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Macros in microRNA target identification

A comparative analysis of in silico, in vitro, and in vivo approaches to microRNA target identification

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MicroRNAs (miRNAs) are short RNA molecules that modulate post-transcriptional gene expression by partial or incomplete base-pairing to the complementary sequences on their target genes. Sequence-based miRNA target gene recognition enables the utilization of computational methods, which are highly informative in identifying a subset of putative miRNA targets from the genome. Subsequently, single miRNA–target gene binding is evaluated experimentally by in vitro assays to validate and quantify the transcriptional or post-transcriptional effects of miRNA–target gene interaction. Although ex vivo approaches are instructive in providing a basis for further analyses, in vivo genetic studies are critical to determine the occurrence and biological relevance of miRNA targets under physiological conditions. In the present review, we summarize the important features of each of the experimental approaches, their technical and biological limitations, and future challenges in light of the complexity of miRNA target gene recognition.

Introduction

Small regulatory RNAs, aptly named microRNAs (miRNAs), are critical regulators of diverse biological phenomena. MiRNAs are typically 21–25 length polynucleotides, untranslated RNA moieties that regulate post-transcriptional gene expression.[1] The primary mechanism of action is base-pairing of miRNA unique "seed sequences" to a complementary sequence on the target genes.[2] MiRNA genes are found either in intergenic regions with independent promoter and regulatory units or within introns of other genes, or even in exons, although this is rare.[3,4] MiRNAs are transcribed by RNA polymerase II as a much larger primary transcript (pri-miRNA), which is sequentially cleaved to form mature miRNA.[5] In the nucleus, pri-miRNAs are processed by the RNaseIII endonuclease Drosha to ssRNA hairpin precursors (pre-miRNA), which are transported across the nuclear envelope into the cytoplasm by exportin5 and processed to mature miRNA by RNase Dicer in the cytoplasm.[6] Although both strands of the miRNA are co-transcribed, only one of the strands, a mature miRNA molecule, is bound by Argonaute (Ago) proteins and secured in the miRNA-induced silencing (RISC) complex, the other strand, known as a passenger strand (miRNA*), is degraded. Alternatively, the two strands are also denoted with the suffix -5p or -3p, according to the end of pre-miRNA on which they are generated.[7] The binding of mature miRNAs dictates post-transcriptional gene silencing through base-pairing of the 5′-seed sequences (two to seven nucleotides) with the 3′-UTR of the target gene.[2] It recently has also been shown that miRNAs can increase target gene expression under specific circumstances.[8-10]

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MiRNA—Miles to Go

The first miRNA, *lin-4*, was identified in 1993; for its role in cell-fate determination in *C. elegans*, to be followed 7 years later by *let-7*.[11,12] The field of miRNA research has made rapid advances, such that it is now clearly established that miRNAs play critical roles in diverse biological processes. An appreciable amount of evolutionary conservation is observed across hundreds of known miRNAs. The conservation of miRNA families through millions of years of selection pressure emphasizes the essential role played by these regulatory molecules.[13] A genome-wide analysis estimates that at least 60% of all genes are regulated by miRNAs.[14] The recent advances in sequencing techniques have enabled the de novo identification of thousands of both conserved and unique miRNAs across several metazoan species, while also making available the sequences of their target mRNAs. Since the first algorithm in 2003, several tools have been developed to predict the biologically relevant miRNA–mRNA target gene interactions. These computational methods are useful in streamlining the putative miRNA target genes, thus enabling researchers to prioritize their research.[15,16] The predictive scoring methods are based on a set of parameters expected to influence the interaction between miRNA–mRNA species. Understanding the predicted strength of miRNA–target genes is essential for subsequent functional analysis. Several in vitro cellular assays have been developed to investigate the phenomenon of target

gene repression by miRNAs.[17] The nature of miRNA gene targeting in animals is particularly complex as: (1) miRNA can bind to partially complementary sequences, leading to a large number of putative targets; (2) the majority of miRNA effects on their target genes show only a modest repression; and (3) distinction of first order effects from second order requires elaborate experimental validation of target genes. A combination of several technical tools often needs to be employed to predict the biological occurrence of miRNA–target gene interactions with greater certainty.

