

**Methodological Reviews** discuss methods that are of broad interest to the community of cardiovascular investigators and that enable a better understanding of cardiovascular biology, particularly recent technologies in which the methods are still in flux and/or not widely known. It is hoped that these articles, written by recognized experts, will be useful to all investigators, but especially to early-career investigators.

## The Art of MicroRNA Research

Eva van Rooij

**Abstract:** Originally identified as moderate biological modifiers, microRNAs have recently emerged as powerful regulators of diverse cellular processes with especially important roles in disease and tissue remodeling. The rapid pace of studies on microRNA regulation and function necessitates the development of suitable techniques for measuring and modulating microRNAs in different model systems. This review summarizes experimental strategies for microRNA research and highlights the strengths and weaknesses of different approaches. The development of more specific and sensitive assays will further illuminate the biology behind microRNAs and will advance opportunities to safely pursue them as therapeutic modalities. (*Circ Res.* 2011;108:219-234.)

**Key Words:** miRNA ■ cardiovascular ■ research methods

Over the past decade, it has become progressively more clear that a large class of small noncoding RNAs, known as microRNAs (miRNAs), function as important regulators of a wide range of cellular processes by modulating gene expression. Within 10 years of research, we have gone from discovering the existence of miRNAs in mammals to exploring their therapeutic applications in numerous diseases. Inherent to the rapid advancements and general excitement surrounding miRNA discoveries is the growing need for applicable and validated experimental tools to enable researchers to accurately study the expression and biological function of miRNAs. This review considers available experimental strategies and summarizes the strengths and weaknesses of different approaches with an emphasis on the involvement of miRNAs in cardiovascular disease.

### MicroRNA Breakthrough Discoveries in a Nutshell

Before the 1990s, miRNAs were an unappreciated class of small RNAs that were only thought to have a relevant function in nonmammalian species. The discovery by Ambros and colleagues on the role of the *lin-4* and *lin-14* genes in temporal control of development in the model organism *Caenorhabditis elegans* rapidly changed these views.<sup>1</sup> Whereas the Ambros laboratory discovered that *lin-4* gene

does not encode a protein product, but instead gives rise to a 61-nt precursor gene that matured to a more abundant 22-nt transcript,<sup>1</sup> the Ruvkun laboratory found that LIN-14 protein synthesis is regulated posttranscriptionally and that LIN-14 levels are inversely proportional to those of *lin-4* RNA.<sup>2</sup> Sequence analysis revealed that the *lin-4* RNA has sequence complementarity to the 3' untranslated region (UTR) of the *lin-14* gene, leading to the hypothesis that *lin-4* regulated LIN-14, in part, through Watson-Crick base pairing, revealing the first miRNA and mRNA target interaction.<sup>1,2</sup>

For 7 years, *lin-4* was considered an anomaly, until the discovery of a second *C. elegans* miRNA, called *let-7*, which repressed *lin-41*, *lin-14*, *lin-28*, *lin-42*, and *daf-12* expression during development.<sup>3</sup> The identification of *let-7* homologs in many vertebrate species including humans<sup>4</sup> stimulated a large cloning effort of small RNAs, demonstrating that miRNAs are evolutionarily conserved across many species and are often ubiquitously expressed.<sup>5-7</sup> Seminal follow-up work by many laboratories unveiled basic concepts of miRNA biogenesis and function (Figure 1).

In 2002, shortly after the expression of mammalian miRNAs was recognized, Calin et al showed a correlation between miRNA abundance and human disease, by indicating an association between the loss of miR-15 and -16 and the occurrence of B-cell leukemia.<sup>8</sup> It was not until 2006 that the first cardiac miRNA-profiling study appeared, linking dysregulation of many

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Non-standard Abbreviations and Acronyms	
<b>AAV</b>	adeno-associated virus
<b>Ago</b>	Argonaute
<b>EGFP</b>	enhanced green fluorescent protein
<b>HCV</b>	hepatitis C virus
<b>HITS-CLIP</b>	high-throughput sequencing to crosslinking immunoprecipitation
<b>ISH</b>	in situ hybridization
<b>LNA</b>	locked nucleic acid
<b>MHC</b>	myosin heavy chain
<b>miR</b>	microRNA
<b>miRNA</b>	microRNA
<b>pri-miRNA</b>	primary microRNA transcript
<b>qPCR</b>	quantitative polymerase chain reaction
<b>RISC</b>	RNA-induced silencing complex
<b>T<sub>m</sub></b>	melting temperature
<b>UTR</b>	untranslated region

different miRNAs to cardiac remodeling in both mice and humans.<sup>9</sup> Today, microarray analysis and deep-sequencing approaches have enabled researchers to correlate the dysregulation of miRNAs to the progression of many different diseases in both animals and humans.

As a logical consequence of the recognition of disease-specific miRNA expression signatures, miRNA researchers started investigating whether these expression changes were causally related to disease. Although many *in vitro* studies indicated prominent and defined functions for miRNAs in different aspects of cell biology, the first genetic evidence for the importance of miRNAs in mammals came from a study showing that homozygous deletion of *Dicer*, a key miRNA processing gene, disrupted prenatal development of the murine embryo through its role in miRNA biogenesis.<sup>10</sup> The conditional *Dicer* allele<sup>11,12</sup> has now been used to study the relevance of miRNAs in many different organ systems, including the heart. In 2007, by deleting *Dicer* under the control of the *Nkx2.5* promoter, it was shown that *Dicer* is essential in prenatal cardiomyocytes for cardiogenesis,<sup>13</sup> whereas a year later, Chen showed that *Dicer* deletion in postnatal myocytes using a Cre recombinase driven by the  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) promoter leads to rapidly progressive dilated cardiomyopathy, heart failure, and lethality.<sup>14</sup>

In that same time frame, the first 3 genetic deletion studies of the function of specific miRNAs were reported, including a muscle-specific miRNA (miR-1-2),<sup>13</sup> a cardiac-specific miRNA (miR-208),<sup>15</sup> and a T-cell-expressed miRNA (miR-155).<sup>16</sup> Targeted deletion of miR-1-2 revealed numerous functions for this miRNA in the heart, such as regulation of cardiac morphogenesis, electric conduction, and cell cycle control.<sup>13</sup> In contrast, genetic deletion of miR-208, which is encoded by an intron of the  $\alpha$ -MHC gene, did not result in an overt phenotype at baseline. However, this miR-208 was found to be required for cardiomyocyte hypertrophy, fibrosis, and expression of  $\beta$ -MHC in response to cardiac disease and

hypothyroidism. Thus, the  $\alpha$ -MHC gene, in addition to encoding a major cardiac contractile protein, regulates cardiac growth and gene expression in response to stress and hormonal signaling through miR-208.<sup>17</sup> Although several miRNA mutant animals have now been shown to induce partial embryonic lethality, eg, the miR-133a-1/-2 double knockout and the miR-126 knockout,<sup>18,19</sup> the lethality of the miR-1-2 mutant animals is exceptional because removal of 1 copy of miR-1, while leaving miR-1-1 intact, is apparently sufficient to drive a phenotype.

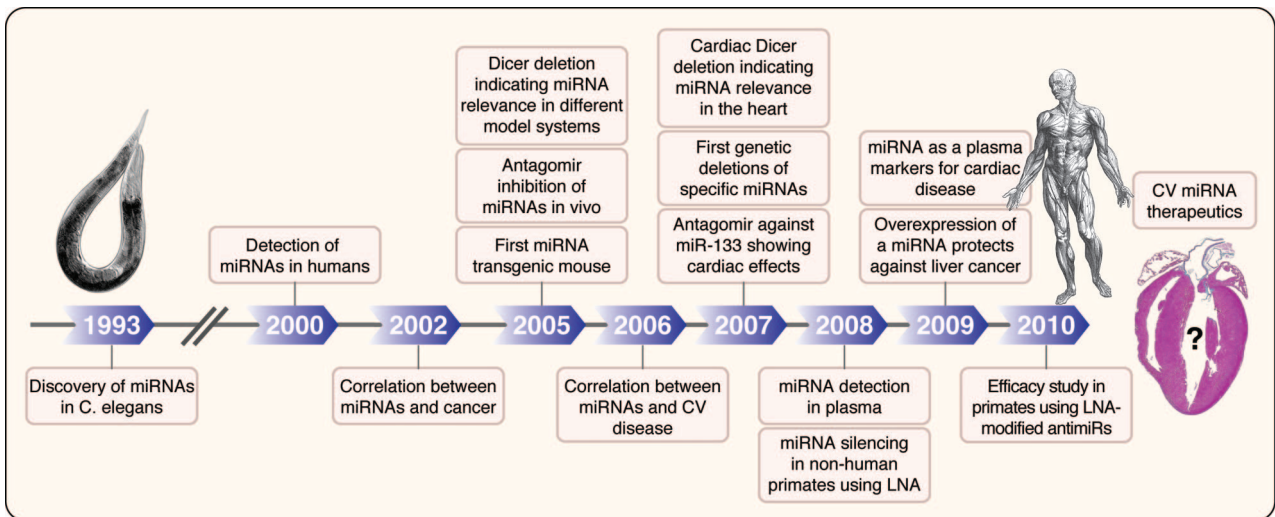
Recently, miRNAs were detected in serum and plasma of humans and animals, opening the possibility of using miRNAs as diagnostic biomarkers of various diseases. The levels of miRNAs in serum are stable, reproducible, and consistent among individuals of the same species.<sup>20</sup> Although initial reports focused on using miRNAs as plasma markers for different forms of cancer, in 2009, Ji et al showed that plasma biomarkers can also detect cardiac injury. Plasma concentrations of miR-208 increased significantly after isoproterenol-induced myocardial injury in rats and showed a similar time course to the concentration of cardiac troponin I, a classic biomarker of myocardial injury.<sup>21</sup> Since then, several reports have correlated miRNAs in plasma with myocardial infarction and heart failure.<sup>22–24</sup>

