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Strategies to determine the biological function of microRNAs

Jan Krützfeldt, Matthew N Poy & Markus Stoffel

MicroRNAs (miRNAs) are regulators of gene expression that control many biological processes in development, differentiation, growth and metabolism. Their expression levels, small size, abundance of repetitive copies in the genome and mode of action pose unique challenges in studies elucidating the function of miRNAs. New technologies for identification, expression profiling and target gene validation, as well as manipulation of miRNA expression *in vivo*, will facilitate the study of their contribution to biological processes and disease. Such information will be crucial to exploit the emerging knowledge of miRNAs for the development of new human therapeutic applications.

miRNAs are short noncoding RNAs that have been identified in the genomes of a wide range of multicellular life forms as well as viruses[1–6]. Like conventional mRNAs, miRNAs are transcribed by polymerase II as long primary transcripts that are capped, polyadenylated and spliced[7]. Unlike mRNAs, miRNAs are processed into duplexes of 19-22 nucleotides (nt) by a two-step process involving nuclear and cytosolic RNase III-type endonucleases, known as Drosha and Dicer, to yield the 'mature' miRNA. In a final step, this RNA duplex is loaded into the RNA-induced silencing complex (RISC), one of the strands is eliminated, and the remaining strand engages in imperfect base pairing with specific sequences in target mRNAs. This induces either degradation of the target mRNA or translational repression[8]. This mechanism resembles the process of RNA interference triggered by double-stranded RNA and uses similar molecular machinery[9]. The elucidation of the mechanism of miRNA function in the regulation of gene expression suggests a gene regulatory model: nuclearly encoded genetic information is not only transcribed and translated into proteins but at the same time regulates these processes through noncoding miRNA by way of sequence-guided interactions with the cognate mRNA. This paradigm adds a new level of regulation and fine-tuning of gene expression that is likely to be important for the maintenance of many, if not all, cellular functions.

In spite of our ability to identify miRNA and elucidate their biogenesis and basic mechanisms of action, very little is known regarding miRNA function in animals. The first known miRNA, *lin-4*, was discovered in 1993 and was shown to have important roles in developmental timing of stage-specific cell lineages[10]. Since then, genetic studies in *Caenorhabditis elegans*, *Drosophila melanogaster* and *Danio rerio* have identified important functions of specific miRNAs in the coordination of cell proliferation and death during development, stress resistance, fat metabolism and brain morphogenesis[11–14]. Relative to less complex model organisms, our knowledge of miRNA function in mammals is much more limited. For the past few years, one primary focus of the investigators studying mammalian miRNAs has been to identify and catalog the complete miRNA inventory and its expression pattern using cloning, bioinformatics and gene expression approaches. With these efforts nearing completion in the near future, the focus is shifting to the elucidation of miRNA function. Several technological advances including bioinformatic prediction algorithms, reporter assays, *in situ* hybridizations, overexpression and silencing technologies, have been developed to deduce miRNA function. Here we address current and future technologies to study miRNA function in mammals, discuss their limitations, and point out their relevance to modern medicine and potential as therapeutic targets for small-molecule inhibitors.

Analyzing the expression of miRNAs

The three approaches most commonly used to identify new miRNAs are forward genetics, directional cloning and bioinformatic analyses. Forward genetics has been used in *C. elegans* and *D. melanogaster* and mandates a mutant phenotype to establish a functional role of small RNAs. Large-scale identification of miRNAs from various cell lines and diverse tissues of mouse, human, fly and zebrafish was made possible when directional cloning techniques were developed[15,16]. To overcome the difficulty of identifying miRNAs expressed in specific (rare) cell types or at low levels, computational algorithms implementing phylogenetic conservation and the structural characteristics of miRNA precursors have been developed[17].