The implication of miRNAs in a number of human diseases warrants a critical need to identify miRNA-regulated genes in biologically relevant contexts.[18,19] Only a small subset of in silico predicted miRNA–target gene pairs has been experimentally validated. The most commonly used cellular assays are based on the principle of studying the functional consequences of artificially manipulating endogenous miRNAs levels. However, as the biological concentrations of these miRNAs in vivo may be several orders of magnitude different than in in vitro conditions, it is essential that the results of ex vivo target recognition techniques be recapitulated in appropriate animal models. Unfortunately, only a few transgenic animals have been specifically designed for miRNA research as a means to provide biological support for ex vivo research findings. Consequently, in vivo biological approaches to microRNA target identification have not been adequately addressed in the literature published so far. In the present review, we discuss the key features of each of the approaches and emphasize how they complement each other. The future of miRNA target identification will require researchers to design experiments with increasing sensitivity to uncover miRNA functional roles within their chosen biological context, such as engineered animal models. This present review's approach and presentation of compiling in silico, in vitro, and in vivo, (a priori) methodologies to microRNA target identification should provide a resource useful toward these endeavors in the future.

Approaches to miRNA Target Identification

Computational methods of miRNA target prediction

The miRNA–mRNA interactions based on sequence complementarity have made available a large number of genes as possible targets for single miRNAs, while also presenting opportunities for in silico prediction of target genes. The available algorithms have been developed based on our existing knowledge of known miRNA–mRNA interactions, to provide a predictive strength on miRNA target genes. The in silico approaches are highly significant and facilitate high-throughput identification of miRNA target genes. However, these methods are complex, as miRNAs often bind to targets with incomplete complementarity, containing mismatches, gaps, and G:U base pairs at multiple positions.[2,20] Since the rules of target recognition are not completely understood, each prediction tool utilizes a unique combination of criterion for miRNA target site prediction. The most commonly utilized criteria by target prediction software are discussed in the following sections.

Seed sequence

The target prediction algorithms identify potential base-pairing between 5′-seed region (nucleotide 2–7) of miRNA with 3′-UTR of the target mRNA. Three primary categories of binding sites can be recognized: (1) canonical sites with seven to eight nucleotide match in the seed region, which are present in the majority of known targets; (2) 3′-supplementary sites at positions 13–16 which provide higher efficacy in target gene downregulation; and (3) 3′-compensatory sites which can compensate for the seed mismatch.[21] The assertion on the two to seven nucleotide seed complementarity is supported, as the 5′-end is the most conserved region of the mammalian miRNA, suggesting its biological relevance.[22] However, the prediction algorithms' search for sequence complementarity between the 5′-end of miRNA and the 3′-UTR of the mRNA vary in the extent of this requirement. For example, TargetScan[23] requires strictly complementary sequences, whereas miRanda allows for one wobble position in the seed region to be compensated with the sequence match in the 3′-end of the miRNA.[24] Another application, DIANA-microT, considers the entire miRNA topology in target gene prediction.[25]

Target site conservation

The conservation of the miRNA binding site in the 3′-UTR of the orthologous genes is a significant feature in predicting miRNA targets. As positive natural selection is expected to act on biologically important miRNA–mRNA interaction, a higher degree of conservation arguably reflects a more reliable prediction. The emphasis on phylogenetic conservations in making target gene predictions is based on seed match (DIANA-microT, PicTar, miRanda, and TargetScanS)[24-27] and calculations on evolutionary distances (EIMMO).[28] However, the computational distinction between identification of targets sites that are functionally preferentially conserved, from those that are expected to be conserved by chance, remains a major challenge to using sequence conservation criterion in target prediction.

Thermodynamic stability

Thermodynamic analysis calculates the energy required for the formation of miRNA–mRNA pairs from a completely dissociated state and is denoted as minimum free energy (MFE) for hybrid formation.[20] This consideration of the stability of miRNA–mRNA base-pairings commonly used by algorithms (DIANA-microT, PITA, and PicTar)[29] is significant in considering that a sufficient time is required for the RISC complex to process its enzymatic activity. The RNA folding programs, such as Vienna package and RNA hybrid, calculate MFE. Additional factors, such as the relative concentration of miRNA and mRNA molecules that affect the energetics of the reaction, remain unavailable for making free energy calculations.

Multiple target sites

The algorithms consider that occurrence of multiple target sites will have a dose-dependent effect on target gene expression. This observation is further extended for a single miRNA and/or the binding of multiple miRNAs on the same gene across species.[30,31] A notable algorithm that extensively considers this

Table 1. Characteristic features of algorithms providing information beyond classical miRNA target gene interaction