Earlier advances in small interfering RNA-based therapeutic strategies facilitated rapid progress toward the therapeutic manipulation of miRNAs. In 2005, Krutzfeldt and colleagues reported on the feasibility of manipulating miRNA levels *in vivo* using intravenous administration of a novel class of chemically engineered oligonucleotides, termed “antagomirs.” These initial experiments validated the *in vivo* efficacy of antagomirs and provided a powerful new research tool to silence specific miRNAs *in vivo*.<sup>25,26</sup> Although these initial reports mainly focused on targeting the liver enriched miR-122, in 2007, Care et al reported for the first time on cardiac silencing of miR-133 using a comparable approach.<sup>27</sup> More recently, other unconjugated chemistries with high binding affinities have been shown to be efficacious *in vivo*, one of them being locked nucleic acid (LNA). Systemic delivery of unconjugated LNA-antimiR potently antagonized the liver-expressed miR-122 in mice and nonhuman primates<sup>28,29</sup> and has now also been shown to be efficacious in chronically infected chimpanzees by suppressing hepatitis C virus (HCV) viremia and improving HCV-induced liver pathology.<sup>30</sup> The potential and possible pitfalls for the therapeutic use of antimiR approaches are discussed in the section Therapeutic MicroRNA Inhibition *In Vivo* (below).

This rapid advancement in new miRNA discoveries has triggered the curiosity of many basic scientists. The remainder of this review serves to outline the fundamental approaches that are most commonly used for miRNA detection, target determination, or miRNA regulation.

### MicroRNA Detection

The integral first step in miRNA research is detection of the miRNA. miRNA biogenesis is governed by many regulatory checkpoints. The primary miRNA transcripts are transcribed as precursor molecules called pri-miRNAs, derived either from annotated transcripts (as the introns of protein coding

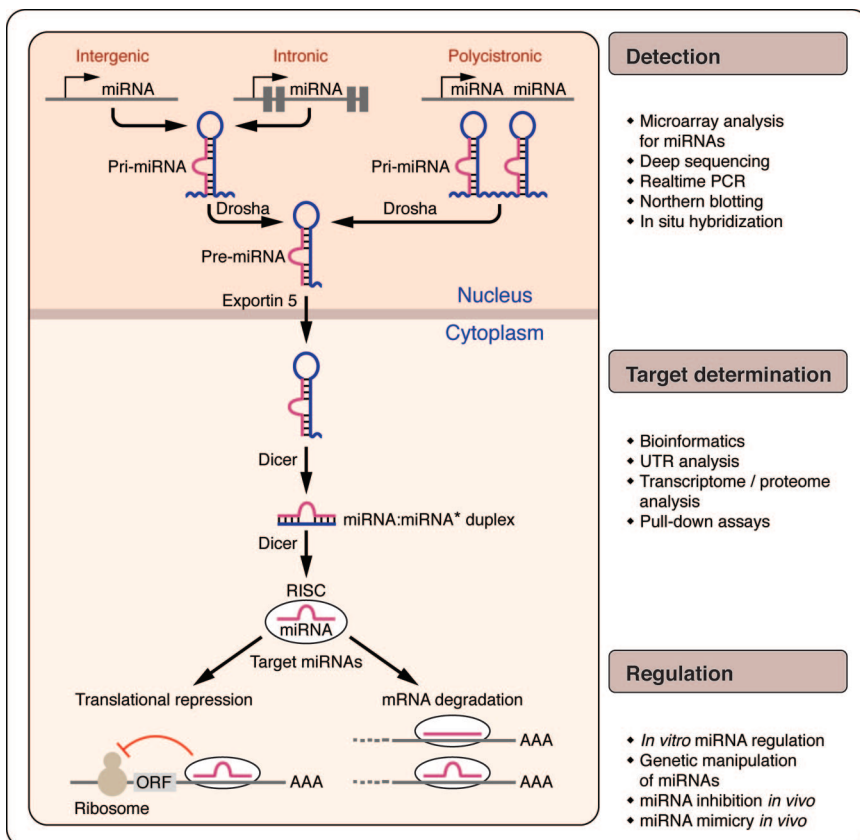


**Figure 1. Breakthrough discoveries in miRNA biology.** Time line indicating seminal discoveries in miRNA biology with a special focus on the cardiovascular field.

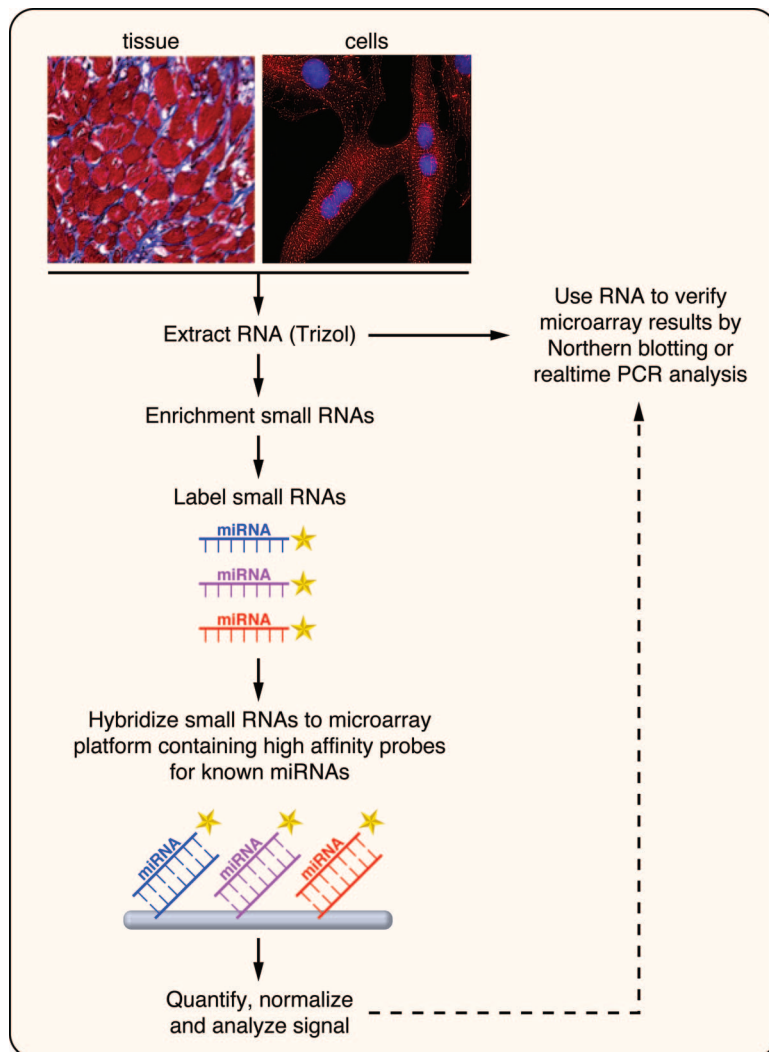
genes, the exons of noncoding genes, or the introns of noncoding genes) or from intergenic regions within the genome and can encode a single or multiple miRNAs.<sup>31,32</sup> Pri-miRNAs fold into hairpin structures containing imperfectly base-paired stems and are processed into 60- to 100-nt hairpins known as pre-miRNAs.<sup>33–35</sup> The pre-miRNAs are exported from the nucleus to the cytoplasm by exportin 5,<sup>36</sup> where they, in general, are cleaved by the endonuclease Dicer to yield imperfect miRNA-miRNA\* duplexes.<sup>37</sup> The miRNA strand is selected to become a mature miRNA, whereas, most

often, the miRNA\* strand is degraded. The mature miRNA is incorporated into the RNA-induced silencing complex (RISC), which recognizes specific targets and induces post-transcriptional gene silencing (Figure 2).<sup>38</sup> However, an alternative biogenesis pathway was recently discovered in which miR-451 enters RISC by direct loading of the pre-miR into RISC after Drosha processing, by skipping further processing by Dicer.<sup>39</sup>

Because the expression of the initial transcript does not linearly correspond to the expression level of mature



**Figure 2. miRNA biogenesis and research tools.** The primary transcripts of miRNAs, called pri-miRNAs, are transcribed as individual miRNA genes, from introns of protein-coding genes, or from polycistronic transcripts. The RNase Drosha further processes the pri-miRNA into 70- to 100-nt, hairpin-shaped precursors, called pre-miRNA, which are exported from the nucleus by exportin 5. In the cytoplasm, the pre-miRNA is cleaved by Dicer into a miRNA:miRNA\* duplex. Assembled into the RISC, the mature miRNA negatively regulates gene expression by either translational repression or mRNA degradation, which is dependent on sequence complementarity between the miRNA and the target mRNA. ORF indicates open reading frame. Commonly used research tools to study miRNAs are indicated on the right and are discussed in more detail in the remainder of the review.



