

REVIEW

# A study of miRNAs targets prediction and experimental validation

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## ABSTRACT

**microRNAs (miRNAs) are 20–24 nucleotide (nt) RNAs that regulate eukaryotic gene expression post-transcriptionally by the degradation or translational inhibition of their target messenger RNAs (mRNAs). To identify miRNA target genes will help a lot by understanding their biological functions. Sophisticated computational approaches for miRNA target prediction, and effective biological techniques for validating these targets now play a central role in elucidating their functions. Owing to the imperfect complementarity of animal miRNAs with their targets, it is difficult to judge the accuracy of the prediction. Complexity of regulation by miRNA-mediated targets at protein and mRNAs levels has made it more challenging to identify the targets. To date, only a few miRNAs targets are confirmed. In this article, we review the methods of miRNA target prediction and the experimental validation for their corresponding mRNA targets in animals.**

**KEYWORDS** microRNA, computational prediction, target, experimental validation

## INTRODUCTION

The miRNAs are a widely distributed class of small non-coding RNAs that play an integral role in gene regulation (Elbashir et al., 2001; Hutvagner and Zamore, 2002). miRNA biogenesis pathway in animals can be divided into two steps (Fig.1). Initially, miRNAs are transcribed by RNA polymerase II as primary miRNAs (pri-miRNAs) with hundreds to thousands of nucleotides in length (Cai et al., 2004; Lee

et al., 2004; Trujillo et al., 2010). Ribonuclease III (RNase III) enzyme Droscha cleaves the flanks of pri-miRNAs to liberate ~70 nucleotide stem-loop structures, called precursor miRNAs (pre-miRNAs). Pre-miRNA hairpins are exported from the nucleus by Exportin-5 (Lee et al., 2003; Engels and Hutvagner, 2006; Flynt and Lai, 2008). In the cytoplasm, the pre-miRNAs are processed into ~22 nucleotide duplex miRNAs (miR/miR\*) by the RNase III enzyme Dicer (Kim, 2004; Lund et al., 2004; Engels and Hutvagner, 2006). Next, one strand of the miRNA duplex is loaded into the RISC (RNA-induced silencing complex) to bind the mRNA target. If the complementarities between the 3'-UTR mRNA and the miRNA are extensive, the target mRNA is degraded; whereas, if the complementarities are partial, the translation of the target mRNA is repressed (Brennecke et al., 2005).

Recent studies have shown that many miRNAs are involved in a variety of biological processes, such as transcriptional gene regulatory network, developmental timing, neuronal synapses formation, cell proliferation, cell death, viral infection, differentiation and tumor metastasis (Sarnow et al., 2006; Hwang and Mendell, 2007; Ma et al., 2007; Bartel, 2009; Nachmani et al., 2009; Xiao and Rajewsky, 2009). Currently, more than 10,000 miRNAs have been identified in the miRBase database (<http://www.mirbase.org/>). Thus, the development of precise and fast assays for miRNA target identification and verification will play a significant role in the study of miRNA functions and the biological processes in which they are involved. Several effective algorithms have been developed for the prediction of miRNAs targets in animals. In this review, we summarize the prediction methods of miRNAs targets and the experimental approaches that have been described for identification of their targets.

