

## Review

# MicroRNA with a MacroFunction

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## ABSTRACT

With the advent of microRNA (miRNA) we are compelled to revise our understanding of the mechanisms underlying gene regulation during health and disease. A miRNA is ~21 ribonucleotides-long, genetically encoded, with a potential to recognize multiple mRNA targets guided by sequence complementarity and RNA-binding proteins. This class of molecules is functionally versatile, with the capacity to specifically inhibit translation initiation or elongation, as well as, induce mRNA degradation, through predominantly targeting the 3'-untranslated regions of mRNA. Early on it was realized that the levels of individual miRNA varied under different developmental, biological, or pathological conditions, thus, implicating these molecules in normal and pathological cellular attributes. In this article we will share our views on how the functions of miRNA conform to our existing knowledge on post-transcriptional regulation of gene expression and disease mechanisms, and their potential as biomarkers and therapeutic targets in diseases.

## THE IMPORTANCE OF BEING miRNA

In computer terms, a macro is a single command that is programmed to replace multiple separate commands that perform a series of actions, thus, simplifying, expediting, and minimizing error. Similarly, the cell employs a single miRNA to regulate post-transcriptional expression of an array of genes that are involved in a particular cellular function. This mechanism will ensure synchronization of the regulatory effects and promptness of the response. Moreover, post-transcriptional regulation of multiple genes via a single miRNA, circumvents the need for transcriptional regulation of individual genes and is, thus, potentially faster and more energy efficient. This is what Beyer et al. refer to as "translation on demand".<sup>1</sup> Not to overlook the fact that regulating RNA with miRNA is unhindered by the need for a translation product. Thus, it becomes clear that the functions of miRNA complement perfectly our current knowledge of post-transcriptional regulation of expression, as will be discussed next.

## POST-TRANSCRIPTIONAL REGULATION BY miRNA

Transcription of a gene does not connote automatic translation of the transcript. In other words, we cannot use relative mRNA levels as predictive measures of relative protein levels of a given gene,<sup>2</sup> which suggest post-transcriptional regulation of gene expression. Post-transcriptional regulation refers to events that limit the availability or accessibility of mRNA for translation. The study by Beyer et al. confirms weak or no correlation between mRNA and protein abundance for the whole cell.<sup>1</sup> Similar results were obtained when the calculations were repeated on separate cellular compartments. But interestingly, a more positive correlation emerged when they grouped functionally related genes. From what we know, an individual miRNA (or a miRNA family) also has the potential to post-transcriptionally regulate a set of specific genes involved in a given cellular function. For example, we observed that miR-1 targets an array of growth-related genes that have been previously implicated in the development of cardiac hypertrophy, which include Ras GTPase-activating protein, cyclin-dependent kinase 9, endothelin, fibronectin, Ras homologue enriched in brain, eukaryotic initiation factor 4E, JunD, quaking, insulin-like growth factor, and Rap1. Concordantly, upon induction of cardiac hypertrophic growth, miR-1 is downregulated, allowing for the up-regulation of these targets.<sup>3</sup> Similarly, when John et al. analyzed individual miRNA for gene ontology terms they observed that some favored certain terms.<sup>4</sup> For example, miR-208 targets were biased

toward “transcription factor”, while miR-105 had a preponderance of “small GTPase mediated signal transduction”. To the best of our knowledge, there is no other class of molecules identified to-date that has the potential to fulfill this function as globally and specifically as observed by miRNA. This supports the idea that miRNA are major post-transcriptional regulators.