**Figure 3. Microarray analysis of miRNAs.** The initial step to perform microarray miRNA analysis is the purification of mature miRNAs from fresh or fixed cells or tissues. After extraction, the miRNAs are enriched and labeled with a dye and then directly hybridized to the arrays with the appropriate probes specific for mature miRNAs. The resulting double-stranded fragments can be easily detected. Whereas some constitutively expressed miRNAs and U6 or tRNA are used for data normalization, validation and comparison, Northern blot, and quantitative RT-PCR analysis on the original starting material can serve to verify the microarray data.

miRNAs, only the determination of the level of the mature miRNA will accurately indicate whether a miRNA is present and/or regulated in its abundance. Currently, various applications are available to determine the abundance of miRNAs. The expression profiles of many different miRNAs in parallel can be measured by microarray analysis or deep sequencing, whereas Northern blotting, real-time RT-PCR, and in situ hybridization (ISH) can be used to determine the level of individual miRNAs. The pros and cons of these specific detection methods are outlined in the next section.

### Microarrays to Detect MicroRNAs

Microarray analysis allows for parallel analysis of large numbers of miRNAs and can be used to detect the presence and/or regulation of a wide range of defined miRNAs. The initial step in miRNA microarray profiling is the purification of RNA or miRNAs from cells or tissue. Many protocols have been developed for the extraction of high-quality RNA using various kits and reagents. Although it is possible to use total RNA for microarray analysis, because small RNAs only make up  $\approx 0.01\%$  of all RNAs, miRNA enrichment increases sensitivity.<sup>40</sup> After extracting RNA, the mature miRNAs can be directly labeled, usually by using T4 RNA ligase, to attach

1 or 2 fluorophore-labeled nucleotides to the 3' end of the miRNA (Figure 3).

In the detection of miRNAs by microarray analysis, appropriate probe design is critical. In all gene expression microarrays, either synthetic oligonucleotides or cDNA fragments are used as capture probes, which ideally have a high specificity and affinity for individual transcripts. Because miRNAs are small ( $\approx 22$  nt) the probe design possibilities to detect a miRNA are limited and based on the miRNA sequence can vary between  $45^{\circ}\text{C}$  and  $74^{\circ}\text{C}$  in their melting temperatures ( $T_m$ ). If a specific hybridization temperature is used to bind the labeled miRNAs to the arrays, the capture probes with the lower  $T_m$  values will yield lower signals, whereas probes with higher  $T_m$  values will show impaired nucleotide discrimination and lower specificity.<sup>41</sup> Because the binding affinities differ among miRNAs, the microarray should in principle not be used to make quantitative statements, but rather serve to determine the relative change in expression between 2 states, for example, nondiseased versus diseased, or should be used to determine the presence of a specific miRNA (Figure 3). However, increasing or reducing the length of the probe based on the physicochemical characteristics of a particular miRNA can result in a better balance for the melting

temperatures of the miRNA probes and make the affinity comparable for all probes, to provide a more accurate array-based analysis.<sup>42</sup>

Several modifications increase the stability of the miRNA: capture probe duplex. 2'-O-methyl modifications of the nucleotides increase the hybridization affinity. Additionally, introducing LNAs, which depending on the position of the LNA moiety in the oligonucleotide, increase the  $T_m$  up to 5°C to 8°C with every LNA monomer, can increase the binding affinity of the capture probe.<sup>43</sup> Although microarray analysis may require optimization, it provides a useful tool to survey the miRNAs that are expressed or dysregulated in a tissue of interest. However, these data should be viewed as a guide and should be confirmed by other detection methods.

Microarray analysis has been widely used to determine the expression of miRNAs in both heart tissue and in cardiac-relevant cell types or to determine whether there is a specific miRNA expression "signature" during a certain disease state. Initially, several profiling studies indicated miRNA dysregulation in cardiac hypertrophy and human idiopathic cardiomyopathy and in response to ischemia in mice, and in 2007, Ikeda et al indicated there to be a disease state-specific miRNA signature pattern correlating to different forms of human heart disease.<sup>44</sup> A comprehensive overview of these studies was provided in a recent review by Small et al.<sup>45</sup> Currently, the available platforms screen for roughly 700 murine and 1000 human miRNAs. In general, tissue samples will result in a better miRNA detection level than cell cultures. Although it is feasible to profile miRNA expression in vitro, there are some confounding factors that should be taken into account when performing miRNA studies in culture (see the section In Vitro MicroRNA Regulation).

Screening for miRNAs in plasma can potentially serve to provide a novel noninvasive biomarker for different diseases. Although most miRNA plasma detection studies for cardiac disease have been performed using real-time PCR for specific miRNAs, Tijssen et al used a microarray approach to screen for differential expression of miRNAs in plasma from either healthy controls or patients suffering from heart failure.<sup>24</sup> These data indicate the feasibility of using a microarray platform to detect changes in plasma miRNAs as a marker for heart disease.

### MicroRNA Detection by Deep Sequencing

In addition to using microarray technology, recently, next generation sequencing platforms such as Genome Analyzer (Illumina Inc) or Genome Sequencer FLX (454 Life Science and Roche Applied Science) became available for the sequencing of small RNA molecules, including miRNAs. Deep sequencing uses massively parallel sequencing, generating millions of small RNA sequence reads from a given sample. The sensitivity of deep sequencing offers an advantage over standard hybridization techniques (microarray), because the wide range of miRNA expression from tens of thousands to just a few molecules per cell complicates the detection of miRNAs expressed at low copy numbers. Unlike profiling of miRNAs by using microarray analysis, deep sequencing measures absolute abundance and, because it is not limited by array content, allows for the discovery of novel miRNAs that

have eluded previous cloning and standard sequencing efforts. A combination of criteria is used to define whether a sequence is a putative new miRNA. The transcript should give rise to a  $\approx$ 22-nt-long transcript that maps precisely to the genome of interest. Additionally, the sequence should be phylogenetically conserved and should be able to form a hairpin structure without large internal loops or bulges.<sup>46</sup> RNA-folding prediction software, such as mfold, can be applied to test whether the sequence can be folded into a miRNA-like hairpin structure.<sup>47</sup> However, because deep sequencing-based expression analysis is still in its infancy, and each sequencing experiment produces up to 3 Gbp of sequence data, there are substantial bioinformatic challenges to appropriately analyze and handle such sequence information.

Although deep sequencing data formed the basis for some of the seminal miRNA expression studies, currently, there are only a few reports containing deep sequencing results from either skeletal or cardiac muscle. Nielsen et al performed deep sequencing on multiple longissimus dorsi samples from crossbred pigs (*Sus scrofa*). After purifying the small RNA fraction, the Illumina Genome Analyzer system was used to analyze the identity and abundance of the miRNAs expressed. Of the 32 million detected reads, 95% were assigned to 212 known microRNAs. The most abundant miRNA was miR-1, accounting for almost 90% of the reads, whereas the second most abundant miRNA was miR-206, with 5.7% of all reads.<sup>48</sup> As has previously been shown for human and rodent cell types,<sup>49–51</sup> the sequences of the majority of the detected miRNAs indicated length and/or end-sequence variations. Based on predicted folding properties, the sequencing identified 35 potential novel miRNAs, although Northern blot analysis for 6 of these did not show the expected sizes for either a mature or precursor miRNA.<sup>48</sup>

The sole report on cardiac deep sequencing was published by Rao et al using the Illumina platform to sequence small RNAs from male and female adult murine hearts. Although it was not disclosed how many different known and unknown miRNAs were detected, the authors showed that 40% of the cardiac reads were miR-1, with other abundant miRNAs being miR-29a, miR-26a, and let-7 family members. Additionally, it was shown that there might be potential gender differences in the expression of cardiac miRNAs.<sup>52</sup> Based on our own deep sequencing experience on diseased versus nondiseased samples in both mice and human cardiac tissue samples, it is expected that roughly 200 miRNAs are expressed in the heart and few novel cardiac miRNAs remain to be annotated (E. van Rooij, unpublished data).

### Real-Time PCR for MicroRNAs

Although global expression profiling assays are useful to provide a broad overview of the presence and regulation of miRNAs, these data need to be confirmed by miRNA-specific approaches to determine their accuracy. By far, the most commonly used method to detect specific miRNAs is real-time PCR analysis. Currently, there are several different approaches for this reaction.<sup>53</sup> The next section briefly summarizes the most commonly used real-time PCR methods and points out potential advantages and disadvantages.