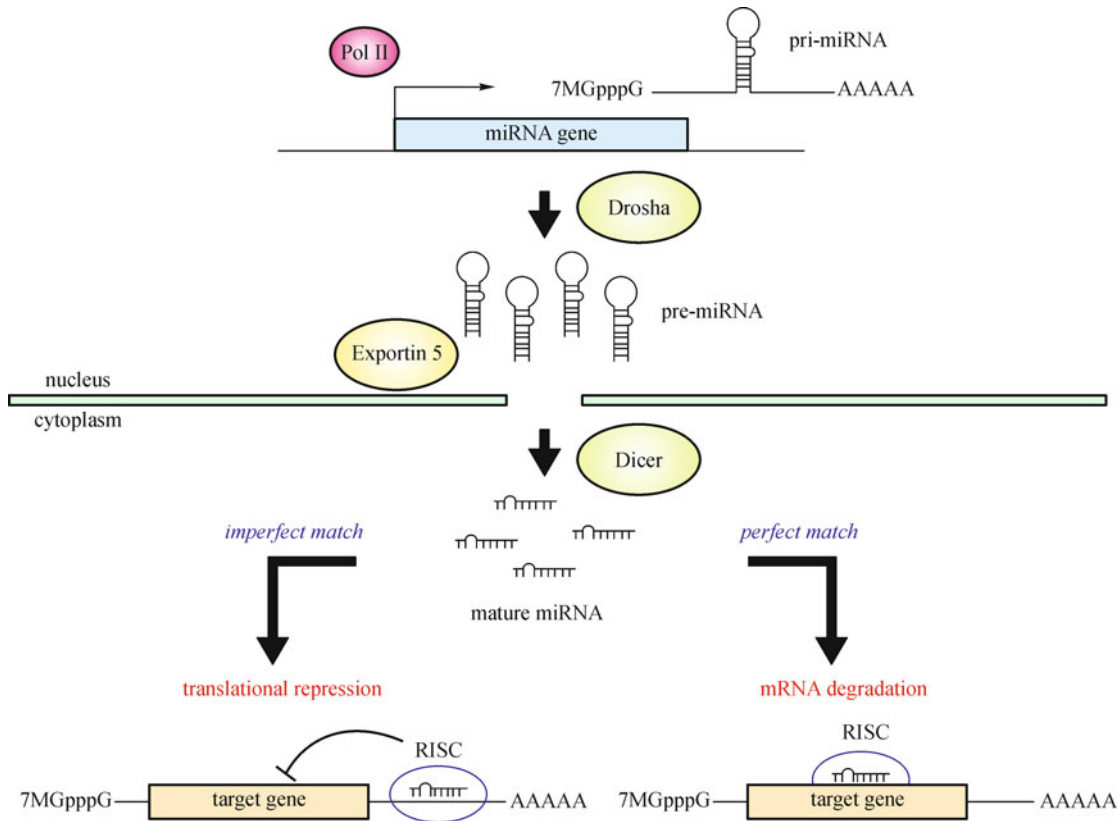


Figure 1. Biogenesis of mature miRNA.

**PREDICTION OF miRNA TARGET**

For miRNAs in animals, the target prediction is more complex because few miRNAs are perfectly complementary to their targets. In the following only animal miRNAs are considered.

**Principles of miRNA target recognition**

The function of a miRNA is ultimately defined by its targets and the effects it has on their expression. Although the detailed target recognition mechanism is still elusive, the consensus suggests that the base pairing of miRNA with its target mRNA is the key. Differences in target complementarities and target location within the mRNA could be related to the silencing mechanism used. The prediction criteria include the following:

1) The miRNA sequence is complementary to the 3'-UTR sequence of potential target mRNAs. Especially, the strong binding of the 5' end (the first eight base pairs) of the mature miRNA to the 3'-UTR sequence is very important for targeting, whereas the G:U wobble pairing reduces the silencing efficiency (Brodersen and Voinnet, 2009). For example, there are three types of target sites: 5'-dominant canonical, 5'-dominant seed only and 3'-compensatory (Fig. 2). They

differ in the level of complementarities of miRNA sequences to the site sequences. In addition to the 3'-UTR regions, base pairing is also observed in a few cases to exist in the 5'-UTR and coding regions (Pillai, 2005; Lee et al., 2009; Tsai et al., 2009).

2) The thermodynamic properties of miRNA-mRNA duplexes are assessed by calculating free-energy ( $\Delta G$ ) of the putative binding. It is an energetically more favorable state when two complementary RNA strands are hybridized. The lower the free energy of two paired RNA strands is, the more energy is needed to disrupt this duplex formation. Thus, an RNA duplex is in a more stable state thermodynamically, which means the binding of the miRNA to the mRNA is stronger, when the free energy is low (Lewis et al., 2005).

3) The conservation of miRNA target sites among different species and its targets are also shown to be conserved (Watanabe et al., 2007). The use of predicted binding sites conserved in multiple species is considered more likely to reduce the number of false positives. Many target prediction algorithms identify orthologous 3'-UTR sequences and then perform conservation analysis across related species. Nevertheless, the sites can also be regarded as conserved if they can just be found somewhere in the sequences but not in aligned positions (Min and Yoon, 2010).