Transcriptional regulation is the main first step in determining the availability of mRNA, but it immediately follows that the mRNA's half-life will dictate its temporal availability for translation of the open reading frame. Wang et al. have reported that in yeast the half-lives of mRNA ranged from 3–90 min.<sup>5</sup> There was no correlation between the half-life and ribosome density (translational activity), but the half-lives of proteins that form a physical complex were very similar. For example, the four core histones have a decay rate of  $t_{1/2} = 7 \pm 2$  min, while 131 ribosomal protein mRNA have a  $t_{1/2} = 22 \pm 6$  min. Similarly, decay rates of mRNA of functionally related genes were also comparable. In general, genes related to metabolism had longer half-lives than those enrolled in regulatory functions, such as initiation of translation. These data reconcile well with the fact that mRNA and protein abundance correlate best within the same functional group of genes. Although mammalian miRNA are commonly known for inhibiting translation vs. inducing mRNA degradation, there is substantial evidence to support the latter as well. Farh et al. showed that predicted mRNA targets of tissue-specific miRNA were lower in the corresponding tissue than they were in others.<sup>6</sup> More convincingly, those targets were, initially, preferentially high in those tissues, before declining, upon the inclining of the targeting miRNA, although they were not completely eliminated. This suggested that miRNA are involved in both mRNA decay, as well as, translational repression. In support, Lim et al. showed that by expressing the muscle-specific miR-1 or the brain-specific miR-124 in HeLa cells the mRNA expression pattern shifted towards the corresponding tissue.<sup>7</sup> The mechanism of mRNA degradation is similar to that employed by siRNA, where the endonuclease ‘Slicer’ mediates mRNA cleavage. Alternatively, miRNA may induce deadenylation, which induces mRNA degradation.<sup>8</sup>

It is well established that in metazoans miRNA inhibits mRNA translation. One study showed that miRNA requires both the Cap and poly(A) tail structures to inhibit translation initiation, but the mechanism remains unknown.<sup>9</sup> It was previously shown that both of these structures act in synergy to enhance translation. This may be explained by the fact that eukaryotic initiation factors 4G (eIF4G), a subunit of the 5' cap-binding initiation factor, binds the polyA-binding protein (PABP) and promotes circularization of the mRNA molecule, a structure that is translationally superior to the linear form.<sup>10</sup> Thus, one can envision a mechanism whereby the 3'UTR-binding miRISC complex may interfere with the formation of this structure and compromise translation. Alternatively, in an intact circular mRNA, the miRISC complex may come into close proximity to the translation initiation complex eIF4F and physically or biochemically hinder its function. But in case the mRNA is already engaged in translation, miRNA also has the capacity to inhibit post-translation initiation steps.<sup>11,12</sup>

Description of post-transcriptional regulation is incomplete without discussing mRNA localization. The cytoskeletal framework provides mRNA with an anchor, as a mean for targeted localization within the cell. By using in situ hybridization and electron microscopy Bassell et al. were able to visualize mRNA that was mainly localized to microfilaments (72%), and partially to vimentin filaments and microtubules in human fibroblasts.<sup>13</sup> Both the poly(A)

mRNA and the polysomes colocalized to the actin crosslinking proteins filamin and  $\alpha$ -actinin and actin-binding elongation factor 1 $\alpha$ . This feature of mRNA is mediated by the 3'UTR. More than a decade ago, Gottlieb et al suggested that mRNA localization evolved by inducing degradation of all un-localized mRNA.<sup>14</sup> In concordance, there is a correlation between translation repression and degradation of some transcripts.<sup>15</sup> Interestingly, one example of a molecule that is involved in this process is a non-coding RNA molecule, Yellow Crescent (YC), found in the ascidian eggs.<sup>16</sup> Swalla et al showed that YC is complimentary to the 3'UTR of proliferating cell nuclear antigen (PCNA), but the two do not exist in the same compartment. This suggested that their association results in degradation of PCNA. This brings to our attention the untapped potential of miRNA to specifically repress un-localized transcripts, earmark them for localization, or maybe aid in regulating the targeting process. Since miRNA have the capacity to both inhibit translation and induce degradation of the mRNA, it is plausible that it exercises both effects on the same transcript in a temporal and spatial manner. For example, if a translationally repressed target does not reach its destination promptly, it must face degradation. More will be revealed when we know if, when, and where miRNA is localized.

## TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL REGULATION OF miRNA

Before discussing the changes of miRNA levels in disease and their potential as diagnostic and therapeutic targets, it is necessary to briefly describe how they themselves are regulated. MiRNA were first discovered by means of large scale cloning and sequencing of the mature forms.<sup>17-19</sup> This group of miRNA was then used as a ‘training-set’ for computer software to generate algorithms for computational prediction of further miRNA (reviewed in ref. 20). The data from that information was then further examined, in an attempt to delineate the transcriptional units of those genes.<sup>21</sup> Out of 232 mammalian miRNA analyzed, approximately 40% existed within introns of coding RNA, 10% within introns of non-coding RNA (ncRNA), and 13% within exons of ncRNA. In addition, a small portion of miRNA exists within exons (3'UTR) of coding RNA and antisense strands of overlapping genes. The remaining miRNA are intergenic, with, as of yet, undefined transcriptional units. MiRNA contained within host genes are expected to be expressed from the same transcriptional unit, as was indeed confirmed for a subset.<sup>21</sup> As for intergenic miRNA, Jegga et al. and Zhou et al. have developed searchable databases for prediction of miRNA core promoters.<sup>22,23</sup> From these studies it was predicted that those genes have features common to RNA polymerase II-dependent promoters, as well as, unique sequence motifs. Besides that, there are only a handful of studies that examine the transcriptional regulation of individual miRNA promoters. Examples include, regulation of mouse miR-1 promoter by serum response factor, myoD, and muscle enhancer factor 2<sup>24</sup> and *Drosophila* miR-1 by twist and muscle enhancer factor 2<sup>25</sup> among others.<sup>26,27</sup> Also, recently a fair number of miRNA present within areas of Alu repeats were found to be transcribed from RNA-polymerase III-dependent promoters.<sup>28</sup> In addition, it was suggested that miRNA found within CpG islands may be regulated by methylation.<sup>29</sup>

The dogma is that the primary miRNA transcripts are processed in the nucleus into the premature form by Droscha, which is then exported to the cytoplasm for further processing by Dicer.<sup>30</sup> But as with other RNA species, miRNA transcripts are not exempt from post-transcriptional

regulation. This was convincingly demonstrated by Thomson et al., where they compared the mature vs. the primary miRNA profile for normal and cancer tissues and found no correlation.<sup>31</sup> Our data also show that in spite of only 200 nucleotides separation between miR-144 and miR-451, the former is undetectable, while the latter is highly expressed and regulated in the heart (unpublished data). It, thus, appears that mature miRNA abundance is regulated at the levels of Drosha and/or Dicer processing. This may be a consequence of regulation of the activity or availability of those enzymes or the targeted localization of the primary or premature miRNA. Thus, for diagnostic or prognostic purposes we must rely on measurements of the mature form.

## THE MECHANISM OF miRNA-TARGET RECOGNITION

To evaluate the significance of a change in the levels of a certain miRNA during health or disease, we have to understand how it selects and targets specific mRNA. Very few miRNA targets have been identified or validated experimentally. But there are multiple searchable databases that have computationally predicted miRNA targets in several species, using various algorithms, which continue to be modified as more targets are being validated experimentally. At present, these databases are vital in guiding us in experimentally validating miRNA targets. The miRanda software uses parameters that include: (1) binding energy between miRNA and its target, (2) asymmetric 5'-3' base-pairing, with more weight on the 5' end, which can, to some degree, be compensated for by stronger complementation with the target at the 3' end. Also, allowing G:U wobble pairing, and (3) the most important criterion is, evolutionary conservation of the sequence and its position in 3'UTRs of human, mouse, and rat genes.<sup>4</sup> A second database, TargetScanS, uses somewhat different parameters, which includes: (1) exact Watson-Crick (W-C) pairing of nucleotides 2-7 of the miRNA to the target, with no weight given to pairing of its 3' end, (2) a conserved adenosine at the first position and/or a W-C match at position 8, (3) disregarding the binding energy and, (4) using the human, mouse, rat, dog and chicken 3'UTR, for determining target site conservation.<sup>32</sup> A third searchable database, PicTar, looks for: (1) a perfect ~7 nucleotide seed at position 1-7 or 2-8, but mismatches are allowed if the free binding energy does not increase,<sup>33</sup> (2) it takes into account the binding energy of the entire miRNA-mRNA duplex, and (3) it includes the genomes of chimpanzee, pufferfish, and zebrafish, in addition to all those listed above, in the alignment for checking conservation, which further reduces false positive predictions. It should be noted, though, that nonconserved miRNA target sites respond equally well to inhibition by miRNA.<sup>6</sup> This first suggested that other factors such as secondary structures, RNA binding protein, and other pairing features must contribute to the *in vivo* specificity of a miRNA. But upon a closer examination, the investigators established that those messages appeared to have specifically evolved to avoided colocalization with the corresponding miRNA. Those genes were dubbed "antitargets". While the three databases overlap in many predicted targets they diverge in others. Thus, it might be beneficial to search all the databases for potential targets of a miRNA of interest, for experimental validation.