A miRNA real-time reaction starts with reverse transcribing RNA into cDNA. The limited length of the mature miRNA ( $\approx 22$  nt), the lack of a common sequence feature like a poly(A) tail, and the fact that the mature miRNA sequence is also present in the pri- and pre-miRNA transcript pose several challenges for appropriate reverse transcription. However, to date, mainly 2 different methods are used for the reverse transcription: miRNA-specific or universal reverse transcription (Figure 4). In the first approach, miRNAs are reverse transcribed individually by using stem-loop-specific reverse transcription primers. Stem-loop primers are designed to have a short single-stranded region that is complementary to the known sequence on the 3' end of the miRNA, a double-stranded part (the stem), and the loop that contains the universal primer-binding sequence. The resulting cDNA is then used as a template for quantitative (q)PCR with 1 miRNA-specific primer and a second universal primer (TaqMan PCR, Applied Biosystems). Stem-loop primers are more difficult to design, but their structure reduces annealing of the primer to pre- and pri-miRNAs, thereby increasing the specificity of the assay (Figure 4A). The second approach first tails all miRNAs with a common sequence and then reverse transcribes the miRNA by using a universal primer. This approach is widely used and especially useful if several different miRNAs need to be analyzed from a small amount of starting material. The 3' ends of all miRNAs are elongated with a poly(A) tail using *Escherichia coli* poly(A) polymerase (miRCURY, Exiqon). A primer consisting of an oligo(dT) sequence with a universal primer-binding sequence at its 5' end is then used to prime reverse transcription and to amplify the target sequences in the qPCR reaction. The stretch of "dT" between the miRNA and the universal sequence of the oligo(dT) primer is defined by using a template binding sequence at the 5' end of the primer that anchors the primer to the 3' end of the miRNA (Figure 4B). Although the methods using the universal reaction tend to be more expensive, because 1 reaction will transcribe all miRNAs in a certain sample, this approach might, in the long run, be more efficient and cost-effective for broader miRNA-profiling studies.

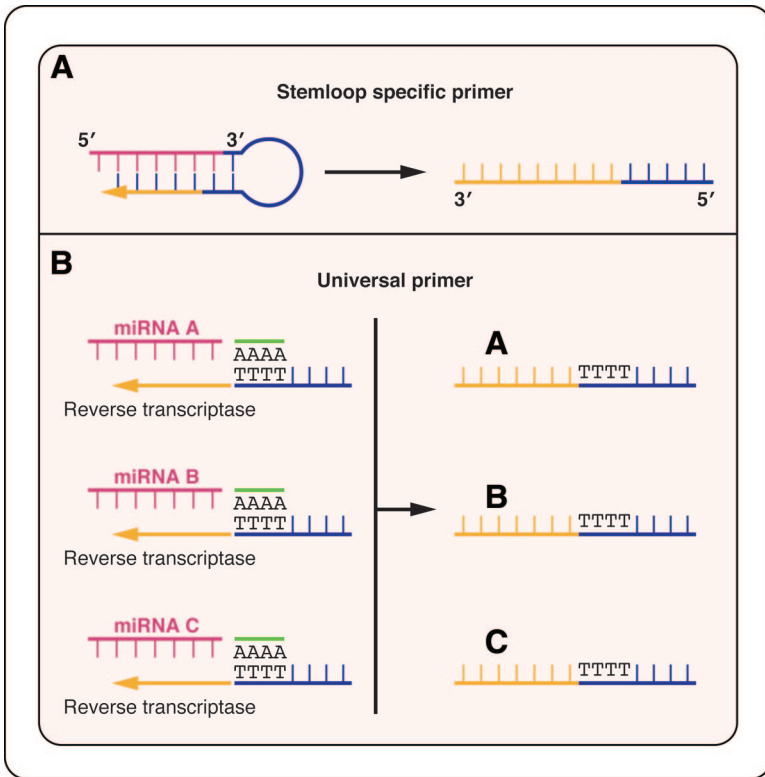
Even though there are several methods to reverse transcribe a miRNA, the specificity and sensitivity of any real-time assay is dependent on the design of the miRNA-specific primer. Because the binding affinity of a primer is determined by the sequence, the GC content of a miRNA determines the  $T_m$  against the complementary sequence of the miRNA-specific primer. One should be cognizant of the fact that the binding affinity of a primer is miRNA-specific and determines the efficiency of miRNA amplification, making it difficult to use qPCR to determine the relative abundance of different miRNAs. One way to circumvent this issue is to run a standard curve in parallel for samples containing known quantities of synthetic miRNA copies. At the same time, the assay is confounded by the existence of closely related miRNAs that often only differ by several bases in sequence. Although the primers are designed to be miRNA-specific, because of the high degree of homology between miRNAs, these assays are susceptible to cross-reactivity among different miRNA family members. As already discussed, LNAs

increase the thermostability of nucleic acid duplexes. One way to increase the  $T_m$  independent of sequence is by incorporating LNAs into an oligonucleotide primer.

### Northern Blot Analysis for MicroRNAs

Northern blotting is widely used to visualize specific miRNAs. Although it is fairly time consuming (at least 2 days) and requires large amounts of RNA (8  $\mu$ g or more), it is the only approach that will visualize the expression of a miRNA, as well as the pre-miRNA (Figure 5A). There are several methods for miRNA Northern blotting that involve comparable procedures. After isolating total RNA from cells or tissue, the small RNAs are fractionated by electrophoresis on a high percentage. After transferring these small RNAs from the gel onto a membrane, the RNA is fixed onto the membrane by UV crosslinking and/or baking the membrane. Because of the small size and the low abundance of miRNA molecules, the use of an oligonucleotide probe with increased sensitivity is essential to detect the miRNA. The miRNA StarFire system from IDT provides an effective means for miRNA detection. In this system, the probe sequence is extended with a hexamer that binds to the 3' end of a provided universal template oligonucleotide that consists of oligo-dT10 sequence at its 5' end. Through a DNA polymerase extension reaction, radiolabeled  $\alpha$ - $^{32}$ P-dATP is added to the miRNA probe, resulting in a miRNA-specific probe containing a radioactive poly(A) (Figure 5B). These probes allow for efficient detection of miRNAs. The ability of the Northern blot probe to distinguish between related miRNAs depends on the level of sequence homology and the position of the base mismatch(es). If the base mismatches are distributed across the length of the miRNA sequence, 3-bp mismatches are sufficient to confer probe specificity (Figure 5A). However, oftentimes, related miRNAs differ by only 1 to 2 bases in their 3' sequence, which makes it difficult to prevent the probe from cross-reacting with the different miRNA family members. As for many assays in miRNA research, the detection affinity depends on the sequence of the mature miRNA. In general, a miRNA that is more GC-rich will bind more efficiently to a detection probe consisting of the complementary reverse sequence. A higher binding affinity and potentially more specificity can be achieved by using LNA-containing Northern blot probes (miRCURY, Exiqon). Using LNA-modified oligonucleotide probes increases the  $T_m$  and, as such, the binding affinity. Standard end-labeling techniques using a T4 kinase with  $\gamma$ - $^{32}$ P-ATP can be used to add a radioactive phosphate to the 3' end of the probe (Figure 5C). Although LNA-modified Northern blot probes are more sensitive than DNA-labeled probes, experience teaches that the poly(A)-labeled oligonucleotide probes result in stronger signal detection.

Additionally, nonradioactive labeling of miRNA probes can be accomplished by direct incorporation of fluorescent tags, crosslinking enzyme molecules directly to nucleic acid, or the incorporation of tagged nucleotides such as biotin or digoxigenin during synthesis of the probe. After using standard blotting and hybridization procedures, labeled probes that hybridize to a target miRNA sequence are detected with streptavidin (biotin) or anti-digoxigenin monoclonal anti-



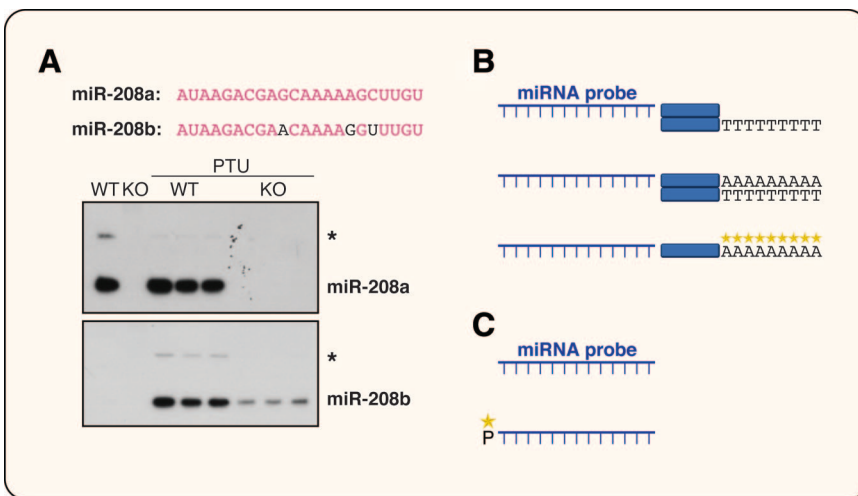
**Figure 4. miRNA-specific reverse transcription.** **A**, In the first approach, miRNAs are reverse transcribed individually by using stem-loop-specific reverse transcription primers that are designed to have a short single-stranded region that is complementary to the known sequence on the 3' end of the miRNA, a double-stranded part (the stem) and the loop that contains the universal primer-binding sequence. The resulting reverse transcription product (cDNA) is then used as a template for qPCR with 1 miRNA-specific primer and a second universal primer (TaqMan PCR, Applied Biosystems). **B**, The second approach first elongates the 3' ends of all miRNAs with a poly(A) tail using *E coli* poly(A) polymerase (miRCURY, Exiqon). A primer consisting of an oligo(dT) sequence with a universal primer-binding sequence at its 5' end is then used to prime reverse transcription and to amplify the target sequences in the qPCR reaction. The stretch of "dT" between the miRNA and the universal sequence of the oligo(dT) primer is defined by using a template binding sequence at the 5' end of the primer that anchors the primer to the 3' end of the miRNA. This approach is especially useful if several different miRNAs need to be analyzed from a small amount of starting material.

body, after which the enzyme activity is usually detected by a chemiluminescent reaction.

