MicroRNA profiling in the dentate gyrus in epileptic rats
The role of miR-187-3p
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Abstract
This study aimed to explore the role of aberrant miRNA expression in epilepsy and to identify more potential genes associated with epileptogenesis.

The miRNA expression profile of GSE49850, which included 20 samples from the rat epileptic dentate gyrus at 7, 14, 30, and 90 days after electrical stimulation and 20 additional samples from sham time-matched controls, was downloaded from the Gene Expression Omnibus database. The significantly differentially expressed miRNAs were identified in stimulated samples at each time point compared to time-matched controls, respectively. The target genes of consistently differentially expressed miRNAs were screened from miRDB and microRNA.org databases, followed by Gene Ontology (GO) and pathway enrichment analysis and regulatory network construction. The overlapping target genes for consistently differentially expressed miRNAs were also identified from these 2 databases. Furthermore, the potential binding sites of miRNAs and their target genes were analyzed.

Rno-miR-187-3p was consistently downregulated in stimulated groups compared with time-matched controls. The predicted target genes of rno-miR-187-3p were enriched in different GO terms and pathways. In addition, 7 overlapping target genes of m0-miR-187-3p were identified, including NFS1, PAQR4, CAND1, DCLK1, PRKAR2A, AKAP3, and KCNK10. These 7 overlapping target genes were determined to have a different number of matched binding sites with rno-miR-187-3p.

Our study suggests that miR-187-3p may play an important role in epilepsy development and progression via regulating numerous target genes, such as NFS1, CAND1, DCLK1, AKAP3, and KCNK10. Determining the underlying mechanism of the role of miR-187-3p in epilepsy may make it a potential therapeutic option.

Abbreviations: AKAP1 = A-Kinase Anchoring Protein 1, AKAP3 = A-Kinase Anchoring Protein 3, BIND = Biomolecular Interaction Network Database, BP = biological process, CAND1 = Cullin Associated And Neddylation Dissociated 1, CC = Cellular component, DCLK1 = Doublecortin Like Kinase 1, FC = Fold change, FDR = false discovery rate, GEO = gene expression omnibus, GO = gene ontology, GRID = general repository for interaction datasets, KCNK10 = Potassium Two Pore Domain Channel Subfamily K Member 10, KEGG = Kyoto Encyclopedia of Genes and Genomes, LIAS = lipoic acid synthetase, MF = molecular function, miRNAs = microRNAs, NCBI = National Center of Biotechnology Information, NFS1 = NFS1 cysteine desulfurase, PAQR4 = Progestin And AdipoQ Receptor Family Member 4, PI3K = Phosphatidylinositol 3-kinase, PRKAR2A = Protein Kinase CAMP-Dependent Type II Regulatory Subunit Alpha, RMA = robust multiarray average, SMAD7 = SMAD Family Member 7.

Keywords: complementary sequence analysis, differentially expressed miRNAs, epilepsy, interaction network, mo-miR-187-3p

1. Introduction
Epilepsy is a serious neurological disorder characterized by spontaneously recurring seizures which are attributed to abnormal and synchronous firing of neurons in the brain.[1] Epilepsy affects approximately 65 million individuals of all ages worldwide, making it second to stroke as one of the most common serious brain disorders.[2] The recent developments in treatment approaches such as surgery,[3] antiepileptic drug therapy,[4] and brain stimulation,[5] have made great advances in the control of seizures. However, almost one-third of all patients with epilepsy still have intractable seizures or adverse effects.[6] One compelling challenge for the treatment of epilepsy is to elucidate the molecular mechanisms of disease development and progression.

MicroRNAs (miRNAs) are a class of endogenous, small, non-coding RNA molecules, which post-transcriptionally fine-tune the expression of their target protein-encoding genes by binding to conserved sequences within their target genes, mainly within the 3′-untranslated region (3′-UTR).[7] There is increasing evidence to support that miRNA changes are involved in the pathophysiology of epilepsy.[8] Using either rodent epilepsy models or human tissue from patients with epilepsy, a number of previous studies have demonstrated that miRNA expression is altered in epilepsy.[9] For instance, Song et al.[10] found that 23 miRNAs were expressed differentially in temporal lobe epilepsy (TLE) rats, including 18 upregulated miRNAs (miR-23a/b included) and 5 downregulated miRNAs (let-7c included).
Moreover, Hu et al.\(^{(9)}\) demonstrated the pattern of increased expression of the pro-apoptotic miR-34a in the hippocampus in post-status epilepticus rats. Additionally, a previous study reported that miR-146a, an inflammation-associated miRNA, was highly expressed in astrocytes in the hippocampus in a rat model of TLE, as well as in hippocampal tissue from TLE patients.\(^{(11)}\) Recently, Henshall\(^{(8)}\) found that miRNAs might play critical roles in the control of cell death, inflammation, synaptic structure, and the immune response in epilepsy. Thus, research on miRNAs has the potential to deliver diagnostics and therapeutics that link directly to how the regulatory molecules influence the pathogenesis of epilepsy.\(^{(8)}\) However, the functional roles of the aberrantly expressed miRNAs and how miRNAs are involved in the process of epileptogenesis via regulating gene expression remain to be explored.