While computational analysis predicted the structural basis for miRNA : target pairing, Brennecke et al experimentally validated some of the parameters applied above. Essentially, they confirmed that nucleotides 2-8 from the 5' end of the miRNA are the most critical in establishing base pairing with the target. This criterion

could be relaxed to 2-5 nucleotides, under conditions where the nucleotides in the 3' end had strong complementarity with the target. Also, the position of the base pairing rather than the pairing energy determined the functional efficiency of the miRNA.<sup>34</sup>

It is worth noting, that another dimension is added to the mechanism of miRNA targeting, by introducing selective miRNA editing. This process converts adenosine to inosine, catalyzed by adenine deaminase in double stranded RNA substrates (reviewed in ref. 35). Luciano et al. were the first to notice that premature miR-22 is edited at multiple sites.<sup>36</sup> It is estimated that ~6% of human miRNA are edited.<sup>37</sup> Importantly, some of the edited sites are within the 'seed' region and result in reassignment of targets.<sup>38</sup>

## miRNA IN DEVELOPMENT AND DISEASE

The first miRNA was discovered in 1993 when Lee et al. found that lin-4, which down-regulates the levels of lin-14 during the development of *C. elegans*, expresses a small non-coding 22 nucleotide RNA.<sup>39</sup> Not until seven years later did Reinhart et al. report the discovery of the second miRNA, let-7, which induces downregulation of lin-41 protein.<sup>40</sup> This was shortly followed by identification of a multitude of human and fly miRNA that were highly conserved.<sup>18</sup> It was, hence, noticed that in each of the heart, brain and liver, there is a distinct tissue-specific miRNA that predominates, which suggested that miRNAs must be involved in tissue specification or differentiation.<sup>41</sup> MiRNA also play a role in stem cell division (reviewed in ref. 42). To-date, more than 4000 miRNA sequences have been identified in a wide range of species. These are annotated and catalogued in a searchable web-based data registry by Wellcome Trust Sanger Institute.<sup>43,44</sup>

MiR-1 has a dominant expression pattern and function in the heart. First identified as a muscle-specific miRNA,<sup>41</sup> its expression is detected as early as embryonic day (E) 8.5 in the mouse heart, and increases with the progression of differentiation.<sup>24</sup> In *Drosophila*, a DmiR-1<sup>KO</sup> mutant dies as a small second in-star larvae, 2-7 d after hatching, from apparent paralysis.<sup>25</sup> Similarly, disrupting miR-1-2 in a homozygous mouse model results in ventricular septal defects and lethality between E15.5 to immediate postnatal.<sup>45</sup> Recently, several reports have also implicated miR-1 in development of cardiac hypertrophy<sup>3,46</sup> and arrhythmias.<sup>47</sup> While, other miRNA are also involved in the progression of hypertrophy, including miR-133,<sup>46</sup> miR-21,<sup>48</sup> miR-208,<sup>49</sup> and miR-195,<sup>50</sup> the emphasis remains on miR-1, which is singularly down-regulated within 24 h of triggering hypertrophy.<sup>3</sup> Notably, adult cardiac hypertrophy starts out as a compensatory growth that is characterized by recapitulating the neonatal heart gene expression profile.<sup>51</sup> Concordantly, our results confirm similar changes in miRNA patterns in adult hypertrophied vs. sham and neonatal vs. adult hearts (unpublished data). Thus, we expected that subsequent progress into heart failure would coincide with a deviation of the miRNA signature from this pattern. To test this idea, we searched for miRNA that were deregulated during the period of transition from hypertrophy to failure in a mouse model. While the results showed an overall continuum of the hypertrophy signature, only one miRNA emerged that sharply demarcated this transition period (manuscript in preparation). Such a molecule would serve as an ideal therapeutic target.