Expression analysis of miRNAs by oligonucleotide microarrays is, in theory, a powerful tool to monitor the whole genome for tissue-specific miRNA expression and regulatory changes in developmental, physiological and disease states, if the method is both sensitive and discriminatory between related miRNAs. Many commercially available miRNA microarrays include the content found in Sanger mirBase 7.0 (http://microrna.sanger.ac.uk), making it possible to measure most expressed miRNAs. Although cross-hybridization and specificity impose important caveats for the first generation of reagents, miRNA microarrays have been used successfully by many groups to study miRNA expression. First

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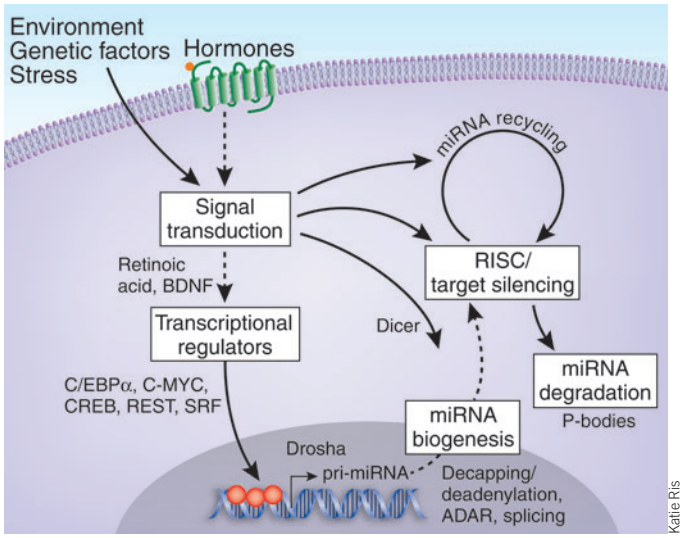

Figure 1 Hypothetical model for the regulation of miRNA expression and function. The schematic indicates how miRNA activity may be regulated by environmental and genetic factors. Extracellular signals could modify activity of an miRNA by affecting its expression, stability (by controlling synthesis or degradation) or cellular localization. Supportive evidence for the model is described in refs. 58–64.

reports using oligonucleotide microarrays to analyze miRNA profiles in multiple tissues confirmed the existence of several tissue-specific miRNAs[18–21]. The restriction of miRNAs to certain tissues may suggest that some sequences have an organ- or cell type–specific function. The factors that control the expression of miRNAs are largely unknown. Promoter elements that could contribute to the expression of the muscle-specific miR-1/miR-133 cluster have been identified[22,23], and several transcription factors have been shown to regulate specific microRNAs (**Fig. 1**). In addition to transcriptional regulation, however, the miRNA biogenesis pathway could be altered at several post-transcriptional levels (**Fig. 1**). It will be important to determine whether any of these regulatory steps are affected in disease states. Microarrays have also been used to study miRNA profiles during differentiation of cells, such as myoblasts[22] or preadipocytes[24]. Levels of *miR-143*, for example, increase when preadipocytes differentiate; this process can be inhibited when *miR-143* function is abolished[24]. Abnormal patterns of miRNA expression have been found in many disease states, most notably human cancer. Both increased and decreased expression of miRNAs have been described in neoplastic tissues. For example, the expression of *miR-15* and *miR-16* is decreased in most B cell chronic lymphocytic leukemia[25]; *miR-143* and *miR-145* are downregulated in colon carcinomas[26]; and *let-7* expression is reduced in small lung carcinomas[27,28]. Furthermore, restoration of the steady state levels of *let-7* can inhibit cell replication in a lung cancer cell line (A549)[28]. Examples of increased expression of miRNAs in cancer include marked upregulation of *miR-155* (ref. 29) and the *miR-17-92* cluster in B cell lymphoma[30]. miRNAs are frequently located in chromosomal regions that are altered in human malignancies owing to genetic instability, deletion and DNA amplification[31], suggesting that miRNAs may have a broad role in cancer. Although microarrays and bead-based flow cytometry have substantially extended our knowledge of miRNA profiles in human cancer, the causal contribution of miRNA in the etiology of malignancies is still uncertain. But increasing evidence suggests that miRNA profiling could contribute to more precise tumor classifications and predict therapeutic outcomes in the future[32]. Taken together, these findings highlight the important role of genome-wide miRNA expression profiling in the functional analysis of cellular differentiation, proliferation, and inherited and acquired disease states.