Prediction method	Characteristic features	Resource
Diana Micro-T	Target prediction made with miRNA or mRNA sequences as input	http://diana.cslab.ece.ntua.gr/microT/
FAME	Experimentally verified miRNA pathways infer biological process affected by miRNAs	http://acgt.cs.tau.ac.il/fame/
Hoctar	Information on host genes regulating expression of its embedded microRNA	http://hoctar.tigem.it/
Magia	Query of miRNA target prediction, analysis of expression profiles, and post-transcription regulatory network	http://gencomp.bio.unipd.it/magia/start/
MaMi	miRNA target prediction based on hybridization energies and secondary structures for the miRNA-mRNA hybrid where parameters can be modified by the user	http://mami.med.harvard.edu/
Microinspector	Identification of potential miRNA binding sites in user-submitted sequences, searching against databases of known miRNA binding sites	http://bioinfo.uni-plovdiv.bg/microinspector/
miR2disease	Information on miRNA association to a disease process	http://www.mir2disease.org/
miRBase	Complete repository of miRNA sequences and targets	http://www.mirbase.org/
mirDIP	mirDIP integrates 12 microRNA prediction data sets from six microRNA prediction databases	http://ophid.utoronto.ca/mirDIP/index.jsp
miRecords	Two main modules, experimentally validated targets, and integrated information across 11 independent prediction softwares	http://mirecords.umn.edu/miRecords/index.php
miRGator	Integrates miRNA expression data with mRNA and protein to interpret the biological functions of miRNAs	http://genome.ewha.ac.kr/miRGator/miRGator.html
miRNAmap	Information on statistics of miRNA sequences and target genes	http://mirnamap.mbc.nctu.edu.tw/
MirPath	Identification of altered molecular pathways by the expression of specific miRNAs	http://83.212.96.7/DianaToolsNew/index.php?r=mirpath
miRTar	Information on the biological function of miRNA-target gene pairs and on miRNA sites on the alternative spliced transcripts	http://mirtar.mbc.nctu.edu.tw/human/
MiRTarBase	Information on experimentally verified miRNA targets by data mining and manually surveying pertinent literature related to functional studies on miRNAs	http://mirtarbase.mbc.nctu.edu.tw/
miRWalk	miRNA-target information on the complete sequence (promoter, 5′-UTR, CDS, and 3′-UTR) and target interaction information across eight other types of prediction software; information on experimentally validated targets	http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html
Patrocles	Polymorphisms on miRNA sequences and target genes	http://www.patrocles.org/
PITA	Secondary structure of the miRNA-mRNA hybrid for target gene prediction	http://genie.weizmann.ac.il/pubs/mir07/index.html
Tarbase	Experimentally validated miRNA targets	http://diana.cslab.ece.ntua.gr/tarbase/
Targetscan	Classical software for miRNA target gene prediction	http://www.targetscan.org/

Table 2. Analytical techniques for miRNA target identification

	Technique	Advantages	Limitations
Gene expression	Microarray	• High-throughput • Highly informative in identifying a subset of target genes	• Unable to distinguish primary targets vs. secondary effects • No evidence of direct miRNA-mRNA interactions • High false-positive and false negative
	RNA Sequencing	• High-throughput • Higher signal-to-noise ratio and high dynamic range • Identification of full-length transcripts includes premature termination sequences and other isoforms	
	CLIP	• Use of argonaute-specific antibodies dictates the evidence of a direct interaction • High-throughput when coupled to microarray or RNA-Sequencing	• Require large quantities of cells or tissue • Optimization of enzymatic and cross linking steps
	LAMP	• Does not need the knowledge of 3′-UTRs • Specific to miRNA	• Effect of adding label to miRNA on its binding is not known
Protein output	SILAC	• Metabolic labeling makes the quantification straight forward • Mammalian cells are labeled easily	• Secondary effects lead to a much higher false-positive rate • Not as high-throughput as transcriptome-based techniques • High set-up cost and not as time efficient
	RPA	• Useful for analyzing a limited amount of clinical samples	
	Translation profiling	• Quantitative data at a greater depth than other techniques	
	Reporter assays	• Easy to adopt • High sensitivity • A wide dynamic range	• Requires prior knowledge of potential miRNA-mRNA interacting pairs • Limited to analysis of single miRNA-mRNA target gene • Caveats of miRNA overexpressio
	Immunoblotting	• Assay the ultimate effect of miRNA activity • Easy to adopt • High sensitivity • A wide dynamic range	

parameter is PicTar.[29] However, the functional consequences of such complex interactions involving multiple tandem binding sites need to be subjected to rigorous experimental verification to improve the predictive scores.

Extension of prediction algorithms

Identification of promising miRNA targets is critical in determining the success of future experiments, so concerted efforts have been made to improve the functionality of prediction algorithms. The computational tools for miRNA target prediction currently available have been appropriately modified to incorporate recent information on miRNA target gene interaction. As listed in **Table 1**, in addition to the conventional prediction of microRNA targets, these tools provide several other valuable features, including information on polymorphisms in miRNAs and target sequences (Patrocles),[32] co-expression of miRNA and their targets (miRGator),[33] analysis of pathways regulated by specific miRNAs (miRPath),[34] and miRNA disease relationships (miR2disease).[35] Further applications, such as miRWalk,[36] link the information across several independent prediction software and enable parallel analysis across multiple databases.