Because of their major roles as oncogenes and tumor suppressors, a group of miRNAs have been dubbed 'oncomirs'.<sup>52</sup> In cancer, the discovery of miRNA is offering answers to previously unresolved questions. For example, after years of futile attempts to identify

Table 1 **Select tissue-specific and disease-related microRNA**

<b>Tissue-Specific</b>	<b>Cardiac Hypertrophy</b>	<b>Cancer</b>	<b>Hypoxia</b>	<b>Athero-sclerosis</b>
miR-1, heart and skeletal muscle <sup>41</sup>	miR-1 ↓ <sup>3</sup>	miR-107 ↑, H&N <sup>70</sup> , colon <sup>71</sup> , pancreas <sup>71,72,73</sup> , stomach <sup>71</sup>	miR-107 ↑ <sup>74</sup>	miR-21 ↑ <sup>75</sup>
miR-122, liver <sup>41</sup>	miR-107 ↑ <sup>3</sup>	miR-15/16 ↓, CLL <sup>53,76</sup>	miR-210 ↑ <sup>74</sup>	
miR-124, brain <sup>41</sup>	miR-133 ↓ <sup>46</sup>	miR-150 ↑, lung <sup>60</sup> , CLL <sup>76</sup>	miR-213 ↑ <sup>74</sup>	
miR-133, heart and skeletal <sup>41</sup>	miR-15a/b ↑ <sup>3</sup>	miR-122 ↓, liver <sup>77</sup>	miR-23a ↑ <sup>74</sup>	
miR-375, Pancreatic islet <sup>78</sup>	miR-150 ↓ <sup>3,48</sup>	miR-125b ↑, H&N <sup>70</sup> , pancreas <sup>73</sup>	miR-24-1 ↑ <sup>74</sup>	
	miR-155 ↓ <sup>3</sup>	miR-143 ↓, colon cancer <sup>79</sup> , pancreas <sup>72</sup> ,	miR-26b ↑ <sup>74</sup>	
	miR-185 ↓ <sup>3, 48</sup>	mi-145 ↓, colon cancer <sup>79</sup>		
	miR-195 ↑ <sup>50</sup>	miR-155 ↑, B cell lymphoma <sup>56</sup> , breast <sup>71</sup> , colon <sup>71</sup> , lung <sup>60</sup> , pancreas <sup>72,73</sup> , CLL <sup>76</sup>		
	miR-199a/b/a* ↑ <sup>3</sup>	miR-199a ↑, lung <sup>60</sup> , pancreas <sup>71,72</sup> , prostate <sup>71</sup>		
	miR-208 ↑ <sup>50</sup>	miR-205 ↑, H&N <sup>70</sup> , pancreas <sup>72</sup> , lung <sup>60</sup>		
	miR-21 ↑ <sup>3,48</sup>	miR-21 ↑, H&N <sup>70</sup> , breast <sup>71</sup> , colon <sup>71</sup> , lung <sup>71</sup> , pancreas <sup>72,73</sup> , prostate <sup>71</sup> , stomach <sup>71</sup> , CLL <sup>76</sup> , liver <sup>77</sup>		
	miR-214 ↑ <sup>3,48</sup>	miR-210 ↑, pancreas <sup>72</sup> ,		
	miR-221 ↑ <sup>3</sup>	miR-213 ↑, pancreas <sup>72</sup> ,		
	miR-222 ↑ <sup>3</sup>	miR-214 ↑, pancreas <sup>71</sup> , prostate <sup>71</sup> , stomach <sup>71</sup> , lung <sup>71</sup>		
	miR-23a ↑ <sup>3</sup>	miR-221 ↑, H&N <sup>70</sup> , colon <sup>71</sup> , pancreas <sup>71,72,73</sup> , stomach		
	miR-23b ↑ <sup>3</sup>	miR-222 ↑, pancreas <sup>72,73</sup>		
	miR-27a ↑ <sup>3,48</sup>	miR-23a ↑, pancreas <sup>72</sup> , liver <sup>77</sup>		
	miR-27b ↑ <sup>3,48</sup>	miR-23b ↑, H&N <sup>70</sup> , pancreas <sup>72</sup> , liver <sup>77</sup>		
	miR-29a ↓ <sup>3,48</sup>	miR-27a ↑, H&N <sup>70</sup>		
		miR-29a ↑, H&N <sup>70</sup>		