Although miRNA expression techniques such as northern blotting, tissue-specific RNA cloning and microarrays have proven to be powerful in determining expression signatures in many organs and cell types, these methods cannot distinguish miRNA expression between cell types in close proximity. Therefore, several techniques have been developed to visualize miRNA expression *in vivo*. Transgenic approaches that use transcriptional fusions of miRNA promoter regions and reporter cassettes driving GFP or β-galactosidase expression have been successfully used in *C. elegans*[33]. A similar method to detect the presence of specific miRNAs in tissues has been the development of 'sensor' transgenes, which constitutively express a reporter gene that contains sequences that are complementary to a given miRNA in the 3′ untranslated region (UTR)[34]. Cells lacking the miRNA that the sensor is designed to detect will express the transgene and allow reporter expression. In contrast, cells expressing the miRNA will lack expression of the reporter gene (for instance, GFP), owing to the perfect complementarity of the miRNA to sequences in the 3′ UTR of the sensor mRNA, which targets it to the RNA interference pathway. This method has potentially excellent spatial and temporal resolution, but it is not known whether it can be used for miRNAs that are expressed at low levels or whether high transgene expression could 'overwhelm' endogenous miRNA expression. The most frequently applied method for visualizing miRNA expression to date is *in situ* hybridization. It has been extensively used to map protein-coding mRNAs, although it does not provide quantitative information. *In situ* hybridization remains challenging with regard to the short sequences of miRNAs, but recent advances in protocols (such as using LNA-modified probes) could help overcome these problems and have already provided information about previously unidentified miRNA expression in mouse embryos[35].

Identification of miRNA targets

In stark contrast to the accumulation of validated miRNA sequences is the lack of experimental evidence identifying their corresponding targets. Several independent groups have established computational algorithms designed to predict target genes of miRNA sequences[36–40]. In general, the basis for these prediction programs is the degree of sequence complementarity between a miRNA and a target UTR, including the presence of a consecutive string of base pairs at the 5′ end of the miRNA known as a 'seed' or 'nucleus', and the cross-species conservation of this binding site. On average, 200 genes are predicted to be regulated by a single miRNA[38,40]. Reviewing the data provided by these algorithms determining candidate targets uncovers the entire gamut of gene categories, such as transcription factors, protein kinases, vesicular trafficking molecules and membrane receptors, suggesting that there is no apparent bias towards one particular function. Predictions of miRNA targets could become even more extensive as recent experimental evidence suggests that binding sites of miRNAs to 3′ UTRs do not necessarily have to be conserved among different species[41,42]. Binding of multiple miRNAs to one target could further increase the complexity of target predictions[38]. To date, several methods have been established to show miRNAs regulating their putative target genes. Most commonly used are luciferase reporter constructs containing the target 3′ UTR with the putative binding site downstream of the reporter coding region. These constructs are used to transfect cells expressing the relevant miRNA, along with vectors carrying mutant versions of binding sites. Evidence for miRNA activity can be established when wild-type reporters have less activity than their respective mutants. A complementary approach is to

