with a microRNA of interest to our in vivo analyses of cardiac microRNA–mRNA interactions.

Conceptually, we compare the RISComes of unprogrammed tissue (in which RISC-Seq will identify direct mRNA targets of all endogenous microRNAs) to those from tissue expressing higher levels of the microRNA of interest (in which the mRNA targets for that microRNA will be further enriched, in comparison with the unprogrammed RISCome). In vivo cardiac RISC programming can be achieved by conventional or inducible cardiomyocyte-specific transgenic overexpression, producing cell-autonomous data. We take care to express the programming microRNA(s) at levels that are observed in either healthy or diseased tissue, to prevent recruitment of nonphysiological mRNA targets to the RISC. Our analyses also evaluate...
The Multiple Degrees of Separation Between a MicroRNA and Its Induced Phenotype

The above discussion suggests how cardiac-directed forced expression of a microRNA can be useful for identifying its
mRNA targets. By extension, this approach can also help define the impact of differential mRNA targeting after genetic reprogramming, as in cardiac hypertrophy or heart failure. The conventional experimental transgenic approach overexpresses a factor, defines the resulting phenotype, and then fills in the intervening biological pathway to establish a plausible mechanism. When a microRNA is overexpressed, the underlying assumption is often that it will suppress a specific messenger RNA that encodes a specific protein, and that suppression of the protein will suppress a specific messenger RNA that encodes a specific protein, and that suppression of the protein produces the phenotype. This linear view, and its relatively modest variations (ie, some proteins regulate gene expression, etc., providing for feed-forward and feed-back regulation) has conceptual appeal because its simplicity and structure lend it to interrogation by molecular perturbation (Figure 4A). The cardiac literature is replete with manuscripts that follow this general experimental and analytic pattern, and much has been learned over the course of these investigations. Using miR-133a as a well-studied example, 6 relatively early manuscripts each reported single or small groups of mRNA targets implicated as the likely explanations for the disconnect between microRNA-targeted mRNA and protein include secondary and tertiary effects noted above, and the inherent bias of conventional proteomics toward the most highly expressed genes, which may not be the direct microRNA targets. Nevertheless, proteomics will be a useful addition to assays of bioinformatically predicted candidate mRNA targets and the point is that conclusions based on an underlying assumption that one miR primarily affects one mRNA whose protein product is largely responsible for an observed phenotype are likely to be significant oversimplifications of the true situation.

The following considerations suggest a more complex interpretation of microRNA actions and support a global/genome-wide approach to their delineation: first, many microRNAs are members of families having several other members with similar sequences; these different family members will have overlapping mRNA targets. For this reason, genetic ablation of one member of a microRNA family will not necessarily have a major effect when other family members remain. miR-133, for which there are 3 family members, provides an example of functional compensation with single gene ablation that was revealed with multigene ablation. Second, as noted above, a single microRNA typically targets dozens or more different mRNAs, and in many instances the effects on individual mRNAs and proteins are modest. Thus, the aggregate phenotype induced by a given microRNA results from the cumulative effects on all of its targets. Because multiple mRNA targets of a given microRNA or microRNA family tend to cluster within specific functional pathways (such as cell growth, differentiation, motility, or programmed death), this action of microRNAs can be considered as modular with respect to biological processes, rather than specific to one or a few mRNAs. Finally, the direct targets of microRNAs may have multiple degrees of separation from the end-organ phenotype, being a culmination of first-, second-, and third-generation effects. Figure 4B schematically depicts a microRNA signaling pathway that takes into account multiple microRNAs acting simultaneously on their mRNA targets, multiple mRNA targets being suppressed by a given microRNA, and reflecting likely avenues of regulatory feedback that can produce indirect secondary and tertiary effects. The best characterized cardiac example of indirect microRNA effects relates to myomiR (miRs-208a, -208b, and -499)–directed cardiac myosin isoform switching in cardiac hypertrophy. These effects are so striking that anti-miR 208a therapy has been shown to prevent hypertensive cardiac remodeling, and yet myosin heavy chain mRNAs are not direct targets of any myomiR. Thus, the major myomiR action on the heart, regulation of myosin isoforms, is indirect.