**In Situ Hybridization**

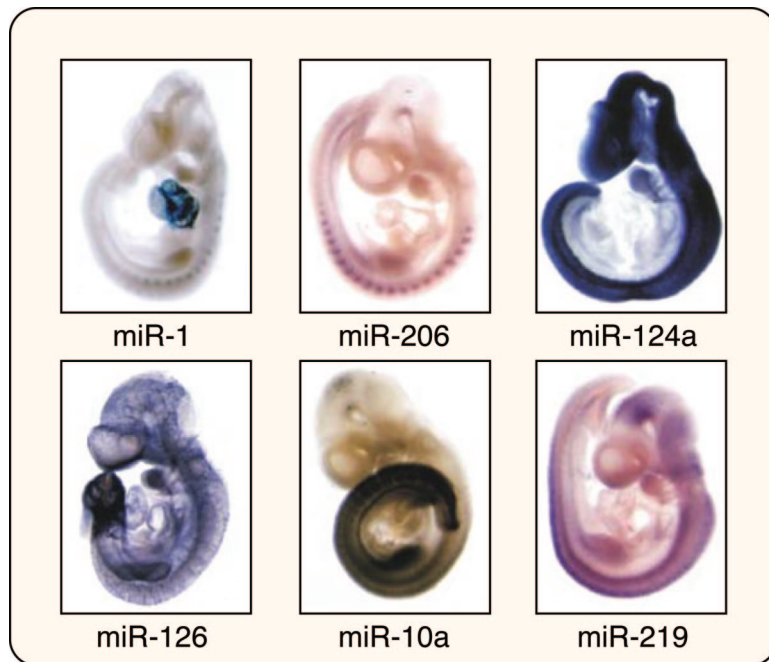
Detection of miRNAs by ISH is technically challenging because of the small size of target sequences. Aboobaker et al described the expression patterns of several precursor miRNA molecules in *Drosophila* using long (~1 kb) RNA

probes antisense to miRNA loci. However, this approach could only detect the longer precursor molecules and not the short mature miRNA.<sup>54</sup> Because of their high binding affinity, LNA-containing probes are able to anneal to miRNAs with high specificity (Exiqon). Kloosterman et al examined the spatial expression patterns of more than 100 zebrafish miRNAs using LNA-modified using digoxigenin-labeled LNA ISH probes and compared these data to microarray data



**Figure 5. Northern blot and oligonucleotide probes.** **A**, Northern blot analysis for 2 related miRNAs indicating the specificity of detection using miRNA-specific detection probes. Detected is the wild-type (WT) expression of miR-208a in the heart, which is absent in the miR-208a knockout animals (KO). Propylthiouracil (PTU) induces the expression of the normally absent miR-208b, an effect that is blunted in the miR-208a KO animals. The miR-208a probe does not detect miR-208b (as seen for the PTU KO animals), and the miR-208b probe does not detect miR-208a (as seen for the WT lane). The ability of the Northern blot probe to distinguish between related miRNAs depends on the level of sequence homology and the position of the base mismatch(es). If the base mismatches are distributed across the length of the miRNA sequence, 3-bp mismatches are

sufficient to confer probe specificity. **Asterisk** indicates the premiR. **B**, The miRNA StarFire system from IDT provides an effective means for miRNA detection. In this system, the probe sequence is extended with a hexamer that binds to the 3' end of a provided universal template oligonucleotide that consists of oligo-dT10 sequence at its 5' end. Through a DNA polymerase extension reaction, radiolabeled  $\alpha$ -<sup>32</sup>PdATP is added to the miRNA probe, resulting in a miRNA-specific probe containing a radioactive poly(A). **C**, A higher binding affinity and potentially more specificity can be achieved by using LNA-containing Northern blot probes (miRCURY, Exiqon). Using LNA-modified oligonucleotide probes, increases the *T<sub>m</sub>* and as such the binding affinity. Standard end-labeling techniques using a T4 kinase with  $\gamma$ -<sup>32</sup>P-ATP can be used to add a radioactive phosphate to the 3' end of the probe.



**Figure 6. Whole-mount miRNA ISH.** Whole-mount in situ detection of miRNAs using LNA probes on mouse embryos showing tissue-specific expression patterns: miR-1, heart, and somites; miR-206, somites; miR-124a, central nervous system; miR-126, blood vessels; miR-10a, posterior trunk; miR-219, midbrain, hindbrain, and spinal cord. Adapted from Kloosterman et al<sup>55</sup> with permission from the Nature Publishing Group.

for the same miRNAs. Whole-mount ISH in both zebrafish and mouse embryos showed that most miRNAs are expressed in a tissue-specific (or even cell-specific) manner during segmentation and later stages but not during early embryonic development. Although some miRNAs were expressed ubiquitously, several miRNAs showed tissue- or cell-specific expression patterns (Figure 6).<sup>55</sup> Using a comparable detection technique, Darnell et al were able to visualize the expression patterns of many different miRNAs in chicken embryos.<sup>56</sup>

Whole-mount ISH can be done in mouse embryos up to embryonic day 11.5, however, at later stages, the increased size and thicker skin inhibits proper penetration and perfusion of the probe into the tissue. To circumvent this problem, several studies performed ISH on dissected tissues<sup>57</sup> or sections. Although it is feasible to perform ISH on paraffin sections using the LNA-modified probes,<sup>58</sup> most studies to date have used cryosections for which several protocols are currently available on line.<sup>59–61</sup>

### MicroRNA Target Determination

The function of a miRNA is ultimately defined by the genes it targets and its effects on their expression. A given miRNA can be predicted to target several hundred genes, and  $\approx 60\%$  of mRNAs have predicted binding sites for 1 or multiple miRNAs in their UTR. Two major silencing mechanisms have been identified for miRNAs: miRNAs can inhibit translation by inhibition of translation initiation or translation elongation or can target mRNAs for degradation.<sup>62–64</sup> Under baseline conditions, miRNAs appear to act as moderate regulators that act as a rheostat to fine tune gene expression, but under conditions of stress or disease, they appear to exert more pronounced functions. Most miRNAs bind to the 3' UTRs of target mRNAs and most commonly form imperfect base heteroduplexes with target sequences. Nucleotides 2 to 8

of the miRNA, termed the “seed” sequence, are essential for target recognition and binding.<sup>65</sup>

One of the most interesting aspects of miRNA biology is that 1 miRNA often regulates multiple genes that are involved in a specific signaling cascade or cellular mechanism, making miRNAs potent biological regulators. However, defining the gene targets through which a miRNA functions is probably also the most tedious aspect of miRNA research. Initial insight into miRNA targets can be obtained bioinformatically through a number of freely available programs that predict potential mRNA targets for individual miRNAs. Because these programs only predict putative targets, it is important to confirm these predictions using miRNA target validation techniques. Because a miRNA can target many different mRNAs in a temporal and cell type-specific manner, often regulates gene expression in a moderate way, and can do so on the mRNA and/or protein level, sensitive and accurate detection methods are crucial to study the function of a particular miRNA. The next section addresses the basic methods available for miRNA target prediction and verification.

### Bioinformatic Prediction of MicroRNA Targets

Bioinformatic target prediction is often the first step toward defining the function of a specific miRNA. Currently, there are a number of freely available programs, such as miRanda (<http://www.microRNA.org>), microCosm (previously known as miRBase targets, <http://www.mirbase.org>), Targetscan (<http://www.targetscan.org>), or PicTar (<http://pictar.mdc-berlin.de>) that will help predict which mRNAs a miRNA can potentially target or which miRNAs might be able to target a certain gene of interest.

These target prediction programs use several characteristics to determine whether a miRNA can potentially target an mRNA. The 5' seed region of the miRNA (bases 2 to 8) must show sequence complementarity to the 3' UTR of a target gene, and the target site within the mRNA should be

conserved among different species. Often, the thermal stability of the mRNA/miRNA duplex, together with the absence of complicated secondary structures surrounding the miRNA binding site, is taken into account to predict whether a miRNA is likely to target an mRNA. However, in addition to these common characteristics in target prediction, there are some distinct differences between the several approaches.

Although all of the prediction algorithms use the seed sequence as the main determinant of target site recognition, PicTar additionally allows for both perfect and imperfect seed complementarity. The perfect seed is defined as perfect Watson–Crick–bp complementarity of 7 nucleotides, starting at either the first or second base of the 5′ end of the miRNA. Imperfect complementarity allows for an insertion or mutation as long as the free energy of binding of the miRNA/mRNA duplex does not increase or does not contain a G·U base-pairing. Both TargetScan and PicTar improve their predictions by taking into account evolutionary conservation. TargetScan also adds a “context score,” which considers features in the surrounding mRNA, including local A·U content and location (near either end of the 3′ UTR is preferred) and improves predictions for nonconserved sequences. mRNAs that have a high context score or multiple predicted miRNA-binding sites are more likely to be true targets. Additionally, TargetScan includes a special class of seed matches with a hexamer match in positions 2 to 7, plus an adenosine at position 1 (reviewed elsewhere<sup>66</sup>).