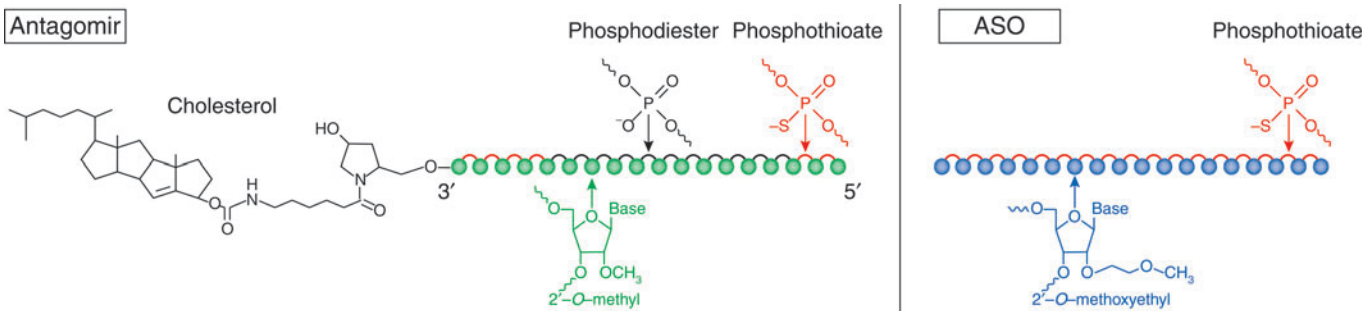

Figure 2 Schematic representation of chemically modified single-stranded RNAs that have been shown to silence miRNAs *in vivo*. Antagomirs[41] are cholesterol-conjugated RNAs with a partially modified phosphorothioate backbone (red) and 2′-*O*-methyl oligoribonucleotide modifications (green). ASOs[55] are 2′-*O*-methoxyethyl phosphorothioate–modified antisense oligonucleotides.

use loss-of-function studies in which the miRNA can be inhibited using antisense 2′-*O*-methyl–modified oligoribonucleotides[22,43,44]. Increased luciferase activity in reporter assays or upregulated gene expression of the endogenous target protein indicates inhibition of miRNA activity. Another approach taken to determine miRNA targets has been to increase the intracellular concentration of a miRNA by transfection of homologous synthetic short interfering RNAs or recombinant adenoviral infection and measure gene expression by microarray. This approach was shown to be effective as many predicted genes were regulated on the array[41,45].

Overexpression of miRNAs *in vitro* and *in vivo*

Induced expression of miRNAs was the initial step in many studies that identified miRNA function in model organisms or mammalian cell systems. Transient overexpression of miRNAs in cell-based assays can be achieved by transfection of double-stranded RNA molecules that mimic the Dicer cleavage product. But long-term studies in cultured cells or mouse tissues depend on DNA plasmids that continuously generate functional miRNA from endogenous or viral promoters. The design of these vector constructs is relatively simple. miRNAs are transcribed by RNA polymerase II, and their primary transcripts contain a 5′ cap and poly(A) tail. This allows overexpression of miRNAs using the same vector constructs that are used for protein-coding mRNAs. Introducing miRNA sequences that were flanked by at least 40 nt from their precursor into DNA plasmids was, in general, sufficient to yield mature miRNAs[46]. Such constructs are being widely used in cultured cells and have even allowed overexpression of different miRNAs from a single precursor[30,47]. Several groups have introduced miRNA-expressing plasmids into adenovirus[41,43] or retrovirus systems[30,46] to overcome the low transfection efficiency of primary cells or to deliver miRNA to mouse tissues *in vivo*. The systemic delivery of miRNAs into mice by intravenous injection of viruses is mostly limited to the liver tissue. Several groups have circumvented this limitation by *ex vivo* gene transfer into hematopoetic stem cells or fetal liver cells and reconstitution into recipient mice[30,46]. Tissue-specific overexpression of a miRNA *in vivo* can also be achieved by generation of transgenic mice. This approach was used to study the function of *miR-1* in cardiogenesis[23]. Studies that are based solely on overexpression of miRNAs must be interpreted with caution. Misexpression of mRNAs could target genes that would otherwise not be affected in a physiologic context, because of either low expression levels of the endogenous miRNA or spatial differences between the miRNA and its target. Therefore, results that are based on induced expression of miRNAs should ideally be confirmed by loss-of-function experiments.