Recently, Bot et al.\(^{(12)}\) observed that complex changes in miRNA and their predicted target genes in the dentate gyrus could participate in several molecular events, especially the immune response and neuronal plasticity in epilepsy. Moreover, Meng et al.\(^{(13)}\) demonstrated that neuronal calcium signaling pathways were associated with the development of epilepsy. However, they did not focus on the miRNAs that were consistently up- or down-regulated at all time points following stimulation in the epileptic dentate gyrus of rats, which could be used as biomarkers. In this study, we downloaded the same miRNA expression profile of GSE49850 used by Bot et al.\(^{(12)}\) and Meng et al.\(^{(14)}\) from a public database, and reanalyzed the data using different methods and tools. Microarray analysis was performed to identify the differentially expressed miRNAs in the rat epileptic dentate gyrus at 7, 14, 30, and 90 days after electrical stimulation compared with those from sham-operated time-matched controls. The miRNAs that were consistently down- or upregulated from 7 days to 90 days were our focus. In addition, target genes of the consistently differentially expressed miRNAs were screened, and subjected to functional enrichment analysis and construction of regulatory network. Furthermore, conserved complementary sites between the miRNAs and their target genes were identified. This research aimed to explore the role of aberrant miRNA expression in epilepsy and to identify more potential genes or pathways associated with epilepsy progression based on rat miRNA expression profiles.

2. Methods

2.1. Microarray data

The Gene Expression Omnibus (GEO) repository at National Center of Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/geo/) is currently the largest fully public gene expression resource, which facilitates the submission, storage and retrieval of microarray and other forms of high-throughput data generated by the scientific community.\(^{(15)}\) In this study, the miRNA expression profile of GSE49850 was downloaded from the GEO database, which was deposited by Bot et al.\(^{(12)}\) The platform is GPL17566 Exiqon miRCURY LNA microRNA array; 7th generation; batch 208500–2, 208510; lot 35009-hsa, mmu & rno (miRBase 19.0). As previously described in the original study by Bot et al.\(^{(12)}\) the status epilepticus was induced by electrical stimulation of the amygdala in adult male Sprague-Dawley rats, which was used as a model of TLE. Time-matched control animals had electrodes implanted but did not receive electrical stimulation. The dentate gyrus was collected at 7, 14, 30, and 90 days after stimulation. The dataset consisted of 40 samples, including 20 samples of electrically stimulated dentate gyrus, at a range of time points (7, 14, 30, and 90 days) after stimulation and 20 additional samples of sham-operated time-matched controls. Each time point included 5 replicates from each group. This study just re-analyzed the microarray data downloaded from public database and performed bioinformatics analysis. The authors declare that no experiments were performed on humans or animals for this investigation. Thus, ethics approval or consent to participate was not applicable.

2.2. Data preprocessing and differentially expressed miRNA screening

The raw data and the probe annotation file were downloaded. Median normalization was performed on the raw data using the robust multiarray average (RMA) algorithm\(^{(16)}\) in R.\(^{(17)}\) The missing values were imputed. The t-test in the multtest package\(^{(16)}\) in R was used to identify the significantly differentially expressed miRNAs in the stimulated groups at 4 time points, which were compared with the respective time-matched controls. Only the miRNAs with \(P < .05\) were considered significantly differentially expressed. The miRNAs that were consistently up- or downregulated at these 4 time points compared with their respective time-matched controls would be our focus and were thus used for the follow-up analysis.

2.3. Prediction of target genes of the focused differentially expressed miRNAs

A growing number of miRNA-related database systems have been developed to provide further insights into miRNAs and their target genes.\(^{(19)}\) For example, miRDB (http://mirdb.org/miRDB/) is an online database for the retrieval of computationally predicted miRNA targets and for miRNA functional annotations with a wiki editing interface.\(^{(4)}\) microRNA.org (http://www.microrna.org/microrna/home.do/) is a comprehensive resource of miRNA target predictions, experimentally observed expression patterns, and target down-regulation scores.\(^{(20)}\)

In the present study, to identify potential target genes of the consistently aberrant miRNAs, we used two databases: miRDB and microRNA.org. The target genes associated with miRNAs were identified from the 2 databases. The common (overlapping) target genes identified from these 2 databases were deemed to be more reliable.