An alternative way to predict miRNA targets is by machine-learning algorithms, which utilizes a database of validated interactions (TarBase[37] and mirRecords[38]) against which it evaluates future predictions. A number of machine-leaning algorithms, such as TargetBoost,[39] TargetSpy,[40] MultiMiTar,[41] and NBmiRTar[42] have been developed. However, the dependence of machine learning algorithms on the quality of training data set of experimentally verified target genes represents a major source of bias.

Challenges to computational methods of target prediction

Computer-based prediction methods are valuable in preliminary identification of miRNA target genes. There are, however, inherent limitations to consider when applying the results of these searches to experimental verification. A majority of the current target prediction methods (with the exception of RNA 22, miRWalk) limit target gene prediction to the annotated 3′-UTR of the genes. A recent study demonstrated that miRNAs target as much as 25% of the sites in the coding region of the gene.[43] Though miRNA target prediction in protein coding regions will likely carry high false positive rates due to background conservation, further studies need to be done to validate and incorporate the possible miRNA target recognition sites outside the 3′-UTR context.

The prediction algorithms, albeit distinct, have overlapping characteristic features. Each method utilizes a unique combination of target prediction criterion, which presents the researcher with an opportunity to compare and evaluate their performance to attain an optimal level of precision. Using a high-throughput proteomic approach, the proposed gene targets of miRNAs were compared among different in silico prediction methods. It was observed that a group of five programs (DIANA-microT, ElMMo, Pictar, TargetScan, and TargetScanS) have a precision of ~50% and sensitivity between 6–12%.[44] In another study, a relative analysis of target prediction algorithms against validated genes in TarBase demonstrated that DIANA-microT, TargetScanS, and PicTar have a precision level > 60%.[45]

It is conceivable that the use of multiple prediction tools lead to a higher confidence in predicting miRNA–target gene

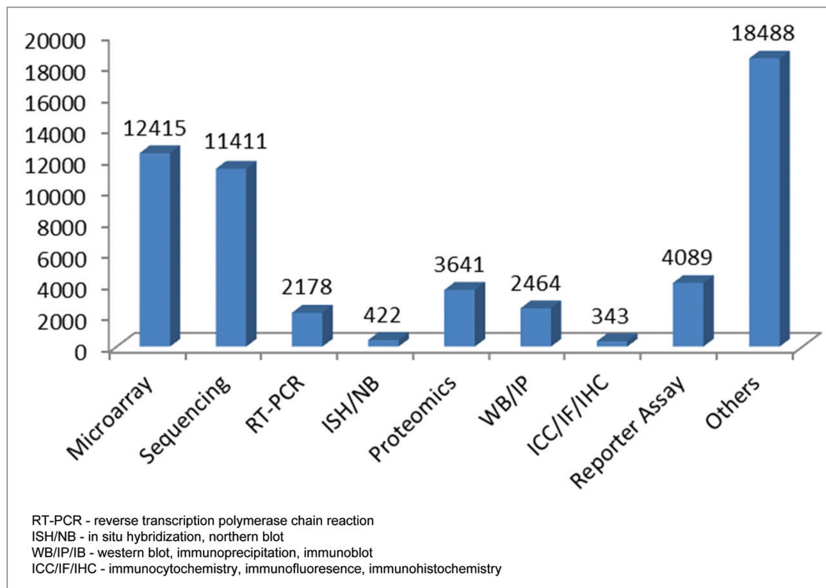

Figure 1. Experimentally verified vertebrate miRNA–target interactions available in miRTarBase (Release 4.5).

pairs. Using computational approaches, the false positives can be eliminated by further experimental analysis, but identification of false negatives is a technically daunting task. Nevertheless, in silico predictions are a proven method to identify a subset of miRNA targets for experimental verification and to further understand the role of miRNAs in gene-regulatory networks.