Although seed pairing is weighed more strongly than pairing elsewhere, the miRanda algorithm aligns a miRNA to the target mRNA to identify highly complementary sequences, whereby allowing for seed G·U wobbles and mismatches. High-scoring targets are then filtered on a secondary criterion of heteroduplex free energy ( $\Delta G$ ), whereas only conserved predictions are considered.<sup>67</sup> Because miRanda does not require exact seed pairing, it predicts sites such as the 2 *let-7* sites in the *Caenorhabditis elegans* gene *lin-41*, which contain either a bulge or a G·U wobble in the seed region.

Combining the results of different target prediction programs to look for overlap in predicted targets between the different programs will result in the highest specificity but lowest sensitivity. On the other hand, combining the results of all programs will lead to the highest sensitivity but lowest specificity. Based on experimentally validated data sets, it has been recommended that intersecting Targetscan and PicTar predictions often results in both high sensitivity and specificity.<sup>68–70</sup>

### In Vitro UTR Analysis

The most commonly used approach to verify a miRNA target site is by cloning the 3′ UTR of a predicted mRNA target into a luciferase reporter. By linking the target UTR to the luciferase reporter, a change in luciferase will indicate whether a miRNA can bind to the UTR and regulate the expression of the gene, whether at the mRNA or protein level.

There are 2 approaches that can serve to determine whether a UTR is sensitive to binding of a specific miRNA. One approach is to transfect a cell line that expresses the miRNA of interest with a reporter containing either the wild-type

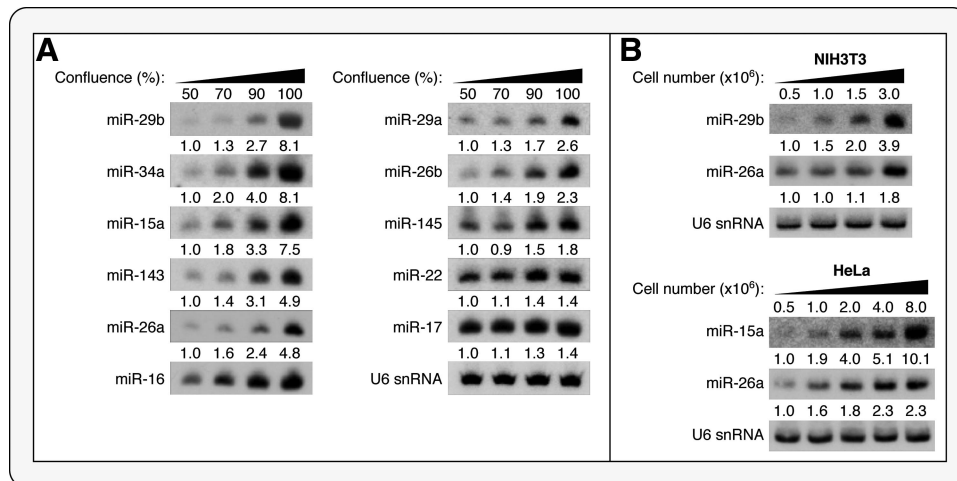
UTR of a target gene or the UTR in which the miRNA binding site is mutated. If the construct containing the wild-type UTR shows a reduction in luciferase expression and this decrease is absent in the mutated version, this likely indicates that the endogenously expressed miRNA is capable of regulating that UTR. In this same setup, one could use a miRNA inhibitor to block miRNA function, which would then lead to an increase in luciferase. Another option would be to transfect cells with both the luciferase reporter construct and increasing amounts of the miRNA of interest by either using an expression construct like pcDNA harboring the full-length sequence of the pre-miRNA or by transfecting in chemically synthesized miRNA mimics. The advantage of introducing a miRNA is that if the miRNA actually targets the binding site in the UTR, a dose-dependent effect on luciferase readout can be determined, with this response being absent with the mutated construct.

To determine whether a UTR is susceptible to miRNA regulation, the complete sequence should be tested to ensure that endogenous sequences that enhance or inhibit miRNA binding and gene regulation located distal to the miRNA-binding site are also present in the reporter construct.<sup>65</sup> Like with many aspects of miRNA biology, target regulation is under the influence of temporal and spatial-specific mechanisms. The cell type, the differentiation state of the cell, and whether a cell is under stress all appear to influence whether a miRNA regulates a target. Thus, to accurately assess whether miRNA/mRNA regulation actually occurs, transfection experiment should be performed in the cell type of interest, where all endogenously expressed cofactors are present.<sup>65</sup> Although overall transfection efficiency is relatively low for cardiomyocytes, changes in luciferase can still serve to indicate the influence of a miRNA on a target UTR. Additionally, one can opt for adenoviral overexpression of the luciferase construct containing the target UTR sequence to enhance delivery of the reporter to cardiomyocytes.

An important discovery regarding miRNA expression was reported by the Mendell group last year. By growing widely used cells at different confluences, they showed that miRNA biogenesis is globally activated with increasing cellular density. The increased abundance of mature miRNAs is associated with enhanced processing by Drosha and more efficient formation of the RISC complex and leads to stronger repression of target mRNAs (Figure 7).<sup>71</sup> These straightforward yet very elegant studies highlight the importance of monitoring confluence for accurate analysis of miRNA expression and function and uncover a caveat in the interpretation of earlier studies where cell density was not closely observed. The increase in miRNA abundance with increased confluence probably also explains why global miRNA abundance is generally higher in tissue than in cell lines.<sup>72</sup> It remains to be determined whether changes in miRNA abundance with increasing cell density have implications for regulatory mechanisms in vivo.

### Transcriptome and Proteome Analysis

Based on the initial findings surrounding LIN-14, it was thought that miRNAs repressed protein levels with little or no influence on mRNA levels.<sup>2</sup> By extension, it was originally



**Figure 7. miRNA biogenesis dependent on cellular density.** **A**, Northern blots demonstrating a widespread upregulation of miRNA expression in HeLa cells at increasing levels of confluence. Representative images of U6 small nuclear RNA (snRNA) are shown. **Numbers below blots** represent relative abundance of each miRNA normalized to U6 expression. **B**, miRNA expression 24 hours after plating HeLa and NIH 3T3 cells at increasing density. Adapted from Hwang et al<sup>71</sup> with permission from the National Academy of Sciences.

thought that monitoring changes in mRNA on miRNA regulation would exclude many miRNA targets that are regulated on the protein level. However, more recently, it has become apparent that miRNAs also decrease the levels of many mRNA targets.<sup>73,74</sup>

To assess the portion of targets that are regulated on the mRNA or protein level, the laboratories of Bartel and Rajewsky performed high-throughput analyses comparing protein and mRNA changes after introducing or deleting individual miRNAs and showed that mRNA destabilization accounts for most of miRNA-mediated gene expression changes.<sup>68,69</sup> Additionally, a recent study by the Bartel group used a deep sequencing approach to study in parallel the effect on mRNA levels and ribosome density and occupancy. For the latter, they treated cells with cycloheximide to arrest translating ribosomes and treated cells with RNase I to degrade regions of the mRNAs not protected by the ribosomes. Next, the ribosome-protected fragments were isolated and processed for Illumina high-throughput sequencing. Unlike with classic proteomics, which preferentially examines the expression of more highly expressed proteins, this method provides quantitative data on thousands of genes that are not detected by general proteomics approaches. Comparing the data from the ribosome profiling with mRNA changes, Guo et al showed that up to 84% of the changes in protein levels induced by miRNA regulation are attributable to changes in mRNA expression.<sup>75</sup>

So, what does this mean for miRNA research? If indeed the majority of miRNA targets are regulated on the mRNA level, this would simplify analyses of targets because protein analysis of a specific gene requires large amounts of material and can be tedious because of the applicability of the antibodies available or the time it requires to generate appropriate antibodies. Although extensive proteomics approaches would allow screening for genome-wide gene expression changes, this work-intensive application is still relatively expensive for the greater audience to apply as standard research tool. However, detecting changes on the

mRNA level is not as straightforward as simply doing a microarray or real-time PCR reaction and looking for changing transcripts with a miRNA binding site but, instead, involves several considerations. First, the changes induced by miRNA-mRNA interaction are spatial and temporal. To be able to appropriately assess whether a miRNA targets an mRNA, it is necessary to manipulate a miRNA in the relevant cell type and examine expression changes at the appropriate time. Second, because miRNAs are moderate regulators and, in some cases, change the expression level of the mRNA target by as little as 35%,<sup>76</sup> the naturally occurring noise in biological samples makes it difficult to accurately measure such relatively subtle changes. Third, the expression level of your miRNA and the abundance of its targets might be influencing the prominence of the expressional changes in miRNA targets. Although Krutzfeldt et al were able to show many changes in mRNAs containing a binding site for miR-122 after in vivo inhibition of miR-122, each liver cell is estimated to contain 66 000 copies of this liver-enriched miRNA,<sup>77</sup> making the outcome of the miRNA repression more likely to be detected than for a miRNA that is hardly there to begin with. Fourth, introducing a miRNA at supra-physiological levels by either transfection of cell cultures or using in vivo transgenesis might potentially induce forced binding of the miRNA to a miRNA binding site and induce changes in gene expression that would not occur naturally. Despite these issues, currently, the best method to validate whether a miRNA targets a gene is by performing mRNA or protein analysis upon miRNA regulation.