### Program for animal miRNA target recognition

Several methods have been developed for computational target prediction. There are differences in the used approaches and implementations. At present, the main used target prediction methods are summarized below in more detail and other available programs are described in Table 1.

#### miRanda

This software was initially designed to predict miRNA target genes in *D. melanogaster* (Enright et al., 2003), but was also used to predict human miRNA targets. MiRanda screened 3'-UTRs, mainly based on sequence matching, miRNA and mRNA duplex thermal stability and conservation of the target site. Based on the above three principles, miRanda successfully predicted many known targets in *D. melanogaster*. When basic parameter settings are used, the approximated false-positive rate was around 24%. John and his colleagues improved the method with a two-step approach for the identification of miRNA targets (John et al., 2004). First, a strict model for the binding sites required almost perfect complementarities in the seed region allowing a single wobble pairing. Second, for the highest scoring alignments, the thermodynamic stability of the complex is calculated and reported. The authors predicted that miRNA genes (about 2000 human genes) regulate the protein production for 10% or more of human genes.

#### TargetScan and TargetScanS

Lewis and his colleagues initially developed TargetScan, a computational method to predict the targets of conserved

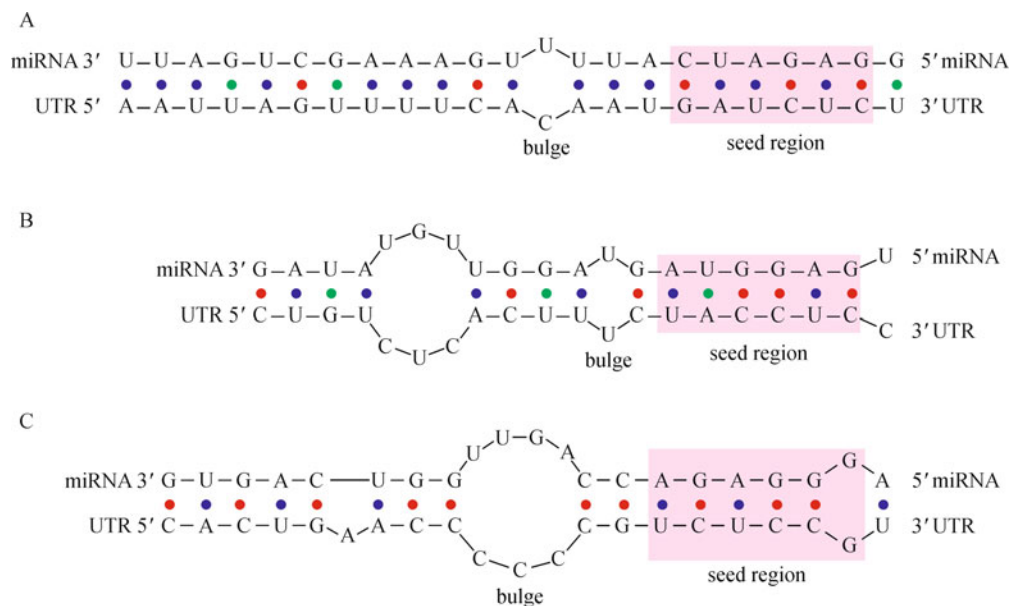
vertebrate miRNAs (Lewis et al., 2003). The program integrates thermodynamics-based modeling of miRNA-mRNA interactions and sequence alignment analysis to predict conserved miRNA binding sites among different species. The 'miRNA seed' is a 7-nucleotide sequence at base 2 to 8 in the 5' end of the miRNAs. It forms perfect base pairing complementary to 'seed matches' which refers to the 3'-UTR heptamer in the target mRNA. This aims at filtering many false positives from the beginning of the prediction process. The estimated false-positive rate varies between 22% and 31%. The method was shown to predict not only known miRNA binding sites but also novel sites. Luciferase reporter constructs validated 11 of the 15 tested sites. TargetScan was then further developed into TargetScanS (Lewis et al., 2005). TargetScanS requires not only target-site conservation in five genomes (human, mouse, rat, dog and chicken), but also requires a six-nucleotide seed (position 2-7) followed by an additional 3' match of adenosines surrounding the miRNA seed. As a result, the estimated false-positive rate was reduced to 22% in mammals, and all known miRNA-target interactions were successfully predicted. TargetScanS succeeded in further increasing its sensitivity by considering the presence of conserved adenosines surrounding the seed miRNA sequence. TargetScanS predicts miRNA targets based on the identification of aligned seed matches and their conservation in several species (Friedman et al., 2009).