2.4. Functional enrichment analysis of target genes

To facilitate the functional enrichment analysis, all target genes identified from the miRDB and microRNA.org databases were subjected to functional enrichment analysis using the Database for Annotation Visualization and Integrated Discovery (DAVID, version 6.8).\(^{(21)}\) Over-represented Gene Ontology (GO) terms (including biological process [BP], molecular function [MF], and cellular component [CC] categories),\(^{(22)}\) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were identified based on a hypergeometric distribution algorithm. The false discovery rate (FDR)\(^{(23)}\) was employed for multiple testing correction using the Benjamini and Hochberg method.\(^{(24)}\) The threshold was set as FDR < .05.

2.5. Construction of the interaction network

The Osprey network visualization system is used to design a complex interaction network to enable visualization and
2.5. Target gene-miRNA binding site analysis

The miRNA gene sequences were collected from the miRBase sequence database (http://www.mirbase.org/). The sequences of the common target genes found in both databases were identified in the NCBI Gene (http://www.ncbi.nlm.nih.gov/gene). Moreover, the BLAST program in NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) was used to search for the binding sites of the miRNAs and their target genes.

3. Results

3.1. Data preprocessing and screening of differentially expressed miRNAs

After data normalization and screening of differentially expressed miRNAs, we found 51 differentially expressed miRNAs in the simulated group at 7 days following electrical stimulation of the amygdala compared with the control group at 7 days. Additionally, 38, 100, and 62 differentially expressed miRNAs were identified in the other simulated groups (at 14, 30, and 90 days) compared with their respective time-matched controls (Supplementary Table 1, http://links.lww.com/MD/B698). The top 5 upregulated miRNAs and downregulated miRNAs in the stimulated group (ordered according to decreasing fold change [FC]) at each time point are shown in Table 1, such as upregulated rno-miR-212-3p at 7 days, downregulated rno-miR-187-5p at 14 days, and downregulated rno-miR-187-3p at 90 days. However, among all the identified differentially expressed miRNAs, only rno-miR-187-3p was consistently downregulated at each time point from 7 days to 90 days. The decrease in rno-miR-187-3p expression was 0.56-fold, 0.62-fold, 0.73-fold, and 0.56-fold of that in the time-matched controls at 7, 14, 30, and 90 days after stimulation, respectively (P<.01) (Table 1). The expression values of rno-miR-187-3p in each sample at each time point are represented in Fig. 1. Thus, we decided to direct our attention toward rno-miR-187-3p.

3.2. Screening rno-miR-187-3p target genes

As miRNAs negatively regulate gene expression by targeting mRNAs at the posttranscriptional level, it is necessary to identify putative target genes for a better understanding of the function of miRNAs. In our study, 15 target genes of rno-miR-187-3p were identified from the miRDB database (there are 2 rat miRNAs found for “rno-miR-187” in the miRDB database, rno-miR-187-3p [previously rno-miR-187] and rno-miR-187-5p [previously rno-miR-187*]; rno-miR-187-3p targets were exactly what we needed). When we searched for potential target genes of rno-miR-187-3p using microRNA.org database, no results were found using rno-miR-187-3p as the direct miRNA identifier. After sequence alignment, we found that the results of rno-miR-187 (corresponding to rno-miR-187-3p currently; rno-miR-187-5p were not included) in the microRNA.org database were exactly what we needed. Thus, a total of 415 target genes of rno-miR-187-3p were identified from the microRNA.org database. Furthermore, 7 overlapping genes for rno-miR-187-3p were identified from these 2 databases, namely, NFS1 Cysteine Desulfurase (NFS1), Progesterin And AdipoQ Receptor Family Member 4 (PAQR4), Cullin Associated And Neddylation Dissociated 1 (CAND1), Double-cortin Like Kinase 1 (DCLK1), Protein Kinase CAMP-Dependent Type II Regulatory Subunit Alpha (PRKAR2A), A-Kinase Anchoring Protein 3 (Akap3), and Potassium Two Pore Domain Channel Subfamily K Member 10 (KCNK10).