In addition to a miRNA regulating many different targets, individual 3' UTRs also contain binding sites for multiple miRNAs, allowing for elaborate and complicated networks in which redundancy and cooperation between miRNAs determine the effect on gene expression. Currently, there are 940 human miRNAs listed in the miRNA database miRBase (<http://www.mirbase.org>), representing >1% of all genes in the human genome. These miRNAs are predicted to target 30% of the human gene pool.

### Biochemical Assays for Target Detection

One way to determine whether an mRNA is targeted by a miRNA is by performing pull-down assays for miRNA-processing proteins and identifying the mRNAs that are bound to these proteins. Pulling down members of the Argonaute (Ago) protein family indicated several mRNA targets<sup>78</sup> and revealed targets for a specific miRNA, miR-124.<sup>76</sup> In 2008, the Darnell laboratory successfully applied HITS-CLIP (high-throughput sequencing to crosslinking immunoprecipitation) to develop a genome-wide map of interactions between the neuron-specific splicing factor Nova and RNA in the mouse brain and revealed that HITS-CLIP provides a robust, unbiased means to identify functional protein–RNA interactions *in vivo*.<sup>79</sup> More recently, the same group applied HITS-CLIP to decode a map of miRNA-binding sites to brain mRNA transcripts by covalently crosslinking native Ago protein–RNA complexes in mouse brain. By simultaneously defining Ago–miRNA and Ago–mRNA interactions and bioinformatically assessing whether these mRNAs contain a miRNA-binding site, the Darnell group was able to validate genome-wide interaction maps for miR-124, and generated additional maps for the 20 most abundant miRNAs present in P13 mouse brain.<sup>80</sup> Interestingly, although these data showed that the Ago–mRNA HITS-CLIP tags were enriched in 3′ UTRs, as expected, additionally an extensive set of tags were identified in other locations, including coding sequences (25%), introns (12%), and noncoding RNAs (4%), suggesting that these sites may provide new insights into miRNA biology.<sup>80</sup> Although the biological meaning behind these findings requires further exploration, these initial data indicate the feasibility of using Ago HITS-CLIP to explore miRNA–mRNA interactions *in vivo*. Although it will require more optimization to apply this approach for all miRNAs in different tissues, and it is not likely to indicate all targets for a specific miRNA, it might enable researchers to get some insight into *in vivo* mRNA regulation by their miRNA of interest.

### Regulating MicroRNAs

The best way to study the functional relevance of a miRNA is by examining phenotypic changes in culture or within an organism in response to regulation of a miRNA. Recently, several strategies for gain- and loss-of-function studies for specific miRNAs both *in vitro* and *in vivo* have been developed. Here, we discuss these methodologies for regulating miRNA levels and compare and contrast the strengths and weaknesses of these approaches.

### In Vitro MicroRNA Regulation

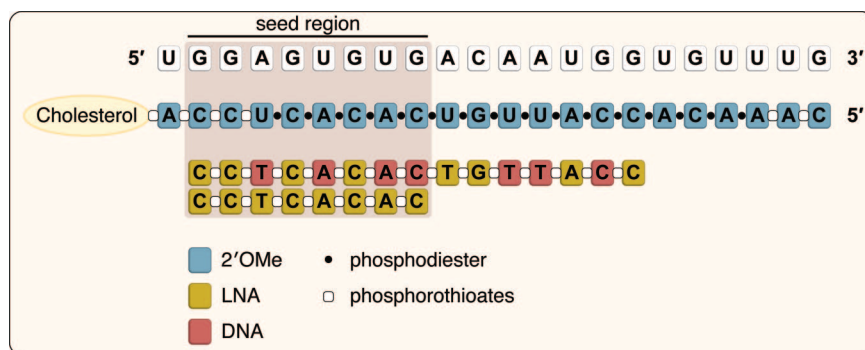
*In vitro* miRNA manipulation can be achieved by straightforward transfection experiments and can serve to determine target regulation or to examine the physiological effect of the miRNA on processes such as the control of cell morphology, differentiation, proliferation, and survival. Whereas miR mimics usually consist of double-stranded oligonucleotide chemistries, anti-miRs are usually single-stranded molecules. The chemical design of these modulators determine the efficiency of miRNA regulation.<sup>81</sup> Because cellular confluence regulates miRNA biogenesis, plating density of the

cultures should be taken into account when studying physiological effects of miRNA modulation (Figure 7).

In addition to modulation miRNA levels by overexpression or deletion, modulation of miRNAs could potentially also be influenced by preventing the miRNA from doing its job by “soaking up” the miRNA. This technique has been named miRNA eraser,<sup>82</sup> sponge,<sup>83</sup> or decoy.<sup>27</sup> A vector expressing miRNA target sites can be used to scavenge a miRNA and prevent it from regulating its natural targets. Most commonly, these vectors harbor multiple miRNA-binding sites downstream of a reporter, such as green fluorescent protein or luciferase, expressed from a strong promoter, whereby the reporter can indicate whether the miRNA is effectively scavenged away by the decoy. Unlike the sponge, which induces a modest decrease of the endogenous miRNAs, erasers are capable of inducing an apparent loss of the miRNA signal.<sup>82</sup> In general, transcripts that contain target sites with perfect complementarity to the miRNA are suppressed to a greater extent than transcripts containing imperfectly complementary targets. Perfectly complementary binding induces mRNA cleavage and prevents the transcript from being translated. On the contrary, miRNAs remain bound longer to imperfect target sites until the mRNA is destabilized by other factors. For this reason, overexpression of imperfect binding sites will more potently reduce the bioavailability of the miRNA and will more effectively inhibit the function of a miRNA. In 2007, the first report on a cardiac application of a miRNA decoy was published by Care et al. To assess the functional consequences of silencing endogenous miR-133 *in vitro*, they infected neonatal mouse cardiac myocytes with an adenoviral vector in which a 3′ UTR with tandem sequences complementary to mouse miR-133 was linked to the enhanced green fluorescent protein (EGFP) reporter gene. The complementary sequences sequestered endogenous miR-133. In cells infected with this decoy adenovirus (AdDecoy), phenylephrine-induced hypertrophy was associated with a marked increase in EGFP expression, compared to that in unstimulated AdDecoy-infected cells.<sup>27</sup> This result indicates that hypertrophic stimuli promote a reduction in miR-133 expression, thus reducing its binding to decoy sequences in the 3′ UTR and enabling EGFP mRNA translation.

### Genetic Manipulation of MicroRNA Levels

To study the function of a specific miRNA *in vivo*, one can use transgenesis or genetic deletion of a specific miRNA or a miRNA cluster. As for any transgenic model, specific promoters can be used to overexpress a miRNA in a cell type-specific manner. Although this approach has proven useful in defining the function of several miRNAs, forced overexpression of a miRNA can potentially result in the regulation of physiologically irrelevant targets if the transgene reaches supraphysiological levels of expression. An elegant way to study the functional relevance of a miRNA is by genetic deletion. Several examples of miRNA knockout animals have now been published and have revealed very specific functions for the deleted miRNAs, especially under diseased conditions.<sup>13,17–19,84–87</sup> However, because 40% of miRNAs are intronic and more than 40% are derived from a polycistronic transcript,<sup>88</sup> a targeting strategy should be



**Figure 8. AntimiR approaches in miRNA research.** Antagomirs are RNA-like oligonucleotides that harbor various modifications for RNase protection and pharmacological properties such as enhanced tissue and cellular uptake. They differ from normal RNA by complete 2'-O-methylation of sugar, phosphorothioate backbone, and a cholesterol moiety at 3' end and are able to induce efficient and long-lasting miRNA inhibition in vivo. Introducing LNA modifications in antimiRs leads to a thermodynamically strong duplex formation with complementary RNAs known because of their high affinity. A fully phosphorothioated,

unconjugated 15-nt LNA/DNA oligo directed against the 5' portion of the mature miRNA containing roughly 50% LNA bases, or an 8-nt fully modified LNA oligomer complementary to the seed region of the miRNA, is sufficient to establish a functional effect in vivo.

designed with care to prevent disrupting transcription of the host gene or flanking miRNAs. Although these models often provide valuable insights, one should consider that genetic deletion of a single miRNA might not result in a phenotypic effect because of redundancy with related miRNAs and that, in some cases, the genetic deletions might be compensated for over the course of a lifetime.

### Therapeutic MicroRNA Inhibition In Vivo

The relative ease by which miRNAs can be manipulated pharmacologically provides interesting therapeutic opportunities. There are several tools available to selectively target miRNA pathways, but, by far, the most widely used approach to regulate miRNA levels in vivo is by using antimiRs. AntimiRs are modified antisense oligonucleotides harboring the full or partial complementary reverse sequence of a mature miRNA that can reduce the endogenous levels of a miRNA. Because miRNAs normally reduce the expression of target genes, antimiRs will result in an increase of expression by relieving this inhibitory effect on gene expression. There are several key requirements for an antimiR chemistry to achieve effective downregulation of a targeted miRNA in vivo. The chemistry needs to be cell-permeable, cannot be rapidly excreted, needs to be stable in vivo, and should bind to the miRNA of interest with high specificity and affinity (reviewed elsewhere<sup>89–91</sup>). Several modifications have been used in vivo to date. These chemical modifications include 2'-O-methyl group-modified oligonucleotides and LNA-modified oligonucleotides, in which the 2'-O-oxygen is bridged to the 4' position via a methylene linker to form a rigid bicycle, locked into a C3'-endo (RNA) sugar conformation.<sup>92</sup> Another chemical modification applied to enhance oligonucleotide stability is the balance between phosphodiester and phosphorothioate linkages between the nucleotides, with phosphorothioate providing more stability to the oligonucleotide and making it more resistant to nucleases.