An unbiased approach to protein regulation by microRNAs would seem to complement the agnostic techniques of microRNA target identification and transcriptional profiling described above. Indeed, global proteomics have been used to connect molecular mechanism with microRNA-conferred phenotype, with some unexpected results. An important finding of the first in vitro global proteomics analyses of noncardiac cells was that the vast majority of proteins regulated by microRNAs are not direct miRNA targets. The likely explanations for the disconnect between microRNA-targeted mRNA and protein include secondary and tertiary effects noted above, and the inherent bias of conventional proteomics toward the most highly expressed genes, which may not be the direct microRNA targets.
steady-state quantification of their specific protein products when determining the mechanistic basis for microRNA-induced phenotypes.

The Pathological Potential for Genetic MicroRNA Variation

The unique ability of microRNAs to impact multiple effectors within a biological pathway optimally positions them as therapeutic targets. MicroRNA-based therapeutics offer an avenue to deal with the inherent plasticity of biological systems, in which critical responses are characteristically mediated through multiple parallel and redundant pathways. Biological redundancy provides an obvious evolutionary advantage: when the primary biological pathway is under attack by a pathological organism or genetic mutation, the essential response can be retained through induction of alternate mechanisms. For the same reasons, the reaction of an organism to experimental manipulation of a single molecular effector does not represent the normal role of that effector in the response. The stimulus–response is more like that of a water balloon than a linear pathway. When one side is pushed in, redundancy in the rest of the structure/pathway compensates and maintains overall systemic integrity. The ability of a single microRNA to coordinate multiple effectors within a given biological pathway avoids this compensation by functionally overlapping factors. Extending the water balloon metaphor, by simultaneously targeting multiple effectors in a given pathway, one can consider microRNAs to adjust the overall volume of water in the balloon.

A largely overlooked aspect of microRNA biology is the potential impact of naturally occurring nucleotide sequence variation on microRNA function, ie, microRNA mutations. It is striking that the nucleotide sequence of mature microRNAs tends to be very highly conserved across species, whereas sequence variability within putative microRNA binding sites in mRNA 3’ UTRs is common.64,65 This makes intuitive sense: nucleotide sequence is the primary determinant of microRNA function because it determines the binding energy of a microRNA–mRNA duplex, and therefore the efficiency of mRNA targeting to RISC and resulting mRNA suppression. With rare exceptions,66 and even though mRNA binding sites far outnumber microRNAs, sequence variation within a microRNA binding site will most likely have modest impact because it will affect the binding of only 1 family of microRNAs to only 1 binding site in only 1 target mRNA. By contrast, sequence variation in a mature microRNA has the potential to alter the mRNA targeting profile of that microRNA for any mRNA with a binding site that either loses or gains sequence complementarity as a consequence of the microRNA mutation. Consistent with this paradigm, a human seed sequence mutation in miR-96 produces heritable hearing loss.67 Recently, we uncovered a nonseed sequence mutation of human miR-499 that alters its mRNA targeting profile and modified the functional and proteomic phenotype induced by miR-499 overexpression in mouse hearts.68 A u to c mutation at nucleotide position 17 altered the pattern of cardiac RISC mRNA enrichment for a subset of mRNA targets whose binding sites had imperfect seed sequence complementarity and tended to have appropriately complementary nucleotides corresponding to miR-499 position 17. These findings demonstrate for the first time that a nonseed sequence mutation can alter microRNA function and the consequent organ/organism phenotype. These results support genetic screening for, and functional analysis of, microRNA sequence variants in human disease.

Final Thoughts

The ongoing explosion of microRNA research reflects a propitious convergence of biology and technology in an area in which nucleotide sequence is the key determinant of function. Next-generation sequencing is being used to globally profile microRNA and mRNA expression, to examine microRNA–mRNA interactions in different pathophysiological contexts, and to discover and evaluate the function of microRNA and binding site mutations. Sophisticated proteomics are being applied to assays of protein expression and posttranslational modification. Computerized algorithms can be used for in silico modeling of microRNA–mRNA interactions and to understand the structural determinants of biological function. Pathway analysis of microRNA–mRNA–protein interactions can be applied to results of genome-wide profiling studies to provide unbiased insight into integrated biological of specific microRNAs. Future efforts will therefore likely have greatest success when they take an agnostic approach, when they integrate multiple techniques, and when they bridge scientific disciplines.

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None.

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