Experimental approaches to miRNA target identification

Experimental identification of the genes regulated by specific miRNAs is essential to elucidate the biological functions of miRNA moieties. The initial studies found that the extent of sequence complementarity between target mRNA and miRNA determined the degree of gene repression, whereas complete sequence complementarity affected mRNA stability, so a partial base pairing was likely to direct only suppression in translational machinery. Recent studies have demonstrated that, in spite of partial miRNA–mRNA base-pairing, many genes are regulated at the mRNA level.[46,47] A study investigating the significance and relative contribution of RNA and protein-based approaches found that mRNA levels account for about 84% of any observed decrease in protein output.[46] The effect of transcript destabilization by miRNAs is mediated by deadenylation, decapping, and subsequent 5′-3′ exonucleolytic cleavage. This effect is facilitated by binding of Ago2 and GW182 proteins to the RISC in humans. Translationally repressed mRNA molecules are sequestered in specialized cytoplasmic structures, known as P-bodies. Translational inhibition is operational if the decrease in protein output is more than that observed for mRNA expression and is primarily observed on the binding of initiation factor elF4E to the 5′-cap of the mRNA molecules. Several findings also support post-initiation suppression of translational machinery by causing premature termination and subsequent ribosome drop-off.[48,49] The molecular determinants of the outcome of miRNA–mRNA base pairing are not clear. Although the answer to the question of whether the phenomenon of transcript destabilization and translational inhibition are mutually exclusive remains elusive, the approaches to study the phenomenon of target repression by miRNAs can be adopted by assaying the abundance of RNA and/or protein molecules as an effect to miRNA manipulation. Alternatively, by utilizing biochemical assays, components of RISC machinery can be investigated. High-throughput technologies serve as an ideal interface between in silico target prediction and the first evidence of a biological interaction. Further analysis on single miRNA–mRNA target genes is important to determine the occurrence of an interaction.

High-throughput target identification

The analysis of changes in global gene expression upon introduction of a specific miRNA is highly informative in rapid identification of a large number of both first- and second-order miRNA target genes. The high-throughput approach for analysis of miRNA-associated expression signatures can be easily adapted to in vitro manipulated cell populations, as well as in vivo animal models of miRNA research.

Microarray profiling is a widely utilized approach to study genome-wide scale changes in gene expression. The miRNA–target gene interactions, which lead to a reduction in mRNA levels, are commonly detected by microarray analysis. The microarray-based approach is supported by the initial findings, which demonstrated that, upon transfection of miR-1 (muscle-specific) and miR-124 (brain-specific) in HeLa cells (which normally do not express these miRNAs), there is a global shift in the expression profile toward a more muscle-tissue- or brain-tissue-like expression, respectively.[47] Recently, advances in sequencing technologies have enabled the use of RNA-sequencing (RNA-seq) as an alternative to gene expression microarrays. As RNA-seq encompasses de novo sequencing of full-length transcripts, it has the advantage of identifying premature termination sequences and identifying changes to a specific isoform. RNA-seq technology, with its higher signal-to-noise ratio and a wider dynamic range, is increasingly being employed as an alternative to microarray hybridization-based transcriptome analysis. High-throughput sequencing of RNA isolated by cross-linking immunoprecipitation (HITS-CLIP) uses UV light for cross-linking, followed by purification, and sequencing of isolated miRNA-mRNA-Ago complexes.[50] A modification to this technique, photoactivable-ribonucleoside-enhanced cross linking and immunoprecipitation (PAR-CLIP), is more efficient in the extent of cross-linking and RNA recovery.[51]

High-throughput proteomic approaches involve various modifications to mass spectrometric analysis. Stable-isotope labeling with amino acids in culture (SILAC) determines the relative abundance of proteins labeled with heavy isotopes (mostly lysine and arginine) and subsequent mass spectrometric analysis.[52] Two-dimensional gel electrophoresis (2D-DIGE) involves isoelectrically

Table 3. Genetic studies to elucidate miRNA functions

miRNA	Biological function	Target gene/s
miR1–2 [65]	Cardiac development and physiology	Hand2, Irx5
miR126[66]	Endothelial cells' response to angiogenic stimuli	Spred1
miR133a[67]	Cardiac growth and function	Srf, Ccnd2
miR140[78]	Cartilage development and homeostasis	Adamts5
miR150[72]	Lymphocyte development	Myb
miR-155[79]	Normal functioning of the immune system - B- and T-lymphocytes and dendritic cells	Maf
miR155[73]	Suppression of *Myc-Igh* translocations in B cells via its target gene AID	Aicda
miR15a/16–1[71]	B cell proliferation	Ccne1,Chk1, Mcm5, Ccnd2, Cdk4, Cdk6, Ccnd3, Cdc25a, Arl2, Igf1r
miR17–92 cluster[80]	B cell survival by repressing pro-apoptotic genes	Bim
miR17–92 cluster[81]	Proliferation of lung progenitor cells	Rbl2
miR182[75]	No significant retinal phenotype	-
miR183 cluster[76]	Retinal function	-
miR206[82]	Onset of neurodegenerative ALS disease	Hdac4
miR-208[68]	Cardiac response to stress and hormonal signaling	Thrap1
miR208a[70]	Muscle gene expression and function	Myh7b,Sox6, Purb,Sp3,Hp1b
miR208a[69]	Cardiac function and conduction	Thrap1, Mstn
miR223[74]	Granulocyte activation and differentiation	Mef2c
miR375[83]	Glucose metabolism-, β-cell turnover	Several genes in growth-promoting pathways