#### DIANA-microT

In 2004, Kiriakidou and his colleagues developed DIANA-microT by combining computational and experimental approaches (Kiriakidou et al., 2004). The algorithm of DIANA-microT searches in the UTRs for stringent seed

**Table 1** The computational program for miRNA target prediction

name of program	organisms	prediction methods	website	references
MicroInspector	any	thermodynamics	imbb.forth.gr/microinspector/	Rusinov et al., 2005
EIMMo	fly	stringent seed pairing, conservation	mirz.unibas.ch/EIMMo3/	Gaidatzis et al., 2007
EMBL	human, mouse, fly, worm, zebrafish	complementary, seed pairing, conservation, free energy	russell.emblheidelberg.de/miRNAs	Stark et al., 2005
PITA	humans, mice, flies, worms	thermodynamics	genie.weizmann.ac.il/pubs/mir07/	Kertesz et al., 2007
GenMiR ++	relationship of miRNA and mRNA expression profiles	Bayesian algorithm based on expression data sets	www.psi.toronto.edu/genmir	Huang et al., 2007
MiTarget	any	Support Vector Machine classifier	/cbit.snu.ac.kr/~miTarget/	Kim et al., 2006
MirWIP	worm	moderately stringent seed pairing, free energy, conservation, site accessibility, total interaction energy, site number	mirtargets.org	Hammell et al., 2008



**Figure 2. Approximate secondary structures of the three main types of target site duplex.** (A) Canonical sites have good or perfect complementarity at both the 5' and 3' ends of the miRNA, with a characteristic bulge in the middle. (B) Dominant seed sites have perfect seed 5' complementarity to the miRNA but poor 3' complementarity. (C) Compensatory sites have a mismatch or wobble in the 5' seed region but compensate through excellent complementarity at the 3' end.

paring (at least 7 consecutive base pairs) to the miRNA. 6 mers or seed matches containing one G:U wobble are also accepted as putative target sites when they are compensated by pairing to the 3' end of the miRNA. Subsequently the conservation of the sites and the binding type are considered for scoring each site. In addition, the putative target sites are compared with the sites identified based on mock sequences to gain scores that include miRNA-specific SNR and the estimation of a precision score. The total score of a target is then calculated by building a weighted sum of the individual scores of each target site on the 3'-UTR (Maragkakis et al., 2009b). Recently the assessment of DIANA-microT 3.0 was found to achieve the highest precision among the most widely used miRNA target prediction programs reaching approximately 66% (Maragkakis et al., 2009a).

### RNAhybrid

RNAhybrid is the first tool that could be used on a single-genome basing on analyzing the secondary structure of the miRNA/mRNA duplex (Rehmsmeier et al., 2004). This method involves a dynamic programming algorithm that calculates the most favorable hybridization of a miRNA in terms of energy and its target mRNA while allowing the user to specify a portion of the miRNA that should form a perfect helix corresponding to the seed site. The statistical significance of the predicted targets is determined using extreme value statistics for minimum free energies normalized for target length, and a Poisson distribution is used to model

multiple binding sites of a miRNA for the same target. The statistical treatment is extended with a comparative analysis of conserved binding sites in orthologous targets of related species. In 2006, the RNAhybrid software was upgraded by Krüger and Rehmsmeier (2006).

### RNA22

RNA22 is presented as a method for identifying miRNA binding sites and their corresponding miRNA:mRNA hetero duplexes (Miranda et al., 2006). It is distinct from previously reported methods in that it obviates the use of a cross-species sequence conservation filter, thus allowing the discovery of miRNA binding sites that may not be presented in closely related species. The user can restrict the number of the results by modifying three parameters. They are i) the maximal free energy, ii) the minimum number of base-pairs between the miRNA and the target and iii) the maximum number of unpaired bases of the miRNA seed region, whereas G:U wobbles count as matches. Note that RNA22 does not consider conservation. The 226 targets predicted by RNA22 were tested in a luciferase reporter gene assay and 168 of them are to be observed for miRNA-dependent repression (Miranda et al., 2006).