Genetic approaches to silencing miRNAs

The development of recombinant technologies to generate loss-of-function mutations in mice has been invaluable in the elucidation of gene function. Several complementary approaches can be used to disrupt miRNA-mediated gene regulation: (i) conditional alleles of the miRNA-processing gene *Dicer1*, leading to deficiency of all mature miRNAs; (ii) knockout of miRNA genes in mice; and (iii) mutation of miRNA target sites in protein-encoding genes. Several mutated alleles of *Dicer1* have been generated in mice, and analysis of their phenotypes indicates that miRNAs maintain an important functional role in many developing tissues. Data showing that *Dicer1*-null embryos arrest early in development (at embryonic day 7.5) with reduced expression of embryonic stem cell markers such as Oct4 suggest that miRNAs are essential for the maintenance of stem cells in the early embryo[48]. Furthermore, conditional inactivation of Dicer by site-specific Cre recombination in the mouse limb bud mesenchyme led to the observation that mature miRNAs are required for cell survival and formation of limb skeletal elements[49]. In the developing lung, Dicer has a key role in regulating lung epithelial morphogenesis[50]. Although these examples highlight the important collective role of mature miRNAs, this approach falls short of providing information on the exact role of each unique miRNA. One approach to study the contribution of individual miRNAs is to restore the expression of specific miRNAs, miRNA families or miRNA clusters in a Dicer-null background. This method has recently been demonstrated in Dicer mutant zebrafish with abnormal morphogenesis during gastrulation, brain formation, somitogenesis and heart development. Injection of *miR-430* miRNA duplexes into developing embryos rescued the brain defects, indicating that this miRNA has an essential role during morphogenesis[14].

miRNAs often exist in families of highly related or even identical sequences. This high degree of redundancy poses unique challenges for the generation of simple loss-of-function approaches. One example, the *let-7* gene family, has been evolutionarily expanded from 1 member in nematodes to 11 members in mice and humans. Also, many miRNAs are clustered together, and the deletion or disruption of individual members may interfere with proper folding and processing of the polycistronic transcript, thereby affecting the expression of neighboring miRNAs. Similar limitations could apply to miRNAs that are located in introns of protein-coding genes[51].

Finally, mutating the miRNA binding sites in a target gene can disrupt miRNA-mediated gene regulation in mice. This approach is applicable when the contribution of specific miRNAs to the expression of a particular target warrants investigation. For example, knock-in mouse models could be generated such that mutations in the

targeted 3′ UTRs confirm the role of the miRNA-target interaction for a miRNA-associated phenotype. This approach will be less viable for simultaneously investigating a larger number of target genes.

miRNA silencing using antisense targeting

Nongenetic approaches have been used to silence miRNA function in cell lines[52], *C. elegans*[53] and *D. melanogaster*[54] using 2′-*O*-methyl–modified oligoribonucleotides that are complementary to the targeted miRNA. These oligos act as irreversible, stoichiometric inhibitors of small RNA function. But additional modifications are needed to deliver miRNA inhibitors to mouse tissues *in vivo*. Silencing of miRNAs in mice has recently been achieved by administration of cholesterol-conjugated single-stranded RNAs complementary to miRNAs, called 'antagomirs'[41], that are stabilized with a partial phosphorothioate backbone in addition to complete 2′-*O*-methyl modifications (**Fig. 2**). When antagomir-122, selective to *miR-122*, a highly enriched miRNA in the liver, was administered to mice by intravenous injection with normal pressure, a marked reduction of endogenous *miR-122* levels was observed in the liver. This technology was also able to reduce the expression of a ubiquitously expressed miRNA, *miR-16*, in most tissues, including liver, lung, kidney, heart, intestine, fat, skin, bone marrow, muscle, ovaries and adrenals. The silencing of miRNAs using antagomirs is dose-dependent, can be observed within 24 h and lasts for at least 3 weeks. Therefore, this technology can assess the effect of acute and long-term miRNA silencing. Antagomirs do not affect the expression of miRNAs in a polycistronic cluster that are not targeted[41], thereby enabling the study of single microRNAs within a common precursor. Four nucleotide mismatches prevented antagomir-122 from affecting miR-122 levels; it remains to be determined whether antagomirs can discriminate between members of miRNA families that differ by less than 4 nt (as in the *let-7* family). The mechanism by which antagomirs are taken up by cells is currently unknown. Antagomirs have no activity in the brain, suggesting that they may not be able to cross the blood-brain barrier. Furthermore, when injected into female mice late in pregnancy they do not silence miRNAs in embryos, indicating that antagomirs do not readily cross the blood-placental barrier (J.K. & M.S., unpublished data). Future studies will need to assess the matter of whether direct administration of antagomirs into the central nervous system or into the embryo will allow the study of miRNA function in the brain or during development.