separating cell populations labeled with different fluorophores and subsequent proteomic analysis.[53] These techniques can be modified for comparison of miRNA mimic/inhibitor-transfected cells. To capture correlative changes in abundance of protein, these data sets are subsequently queried to identify overrepresentation of targets containing seed-sequences to miRNA to distinguish second order effects of miRNA function. However, while high-throughput analysis can unequivocally establish global effects due to miRNA, there remains a challenge to appropriately interpret the massive amount of data generated and segregate the observed changes due to a direct miRNA–target interaction from the secondary changes in gene-regulatory network. Regardless, validation of high-throughput data using cellular assays is essential for miRNA–mRNA gene-specific interactions. MiRNA-associated effects on target gene expression can be studied by approaches such as qRT-PCR, luciferase assay, western blotting, and immunohistochemical analysis. Alternatively, several biochemical assays have been developed to investigate the RISC complex for direct evidence of an interaction between a specific miRNA and its target gene.[17,54] A combination of these techniques often is adapted to obtain a more definitive answer on miRNA–mRNA interactions.

In vitro biochemical assays

In vitro studies on isolated cell populations that are amenable to experimental manipulation are useful in identification of the molecular mechanisms of miRNA function. Biochemical assays often utilize either labeling or isolation with Ago-specific antibodies or exogenous miRNA mimic. Whereas, HITS-CLIP and PAR-CLIP capture the entire mRNA cellular pool with antibodies to Ago protein, labeling the miRNA and analysis of the associated transcripts is another commonly used experimental approach. Labeled miRNA pull-down assay (LAMP) involves the transfection of a tagged-miRNA (biotin/digoxygenin) and isolating the miRNA–mRNA complex using an antibody against the tag.[55] The assay has the advantage of not needing the genomic information on 3′-UTRs. Parallel analysis of RNA ends (PARE), also known as degradome sequencing, is a recently developed modification to RACE, which also identifies direct Ago2 RISC-associated cleavage targets.[56] It involves ligation of an RNA adaptor (containing *Mme*I sites) to 5′-monophosphorylated products of miRNA-induced cleavage. The ligated products are reverse-transcribed and cleaved with *Mme*I. The 5′-fragments are ligated to a 3′-double-stranded DNA adaptor and PCR-amplified and subjected to deep-sequencing.

Another approach to the analysis of translationally active ribosome-associated mRNAs can be used to identify the direct effects of miRNA-induced translational repression. This technique, known as "translational profiling," involves the separation of ribosome-bound and -unbound mRNAs populations on sucrose gradient, labeling, and hybridization to microarrays.[57] These techniques, however, do not measure protein levels they yield quantitative data at a greater depth than other direct protein-based approaches. Reverse-phase protein arrays (RPA) have also been applied to probe the complex mixtures of proteins with antibodies to target genes that have been predicted computationally. Such an approach is particularly useful for analyzing limited amounts of clinical samples.[58]