The 2'-O-methyl group modification is used most often to improve nuclease resistance and improve binding affinity to RNA compared with unmodified sequences. In 2005, Krutzfeldt et al reported on the first mammalian in vivo study using these so-called “antagomirs” to inhibit miR-122, a liver-specific miRNA.<sup>26</sup> These chemically modified oligonucleotides are complementary to the mature miRNA sequence and are conjugated to cholesterol to facilitate cellular uptake

(Figure 8). Systemic delivery of an antagomir via intravenous injection is sufficient to efficiently reduce the level of the miRNA of interest in multiple tissues for an extended period of time and resulted in upregulation of genes involved in cholesterol biosynthesis. Although the required doses are quite high, a single intravenous bolus injection of an antagomir is sufficient to inhibit the function of its target miRNA for weeks. These lines of evidence validate the efficacy of antagomirs in vivo and probably founded the basis for the antagomir being the most commonly used antisense oligonucleotide to silence miRNAs in research studies thus far. Two cardiac reports on the feasibility of using antagomirs for cardiac disease implications have come from the groups of Condorelli<sup>27</sup> and Engelhardt.<sup>93</sup> Care et al indicated a decrease in miR-133 in both mouse and human models of hypertrophy. Treatment with an antagomir against miR-133 resulted in cardiac growth.<sup>27</sup> The group of Engelhardt used an antagomir against miR-21 and showed that inhibition of miR-21 in the heart reduced cardiac fibrosis and thereby blunted cardiac remodeling in response to stress.<sup>93</sup> These studies were important in that they showed that miRNA regulation can have an influence on heart disease and, at the same time, indicated that cardiac miRNAs can be targeted with an antimiR approach.

Recently, the therapeutic applicability of LNA-antimiR technology has been reported in rodents and nonhuman primates. The LNA modification leads to a thermodynamically strong duplex formation with complementary RNA, and systemic delivery of unconjugated LNA-antimiR potently antagonized the liver-expressed miR-122 in mice and nonhuman primates. Acute administration by intravenous injections of relatively low doses of LNA-antimiR into African green monkeys resulted in uptake of the LNA-antimiR in the cytoplasm of primate hepatocytes and formation of stable heteroduplexes between the LNA-antimiR and miR-122. This was accompanied by depletion of mature miR-122 and dose-dependent lowering of plasma cholesterol.<sup>28–30</sup> LNA derivatives also have shown efficacy in chronically infected chimpanzees by suppressing HCV viremia and improving HCV-induced liver pathology<sup>30</sup> and are being evaluated in the first human clinical trials of miRNA inhibition (Santaris Pharma, ClinicalTrials.gov). Whereas antagomirs are complementary to the full mature miRNA sequence, the high

binding affinity for LNA-containing antisense oligonucleotides allows inhibition with shorter LNA oligonucleotides (Figure 8). A fully phosphorothioated LNA/DNA oligo directed against the 5' portion of the mature miRNA, containing roughly 50% LNA bases, or 8-nt fully modified LNA oligomer complementary to the seed region of the miRNA is sufficient to establish a functional effect *in vivo*. Recently, it was shown that an LNA-containing anti-miR-targeting miR-33 is able to increase the levels of high-density lipoprotein cholesterol *in vivo*.<sup>94,95</sup> Although both the anti-miR and LNA-modified oligos can effectively target a miRNA, the LNA-modified chemistries require lower doses based on their higher binding affinity. The 8-mer fully modified LNA oligomer directed against the seed region of a miRNA can additionally be functional for targeting multiple miRNA family members at once. Gene expression analysis indicates that the shorter LNA-containing chemistries do not induce off-target gene expression changes.<sup>28</sup>

Anti-miR detection assays by either Northern blot analysis, ELISA-based detection methods, or radioactive labeling of the anti-miRs have shown that anti-miRs mostly end up in the liver and kidney (as is true for most single-stranded oligonucleotide chemistries), but they are capable of targeting the heart.<sup>25–28</sup> Although there are individual beneficial characteristics to these different approaches, considering that these anti-miR chemistries are able to efficiently inhibit the target miRNA *in vivo* and establish a functional effect, anti-miR oligonucleotide might be a feasible approach for future development of miRNA-based therapeutics.

Like other oligonucleotide chemistries, anti-miRs can be dissolved in saline and injected into the animal intravenously or subcutaneously. Although intravenous delivery through the tail vein of mice has been the most commonly used route of administration, based on our own experience, one can target the heart as well through intraperitoneal or subcutaneous delivery of the anti-miRs. However, although these anti-miR approaches are very effective in inhibiting a miRNA *in vivo*, one should consider that systemic delivery might induce unknown off-target effects and that the effect observed might be attributable to effects outside of the target tissue. Although currently unknown, this might potentially explain the discrepancy between the cardiac phenotype of the genetic deletion of miR-133a and miR-21, and the miR-133a and miR-21 anti-miR study.

An additional potential artifact can be introduced by the *in vivo* sequestering of the anti-miRs into extracellular or intracellular compartments (eg, endosomes). Although systemic delivery results in efficient uptake of anti-miR in the cells, with the anti-miRs being taken up by endosomes that slowly release the anti-miR endogenously, anti-miRs only inhibit miRNAs when released from these compartments. However, tissue homogenization after systemic delivery can cause these captured anti-miRs to be released and bind to the mature miRNA, causing an overestimation of binding efficiency of the anti-miR *in vivo*. For this reason, it is essential to look for biological target readouts rather than simply measuring miRNA inhibition.

### MicroRNA Mimicry In Vivo

In addition to the anti-miRs, there is also the opportunity to mimic or reexpress miRNAs by using synthetic RNA duplexes designed to mimic the endogenous functions of the miRNA of interest, with modifications for stability and cellular uptake. The “guide strand” is identical to the miRNA of interest, whereas the “passenger strand” is modified and typically linked to a molecule such as cholesterol for enhanced cellular uptake. However, it should be noted that although this method would replace the miRNA levels lost during disease progression, it will also result in the uptake by tissues that do not normally express the miRNA of interest, resulting in potential off target effects. Even more so than for anti-miR approaches delivery to the appropriate cell type or tissue is an important aspect of effective miRNA mimicry to prevent unwanted side effects.

Another way to increase the level of a miRNA is by the use of adeno-associated viruses (AAVs). Delivered in viral vectors, the miRNA of interest can be continually expressed, resulting in robust replacement expression of miRNAs down-regulated during disease. Additionally, the availability of a number of different AAV serotypes allows for the potential of tissue-specific expression because of the natural tropism toward different organs of each individual AAV serotype, as well as the different cellular receptors with which each AAV serotype interacts. The use of tissue-specific promoters for expression allows for further specificity in addition to the AAV serotype. Furthermore, AAV is currently in use in a number of clinical trials for gene therapy, of which the safety profiles have looked quite positive. In line with this, Kota et al recently showed AAV-mediated delivery of miR-26a blunts tumor genesis in a mouse model of liver cancer.<sup>96</sup> Although systemic viral delivery of miRNAs to the heart during disease has not been performed yet, there have been a number of studies using AAV9 to successfully deliver RNA interference to cardiac tissue and effectively restore cardiac function during disease in rodents (reviewed elsewhere<sup>97</sup>).

### Concluding Remarks

The incredible enthusiasm for miRNAs as a novel class of functional regulators of tissue maintenance and stress responses demands for appropriate and reliable research tools. This review summarizes many of the currently available basic tools and describes the most widely used approaches for miRNA research to date. Although it will be the combination of several validated research methods that will enable researchers to validate the relevance or contribution of a miRNA to a certain phenotype, some caution should be taken to circumvent erroneous interpretation of data.

Detecting a miRNA appropriately can be tedious based on the size of the miRNA and the existence of related miRNA with a high degree in sequence homology. Applying multiple methods in parallel will increase the likelihood of proper reflection of the presence or regulation of a miRNA. Another key take-home message should be that miRNAs do not function through a single gene target. The combined regulation of many different genes determines the functionality of a miRNA. Although there are exceptions of genes that show prominent expressional regulation upon changes in miRNA

levels, oftentimes, the regulatory changes are small and might get lost in the biological noise when using a small number of samples. One should be especially cautious when using in vitro systems to study miRNA phenotypes because the biogenesis might be different from what is happening in vivo. Lastly, efficacy of antimiR tools using systemic delivery should be validated using downstream target readout rather than miRNA knockdown, because the knockdown indicated by real-time PCR or Northern blotting might not reflect the endogenous interaction between the antimiR and the miRNA.

Taking these and the additional issues outlined in this review into account will allow for more accurate examination of a miRNA to guide one in understanding its biological significance. Further elucidation of miRNA biogenesis and functionality will enable the development of more specific and sensitive assays. Enhancing the art of performing research surrounding these exceptionally exciting novel gene regulators will advance our understanding of miRNAs and their specific functions and will augment the opportunities to safely pursue them as therapeutic modalities.

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