An alternative strategy to target miRNAs *in vivo* has recently been reported using antisense oligonucleotides (ASOs)[55]. ASOs are unconjugated single-stranded RNAs that carry complete phosphorothioate backbones and 2′-*O*-methoxyethyl modifications (**Fig. 2**). In this study, *miR-122* was efficiently inhibited in the liver after a 4-week treatment period and *miR-122* levels were markedly reduced. It is currently unknown whether ASOs allow for acute studies on miRNA function *in vivo* as time kinetics have not yet been reported. It also remains to be determined whether ASOs can inhibit miRNA function in extrahepatic tissues. But studies using either antagomirs or ASOs show notable similarities in terms of target identifications of *miR-122*, and both studies suggest that endogenous *miR-122* has a role in lipid metabolism of the liver.

Unbiased approaches to study miRNA function

The technologies described above provide the framework in which miRNA function can now be studied *in vitro* and *in vivo*. Genome-wide expression profiling of cells and tissues in different stages of development or differentiation, metabolic conditions, and disease models using miRNA-specific microarrays may uncover clues about specific miRNAs that are intrinsically involved in these processes. Collective silencing of miRNAs by conditional Dicer mutants can furthermore provide important phenotypic information. Perturbation of miRNA expression, including overexpression and silencing, is a powerful approach to study miRNA function. These studies should ideally be done in primary cells or *in vivo* and coupled to phenotypic and gene-expression studies (**Fig. 3**). It has been suggested that miRNAs modulate translational gene expression, but recent evidence has shown that miRNAs also regulate the steady-state mRNAs of many targets[41,45,56]. The effects of miRNAs on the target mRNA levels are usually modest but can be measured reliably using state-of-the-art microarray gene-expression technologies. Silencing of *miR-122* in the liver using antagomirs identified a large number of transcripts that were upregulated compared with controls using a scrambled antagomir[41]. Many of these transcripts had at least one *miR-122* recognition motif that corresponded to nucleotides 2–7 of the miRNA, suggesting that they could represent direct miRNA targets. Most of these genes were not predicted by existing target prediction algorithms, a finding that can be explained by stringent criteria concerning the evolutionary conservation of putative binding sites. Therefore, genome-wide expression analysis of loss-of-function and overexpression models constitutes an unbiased approach of identifying miRNA targets and has the advantage of not relying on a prespecified computational model. In addition to identifying direct targets, this analysis can also uncover a positive relationship between the expression of protein-coding genes and miRNAs. For example, silencing of *miR-122* in the liver identified a substantial number of genes that were downregulated[41]. The annotation of these genes in Gene Ontology categories identified cholesterol biosynthesis as the top group and comprised a total of eight genes, including the rate-limiting enzyme 3-hydroxy-3-methylglutaryl-CoA-reductase, which was markedly downregulated. Silencing of *miR-122* in these mice also lowered plasma cholesterol levels[41]. Overexpression of *miR-122* in the liver using a recombinant adenovirus resulted in increased expression of cholesterol biosynthesis genes. Even though the precise mechanism by which miRNAs activate gene expression is not understood, this example shows that manipulation of miRNA gene expression, coupled with gene-expression profiling, computational approaches and phenotyping, can uncover effects of miRNAs that are likely indirect (such as inhibition of a transcriptional repressor).