Select miRNA were compiled from various reports in an attempt to highlight some of the differences and similarities in disease patterns, as well as, the involvement of tissue specific miRNA in pathogenesis. H&N, head and neck; CLL, chronic lymphocytic leukemia.

the gene(s) in the deleted 13q14 region that participate in the pathogenesis of chronic lymphocytic leukemia (CLL), Calin et al. discovered that miR-15 and miR-16 are located within this sequence and that both are downregulated in ~68% of cases.<sup>53</sup> These miRNA molecules are now considered tumor suppressors, one of their targets being Bcl2, the level of which reciprocally correlates with that of miR-15/16 in CLL.<sup>54</sup> Calin et al. have then gone on to show that 52.5% of miRNA are located in previously established cancer-associated regions.<sup>55</sup> In contrast, over-expression of miRNA is also associated with cancer. For example, miR-155 is upregulated in B cell lymphoma.<sup>56</sup> Notably, tissue-specific over-expression of miR-155 in a transgenic mouse model, proved it sufficient for induction of lymphoblastic leukemia.<sup>57</sup> Overall, unique miRNA expression profiles have been detected in chronic lymphocytic leukemia,<sup>58</sup> breast,<sup>59</sup> and lung cancers.<sup>60</sup>

Although we have learned much about the miRNA signatures in cardiac hypertrophy and cancer we have very little knowledge about the targets and functions of individual miRNA deregulated during these diseases. This is indeed quite a challenging task. But until we learn more about function, we cannot use miRNA successfully as therapeutic targets. In an attempt to gain some insight, we examined the differences between the miRNA expression patterns that emerged from studies of various diseases. Accordingly, we tabulated some of

the miRNA that were emphasized in cardiac hypertrophy, different cancers, hypoxia, and atherosclerosis (Table 1). We searched for trends, such as an association between certain miRNA and known disease mechanisms. For example, it was immediately noticed that miR-21 is upregulated in all cancer forms, as well as, cardiac hypertrophy and atherosclerosis, but not hypoxia. Unlike cancer or atherosclerosis, cardiac hypertrophy is a process of cellular growth in the absence of cell cycling. Thus, by deduction, we may predict that miR-21 may be related to a process of enhanced global protein synthesis that underlies hypertrophy and proliferation. On the other hand, we could also infer that miR-21 does not play a direct role in the cell cycle or hypoxia. In contrast, miR-15/16 cluster is deleted in chronic lymphocytic leukemia (CLL), but remains high and unchanged in the heart during hypertrophy, which later shows a further increase in miR-15b in late hypertrophy and failure. This supports a role for the miRNA cluster in cell cycle, as indeed has been established recently. Another trend involves down-regulation of tissue-specific miRNA (miR-1, miR-133, miR-122) in disease states. Overall, such comparisons may also prove useful when evaluating miRNA therapeutic agents and their side effects.

Besides cardiovascular diseases and cancer very little is known about miRNA in other disorders. But there is little doubt that it will soon prove that miRNA have a role in almost every aspect of a cell's

function, as seen in its emerging role in regulating metabolism and the lifespan (reviewed in ref. 61).

## THE DIAGNOSTIC AND PROGNOSTIC POTENTIAL OF miRNA

Within the past five years we have seen the rise and fall of cDNA microarrays as diagnostic and prognostic measures in disease.<sup>62-64</sup> While much work is still being done in an attempt to optimize and standardize the methodology, there will remain inconsistencies inherent to the characteristics of mRNA itself: having multiple isoforms and splice variants, and a notoriously unstable nature.