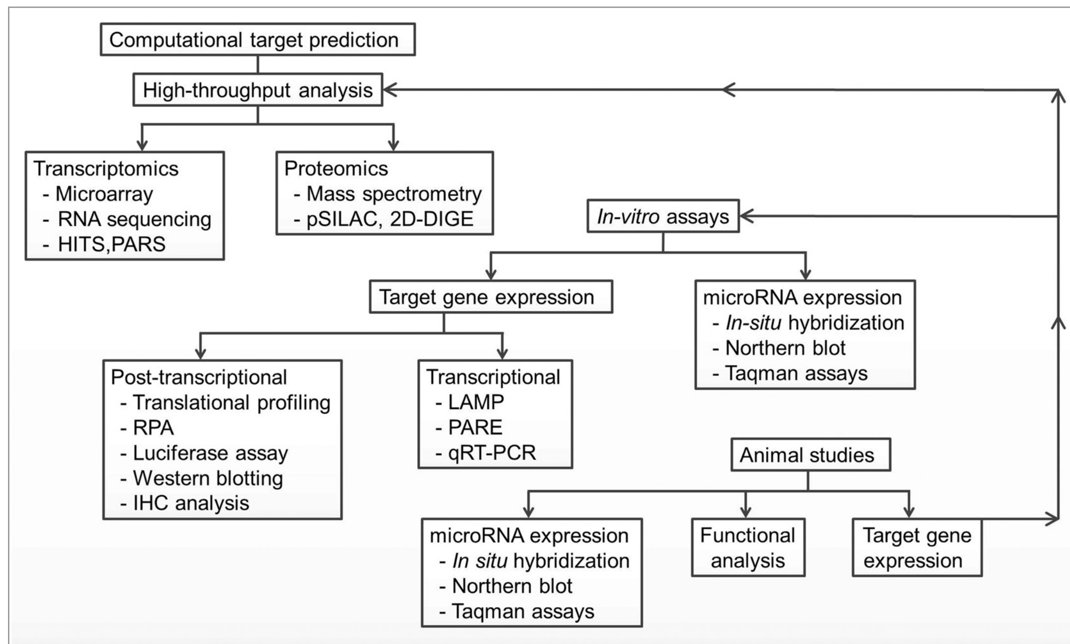

Figure 2. Flowchart summarizing the key approaches and major techniques in miRNA target identification.

A comparative analysis of various transcriptional and post-transcriptional approaches is shown in **Table 2**. The analysis of the number of miRNA target genes identified with major experimental approaches has been made using databases, such as TarBase and miRTarBase. We further analyzed the complete list of experimentally verified miRNA–target interactions available in miRTarBase (Release 4.5) and have graphically represented the data in **Figure 1**.

The caveats of ex vivo experimental techniques

Ex vivo studies provide excellent model systems to study the effects of miRNA manipulation on target gene expression. This includes the opportunity to artificially manipulate the concentrations of endogenous miRNAs with the use of double-stranded mimics and miRNA inhibitors, and to further validate the findings by investigating the rescue effects on mutations of these miRNA sequences. These cellular assays are based on the principle that loss-of-function or gain-of-function of specific miRNAs is associated with a corresponding change in the expression of a putative target. Although this seems plausible, a major caveat of such an assumption is that transfection with miRNA mimic or inhibitor often leads to supra-physiological levels that may perturb the endogenous gene-regulatory networks.[59] Recently, an analysis comparing miRNA overexpression (representing global mRNA changes due to miRNA transfection) to sequence data obtained from CLIP-based approaches (which represent a more direct interaction of target mRNAs with endogenous miRNAs and AGO proteins) supports the notion that alteration in miRNA levels has effects on the predictive power of miRNA–target interactions. Indeed, each of these methodologies were biased on detecting miRNA targets on different, yet complementary, parameters of miRNA target prediction.[60] It has been demonstrated that target genes for endogenous miRNAs have significantly higher levels of expression upon transfection with small RNA molecules.[59] Another study investigating the target genes of miR140 by both inhibition and overexpression, showed that although each affected the expression of hundreds of genes, only 49 overlapping genes were identified between the two groups.[61] Therefore, artificial concentrations might lead to the formation of erroneous miRNA–mRNA pairs that may not be relevant in complex biological situations. Further, the gene-regulatory networks operating within a cell may be highly complex.

While a wealth of information is available on miRNA regulation of target genes, not much is known about how miRNAs themselves are regulated. In physiological conditions, the cellular signaling pathways are highly complex and not linear, as investigated by in vitro experiments. For example, the activity of a miRNA and its target gene could in turn be a regulated by a common transcription factor. Thus, the effect of a miRNA on a joint target gene will be the combined effect of the transcription factor regulating the miRNA and the target protein-coding genes. Such interactions have been commonly observed and can be predicted in silico by a sequence-based prediction program.[62] An experimental verification of such an interaction is technically daunting and, when possible, is limited to analysis of only a few gene regulatory networks at a given time.

The significance of animal studies

MiRNAs play critical roles in maintaining cellular homeostasis by regulating key biological functions. A deregulated miRNA expression is therefore observed in many pathological conditions.[63,64] miRNAs often target multiple genes in a common regulatory network, so miRNA-based therapy offers a distinct therapeutic advantage over other approaches. Though the ongoing research in the field of miRNA biology has been successful in demonstrating the translational potential of miRNAs, the current utilization of miRNAs in clinics is primarily limited to expression profiling for diagnostic or prognostic purposes.[63]

The animal studies on miRNA done so far have established a critical role of miRNAs in several biological processes **(Table 3)**, while also demonstrating the complexity of miRNA-regulated pathways in physiological conditions. The knowledge obtained from in-depth analysis in appropriate animal models can be translated into designing miRNA-based therapeutics.