### TargetBoost

Saetrom and his colleagues proposed a machine learning algorithm called TargetBoost that works on a set of validated

miRNA targets in lower organisms to create weighted sequence motifs that capture the binding characteristics between miRNAs and their targets (Saetrom et al., 2005). The central idea underlying TargetBoost is to find differential DNA nucleotide sequence patterns from training data, which can discriminate true and false target sites best. It is also a machine-learning method that combines genetic programming with boosting, and tries to learn the hidden rules of miRNA: target site hybridization instead of relying on criteria based on sequence complementarities, thermodynamic stability or evolutionary conservation. The authors showed that TargetBoost's weighted sequence motif approach is favorable to the use of both the duplex stability and the sequence complementarities steps.

### PicTar

PicTar uses the criteria of co-expression in space and time of miRNAs and their targets (Krek et al., 2005). It identifies two types of miRNA:target interactions: i) those with perfect complementarities between the seed region of the miRNA (7 nt starting at position 1 or 2 of the miRNA's 5' end) and the 3'-UTR target site and ii) those for which the perfect complementarities are interrupted by at most one nucleotide bulge, mismatch, or G:U wobble. In both the instances, the algorithm requires that the binding stability of the putative miRNA:target interaction, as measured by thermodynamic binding energy, exceed a specified threshold. The experimental validation of 7 out of 13 tested predicted targets, as well as the confirmation of eight of nine previously known targets, demonstrate the efficiency of the algorithm (Krek et al., 2005).

### TargetSpy

TargetSpy is a novel computational approach for the prediction of target sites regardless of the presence of a seed match (Sturm et al., 2010). It is based on machine learning and automatic feature selection using a wide spectrum of compositional, structural, and base pairing features covering current biologic knowledge and does not rely on evolutionary conservation, which allows for detection of species-specific interactions and makes TargetSpy suitable for analyzing unconserved genomic sequences. Features of the algorithm include: i) no seed match requirement, ii) seed match requirement, and iii) conserved seed match requirement. The results showed that TargetSpy predicts 26–112 functional target sites without a seed match per miRNA that are missed by all other currently available tools algorithms. The author has demonstrated that the application of TargetSpy in combination with upcoming deep sequencing data results in a powerful miRNA target site prediction method.

The different algorithms above have their own characteristics. Different methods provide various predictions, and the

degree of overlap between different lists of predicted targets is sometimes poor or null (Sethupathy et al., 2006). However, which of them have the best performance? How reliable are their results? These questions are difficult to answer.

## EXPERIMENTAL IDENTIFICATION OF miRNA TARGETS

Compared with the miRNA target gene prediction methods, the availability of approaches for the experimental verification of miRNA target genes is much more challenging. The traditional validation methods of target gene include: mutation studies (O'Donnell et al., 2005), gene-silencing techniques (Poy et al., 2004; Johnson et al., 2005), and classic genetic studies (Lee et al., 1993; Johnston and Hobert, 2003). The current main experimental identification approaches are described here.

### Reporter gene assay

The most common method for miRNA-mRNA interaction analysis is the reporter gene assay, which provides direct evidence about the functionality of a miRNA:mRNA pair. The experimental approach is to clone the 3'-UTR of the target gene of interest immediately downstream of the reporter gene luciferase (Photinus or Renilla) or green fluorescent protein (GFP) open reading frame sequence contained in the reporter plasmid and the reporter gene under the control of a ubiquitous promoters (Aravin and Tuschl, 2005; Lee et al., 2008; Ørom and Lund, 2010). The reason is that binding of a given miRNA to its specific mRNA target site will repress reporter protein production, thereby reducing activity/expression that can be measured and compared to a control by introducing into a cell line of interest. Liu and his colleagues used this method to study the function of the miR-16 family. The studies showed that miR-16 family can regulate *ccnd1* gene. They inserted the 3'-UTR of the *ccnd1* gene into a luciferase report gene vector, and co-transfected it with a miRNA expression library into HepG2 cells. Then the function of the miRNA that significantly reduced luciferase expression was validated *in vivo* (Liu et al., 2008).