So what does miRNA offer us in the way of a diagnostic and prognostic tool that mRNA does not? While a single mRNA generally dictates the translation of a single protein, a single miRNA molecule has the capacity to regulate the translation of an array of genes governing a certain function.<sup>3,4</sup> The significance of which is underscored upon recalling that the underlying apparatus, generally regulating normal cellular functions and pathologies, consist of a consortium of genes. Those genes frequently exhibit deliberate structural and functional redundancy, to ensure a failsafe operation. More importantly, changes in a gene's mRNA levels can be counter-balanced by post-transcriptional regulation. Thus, we must come to the realization that changes in the levels of mRNA are not necessarily predictive of functional outcome. Using the counter argument, it is expected that miRNA levels are more tightly regulated and less variable, as a single miRNA has the capacity to regulate a cellular function. Moreover, mature miRNA are 18–25 nt long, end products, that have developed beyond post-transcriptional regulation and are inherently very stable. Thus, it is expected that miRNA provide us with a more superior predictive parameter in diseases. When Lu et al. put this idea to the test; they found that miRNA profiling was highly accurate in predicting the differentiation state of tumors and in classification of poorly differentiated tumors, a fact that they could not determine by mRNA profiling.<sup>65</sup> But clearly, more studies are needed to further validate the predictive powers of miRNA in cancer.

We also hope that miRNA might aid in the diagnosis, prognosis, and treatment of heart diseases, which remain the leading cause of death in the United States. Currently, diagnosis and prognosis of heart failure relies mainly on clinical signs and symptoms, aided with only a handful of circulating biomarkers.<sup>66</sup> Unfortunately, there "isn't a single patient characteristic that can be used to reliably predict a patient's outcome".<sup>67</sup> This is probably due to the fact that the utility of more direct biomarkers is hindered by the impracticality of routine cardiac biopsies. Alternatively, miRNA profiling of other secondarily affected tissues, in particular, the liver, might provide a promising substitute for establishing miRNA biomarkers in heart failure. We are currently in the process of testing this hypothesis in a mouse model of heart failure.

## MIRNA AS THERAPEUTIC TARGETS

MiRNA are characterized by targeting a functional group of genes, versus a single mRNA, which renders them a potentially powerful therapeutic target. As an example, it proved more effective to simultaneously, rather than individually, inhibit the cell cycle-related miR-16 targets.<sup>68</sup> Also, it becomes clear that before therapeutically targeting a miRNA, we require knowledge of its gene targets, function, and tissue distribution. Since the discovery of mammalian miRNA is still in its early phase, we have very little information in that regard. In the mean time, however, Krutzfeldt et al. developed a method for antagonizing miRNA function in vivo.<sup>69</sup>

The design involves synthesis of a miRNA-specific, anti-sense, cholesteryl-conjugated, 2'-*O*-methyl oligoribonucleotide, which was dubbed 'antagomir'. This nonhydrolysable oligoribonucleotide is cell permeable and inhibits the miRNA by inducing its elimination. Hence, it offers a long-lived effect and circumvents the need for a cell delivery vehicle. This approach, though, is not suitable for replenishing depleted miRNA. That is because it would require delivery of the premature miRNA in its native form, as to allow for proper processing and incorporation into the RISC complex. Thus, other known delivery methods employed in gene therapy, including viral vectors and liposomes, will have to serve as substitutes in this case.

## CONCLUDING REMARKS

As pointed out in the above discussion, we remain in dire need for new biomarkers and therapeutic targets for the two leading causes of death in the United States, cardiovascular diseases and cancer. MiRNA offer us that prospect. But in order to maximally exploit their potential, we must continue to dissect their function in great detail. It is still necessary to identify the specific mode of transcriptional and post-transcriptional regulation of individual miRNA, their cellular localization, targets, and overall function, in parallel to, continuing to devise modes for in vivo delivery of miRNA and anti-miRNA.

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