Sequence-based target gene predictions and in vitro approaches to manipulate miRNA functions in isolated cell populations have identified several miRNA–mRNA interacting pairs, yet in vivo identification of true miRNA targets is challenging. Animal studies investigating miRNA function in vivo have underscored miRNAs as critical mediators of biological processes, such as cardiovascular development (miR1-2,[65] miR126,[66] miR133a,[67] miR208,[68] miR208a[69,70]) and immune function (miR15a/16-1,[71] miR150,[72] miR155,[73] miR223[74]). Interestingly, certain miRNAs have undergone evolutionary processes to regulate the expression of critical target genes and are likely to produce greater changes in target gene expression. Consequently, a disruption of such miRNA–target gene interaction has more discernible phenotypic consequences. This is exemplified by miR150 regulation of c-Myb, which plays key role in lymphocyte development.[72] Alternatively, a single miRNA can target multiple genes in the same pathway, while a target gene may be regulated by several miRNAs. In physiological conditions, miRNA-regulated pathways are often redundant and highly complex.[29] A few of the studies highlighting the complexity of miRNA target recognition in in vivo conditions are discussed in the following section.

Complexity of miRNA regulation

The phenotypic consequences of miRNA targeting are subtle. Therefore, when extrapolating the rules of miRNA target repression to in vivo conditions, it is not surprising that the identification of such small changes in gene expression in a heterogeneous environment is challenging. It is estimated that 90% of evolutionarily conserved miRNA–mRNAs interactions involve a single target site and are expected to downregulate target mRNAs by less than 50%.[21] In addition, most mRNAs that have one miRNA site also harbor another site to some other miRNA. Thus, assuming active regulation by other co-expressed miRNAs, perturbing target interactions for one miRNA may not have an appreciable effect on target gene expression. The miRNA-perturbed pathways are also believed to be more tolerated due to gene regulatory buffering.[21] The targeted deletion of miR182 with abundant retinal expression did not show an upregulation in the expression of target genes by microarray analysis, and the cellular retinal structure was also normal.[75] Interestingly, miR182 is within a polycistronic miRNA cluster that includes miR182, miR183, and miR96. These miRNAs are co-transcribed with similar tissue expression patterns and seed sequences.[75] A possible explanation could be that they exhibit redundancy in function and compensation. Indeed, germline deletion of the miR182, miR183, and miR96 cluster exhibited a robust retinal phenotype, consistent with the idea of functional redundancy and compensation among miR182, miR183, and miR96.[76]

It is important to note that the genetic studies on targeted deletion of miRNAs are effects of deregulated expression of several genes. Thus, the net effect of a miRNA on a specific gene is the consequence of its direct effect on its target genes and/or indirect effect on other genes that are also miRNA targets and may also influence the expression of the gene in study. The relative contribution of miR155-AID (activation-induced cytidine deaminase) signaling and indirect signaling pathways in Myc-Igh translocations was analyzed by a mutation in miR155 binding site in the 3′-UTR of its target AID. Though similar levels of AID RNA and protein were observed in miR155$^{-/-}$ and AID155 mice, higher translocations were observed in miR155$^{-/-}$ mice suggesting that miR155 regulates other genes that also act to suppress Myc-Igh translocations.[73] Another study investigating the role of miR143/145 in cardiac muscle development found contradicting roles for miR143/145 in in vitro model assays of carotid artery injury model to those observed in miR143/145 mutant mice.[77] A study demonstrating 3-fold higher miR208 in transgenic hearts showed that, in wild-type hearts, the upregulated genes in the microarray were not predicted to be the direct targets of miR208.[68] These findings imply that either the computational algorithms are unable to identify a significant proportion of target genes, or that miRNA-mediated effects are too subtle to be identified by our experimental approach. These findings further emphasize the significance of in vivo studies in transgenic models for an in-depth understanding of miRNA functions in disease processes.

A schematic representation of the various approaches to identify miRNA target genes and a generalized hierarchical order are shown in **Figure 2**.

Summary

In a relatively short period since the significance of miRNAs was realized in 2000, miRNA function has come to be considered a biologic modulator (within the context of most major biomedical research questions), as evidenced by its increasing prevalence in the scientific literature. However, recent findings also challenge the dogma of prevalent molecular mechanisms of miRNA action and its role in various life processes. A future challenge in the field will be assigning functions of both evolutionally conserved miRNAs and species-specific miRNAs in normal and disease biology. The present approaches to miRNA target identification (in silico, in vitro, and in vivo) each have advantages and limitations. While computational methods streamline possible target sets, cell-based assays are more amenable to artificial manipulation in deciphering the molecular mechanisms of miRNA action. In spite of robust ex vivo analytical techniques of miRNA target identification, it is important to realize that an experimentally verified miRNA–target gene interaction may have no functional consequences in the complex microenvironment under in vivo physiological conditions. Future methodological and computational advancements in primary, secondary, and higher-order functional assessments of miRNA function(s) using appropriate miRNA/siRNA animal models, along with high-throughput quantitation to assess global miRNA and target gene levels, will be necessary to advance the field and are essential to assess the potential and pitfalls of any envisioned miRNA-based therapeutics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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