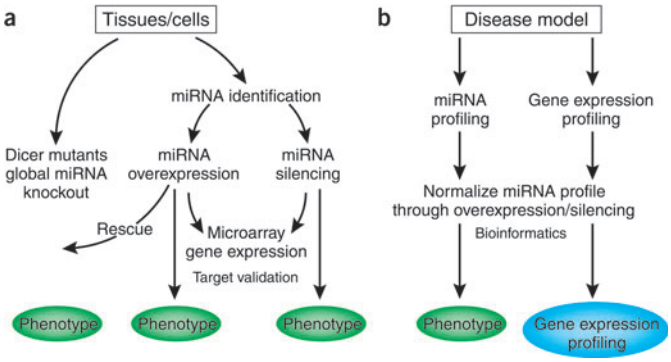

Figure 3 Experimental strategies to study miRNA function using molecular, genetic and bioinformatic techniques. (**a**) Identification of miRNAs in distinct cell types can be achieved by microarray expression profiling or random sequencing of miRNAs. The effect of collective silencing of miRNA expression can be studied by inactivation of Dicer. Alternatively, the expression of specific miRNAs can be manipulated by either overexpression or gene silencing. Gene-expression analysis can then be used to validate miRNA targets and analyze phenotypes. (**b**) Strategies to study miRNA function in disease states. Expression profiling can be used to test whether miRNA levels are altered in specific disease states. miRNA profiles can then be restored *in vivo* by overexpression or silencing of specific miRNAs. The effect on the disease phenotype and gene expression levels can then be assessed.

Future directions and challenges

miRNAs, like transcription factors, are an abundant class of gene-regulatory molecules in animal cells. Their unique mode of action requires new analytical strategies. The technology to systematically identify the miRNA target genes and to study the biological effects of overexpression or silencing of specific miRNAs is now available. It will also be important to elucidate signaling pathways that affect miRNA function as well as environmental or genetic factors that affect miRNA expression. Once this information is available, a rational approach can be taken to design new therapeutic strategies that aim to correct inherited and acquired diseases. Because the effect of single miRNAs on gene expression and other phenotypic traits may be modest, an important future step will be to study combinatorial effects of multiple miRNAs on target gene expression. Many 3′ UTRs of mRNAs contain multiple ubiquitous as well as tissue-specific miRNA target sites. *In vitro* experimental evidence suggests that different miRNAs can act synergistically to inhibit gene expression[38]. The elucidation of how global miRNA expression contributes to phenotypic outcomes will be important to interfere with disease-relevant pathways. The use of chemically modified RNAs may be an effective strategy to manipulate miRNA expression. Modified short interfering RNAs have been shown to silence target gene expression in mice[57], and such synthetic short interfering RNAs that are homologous to miRNAs may also prove effective for ectopic miRNA expression or increasing miRNA activity. Conversely, miRNA function can be inhibited by chemically modified RNA analogs with complementary sequence to miRNAs. These compounds selectively reduce the detectable steady-state abundance of miRNAs and inhibit their function[41,55]. A molecular understanding of how double- and single-stranded RNA are taken up by cells will be important to develop drugs that can enhance or inhibit miRNA function. For instance, it may be possible to conjugate modified RNAs to ligands that recognize specific cell surface receptors and mediate selective delivery to certain cells. This may also result in a reduction of the dose that is required to silence miRNA expression by chemically modified RNAs. In addition, different routes of application may help directing miRNA inhibitors to tissues (*e.g.*, brain) that have been difficult to target.

COMPETING INTERESTS STATEMENT
The authors declare competing financial interests (see the *Nature Genetics* website for details).

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