### Protein level analyses

A more global approach to find target genes is to use proteomics to uncover target proteins that are affected as a consequence of miRNA expression in target cells. pSILAC (pulsed stable isotope labeling with amino acids in cell culture) is a method that directly measures changes in protein production induced by overexpression of miRNAs (Vinther et al., 2006; Selbach et al., 2008). This experimental approach is appealing as it may identify targets regulated both by transcript destabilization and translational repression. Vinther and his colleagues used this method to investigate the

effect of miR-1 on the HeLa cell proteome (Vinther et al., 2006). The results showed that the expressions of 12 out of 504 investigated proteins was repressed by miR-1 transfection. Selbach and his colleagues also used pSILAC to study the miRNAs miR-1, miR-30, miR-155, miR-16 and let-7b, and knock-down of let-7b in HeLa cells in hopes to observe more instances of specific translational inhibition (Selbach et al., 2008). Proteins that are altered as a probable consequence of higher miRNA expression may be purified from gels and subsequently identified using MALDI (matrix-assisted laser desorption/ionization) technology (Banerjee et al., 2008). Another approach to verify a miRNA-target interaction would be to knockout the miRNA gene and examine the effects on protein changes (Baek et al., 2008). An integrated detection approach, which combines computational prediction with high-throughput biologic validation, has been most effective in discovery of miRNAs.

### Other biochemical approaches

To determine whether the miRNA induces translational repression or mRNA cleavage, it is necessary to measure mRNA levels as well. Further techniques to observe miRNA-mRNA interactions are microarray analysis. Microarrays measure changes of mRNA levels and Easow and his colleagues analyzed miRNA target genes by the purifying miRNP with associated miRNAs and bound mRNA targets with microarrays method (Easow et al., 2007). Regulation of these mRNAs by the miRNA was then validated. Recently, an improved method called PAR-CLIP (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation) was developed (Hafner et al., 2010). The author uncovered tens of thousands of binding sites for several important RBPs and RNPs and assessed the regulatory impact of binding on their targets. A possible experimental procedure "pull-down" strategy was developed to detect targets (Beitzinger et al., 2007). Recently, immunoprecipitation of RISC components has been used to identify mRNAs targeted by miRNAs (Zhang et al., 2007). Moreover, high-throughput sequencing of RNAs isolated by crosslinking immunoprecipitation (HITS-CLIP) has been used in order to identify and sequence specific miRNA binding sites on targeted mRNAs (Chi et al., 2009). The investigators demonstrated that by using antibodies against members of the Argonaute (Ago) protein family, a group of proteins known to bind to miRNAs, and to partially complementary sequences in the 3'-UTR of specific target mRNAs, they could co-immunoprecipitate Ago bound mRNAs. They analyzed six randomly selected Ago1-bound mRNAs and validated five of them as miRNA targets (Hassan et al., 2010). Alternatively quantitative RT-PCR can also be used to monitor changes in mRNA levels after a miRNA has been introduced in a cell (Boissonneault et al., 2008). Recently, a target identification approach was first developed based on tandem affinity purification, in which mRNA/miRNA

complexes are sequentially pulled down via the Ago moiety and then via the miRNA (Nonne et al., 2010).

### PERSPECTIVES

In recent years, the study of miRNAs has become an important area in the field of life sciences. The increasing interest in miRNA regulatory function triggered the development of many computational approaches for miRNA target prediction. Prediction and identification of miRNA target genes is the basis for the functional study of miRNAs. The rapid development of computational methods for miRNA target prediction is promising for future research. Computational approaches, which excel in the handling of genomic, transcriptomic, and proteomic data, should provide invaluable tools for identification of the relative position of miRNAs in various biologic networks.

Research of miRNA target genes may be more complex than imagined. Continuous efforts were needed to discover numerous rules for the interaction of miRNAs and targets. Currently some types of computational tools algorithms exploiting this novel information will undoubtedly be released. Proper integration of these prediction algorithms can significantly improve the prediction accuracy. The present scarcity of confidently validated miRNA targets, not only establishing miRNA-target associations, but specifically pinpointing the hybridization sites, is the greatest obstacle to the development of better prediction methods and the systematic assessment of the performance of current tools. This question requires more complex computational approaches that will identify precisely and predict miRNA regulatory networks and will model the interplay between miRNAs (Ivanovska and Cleary, 2008). Taking together, miRNAs regulatory role in the biologic functions and identification of targets will be explored through integrating computational and experimental approaches. The validation of miRNA targets will expand recognition and spectrum of miRNA network.

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