3.3. Functional enrichment analysis of all target genes of rno-miR-187-3p

After pathway and GO analyses, we found that the target genes of rno-miR-187-3p were enriched in different GO terms and pathways. The top 5 GO terms in each category and all the pathways enriched

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Table 1

The top 5 upregulated miRNAs and downregulated miRNAs between the stimulated and control group at each time point.

| The day after stimulation | microRNA   | log2FC | P      |
|---------------------------|------------|--------|--------|
| 7 day                     | rno-miR-21-3p | 0.9198 | .0006732 |
|                           | rno-miR-212-3p | 0.875  | .000249 |
|                           | rno-miR-132-3p | 0.803  | .0002784 |
|                           | rno-miR-370-5p | 0.5902 | .001574 |
|                           | rno-miR-142-3p | 0.8628 | .0073172 |
|                           | rno-miR-7a-5p  | -0.9262| .001465 |
|                           | rno-miR-551b-3p | -0.8474| .002375 |
|                           | rno-miR-187-3p | -0.842 | .0031653 |
|                           | rno-miR-33-3p  | -0.8008| .0035162 |
|                           | rno-miR-30d-5p | -0.7782| .003545 |
| 14 day                    | rno-miR-212-3p | 0.5488 | .010968 |
|                           | rno-miR-132-3p | 0.4042 | .033279 |
|                           | rno-miR-132-3p | 0.5124 | .046485 |
|                           | rno-miR-212-5p | 0.359  | .043281 |
|                           | rno-miR-34c-5p | 0.1944 | .013977 |
|                           | rno-miR-30a-5p | -0.7486| .01905 |
|                           | rno-miR-3b-5p  | -0.6996| .025855 |
|                           | rno-miR-187-3p | -0.695 | .008891 |
|                           | rno-miR-363-5p | -0.563  | .030733 |
|                           | rno-miR-30b-5p | -0.4562| .007602 |
|                           | rno-miR-187-5p | -0.481 | .000203 |
| 30 day                    | rno-miR-146a-5p | 0.702  | .00456174 |
|                           | rno-miR-132-5p | 0.6874 | .000691 |
|                           | rno-miR-21-5p  | 0.64   | .0007029 |
|                           | rno-miR-212-3p | 0.6154 | .0019641 |
|                           | rno-miR-23a-3p | 0.5174 | .0167972 |
|                           | rno-miR-23b-3p | -0.7364| .0019058 |
|                           | rno-miR-352    | -0.5116| .0025167 |
|                           | rno-miR-39a-5p | -0.481 | .0025356 |
|                           | rno-miR-187-5p | -0.481 | .01308175 |
|                           | rno-miR-30b-5p | -0.4563| .00389951 |
| 90 day                    | rno-miR-742-3p | 0.3104 | .008055 |
|                           | rno-miR-382-3p | 0.3078 | .021826 |
|                           | rno-miR-34c-5p | 0.2422 | .000515 |
|                           | rno-miR-499-3p | 0.1894 | .029416 |
|                           | rno-miR-489-3p | 0.1736 | .014029 |
|                           | rno-miR-187-3p | -0.8388| .010097 |
|                           | rno-miR-451-5p | -0.5338| .039916 |
|                           | rno-miR-708-5p | -0.4822| .023886 |
|                           | rno-miR-146b-5p| -0.4   | .010581 |
|                           | rno-miR-190a-5p| -0.3984| .038816 |

The upregulated miRNAs and downregulated miRNAs were ordered according to decreasing (log2FC), respectively. FC = fold change, miRNAs = microRNAs.
by all target genes are shown in Table 2. From these results, we found that the target genes of rno-miR-187-3p were related to positive regulation of cell proliferation, protein binding, and kinase activity. Moreover, the target genes of rno-miR-187-3p were involved in different pathways such as the hypoxia-inducible factor 1 (HIF-1) signaling pathway, long-term depression, and the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway.

### 3.4. Interaction network construction of rno-miR-187-3p and all target genes

Interactions between rno-miR-187-3p and all of its potential target genes, and interactions between these target genes, are presented in the network as shown in Fig. 2. rno-miR-187-3p was the center of the network, and we found that rno-miR-187-3p could regulate 430 genes. Additionally, from this interaction network, 80 interaction pairs among the target genes could also be identified. Therefore, one of the overlapping target genes, PRKAR2A, could interact with A-Kinase Anchoring Protein 1 (AKAP1), a member of the AKAP family.

### 3.5. Analysis of rno-miR-187-3p and its target gene binding sites

To understand the mechanism of the miR-187-3p-target gene interactions, the miR-187-3p sequence and the sequence of the overlapping target genes were subjected to sequence alignment. From these results, we found that the overlapping predicted target genes NFS1, PAQR4, CAND1, DCLK1, PRKAR2A, AKAP3, and KCNK10 had a different number of matched binding sites responsive to rno-miR-187-3p. In detail, NFS1, PAQR4, CAND1, DCLK1, PRKAR2A, AKAP3, and KCNK10 had 1, 1, 3, 2, 1, and 5 matched binding sites responsive to rno-miR-187-3p, respectively.

### 4. Discussion

Epilepsy is a chronic disease and places both a health and social burden on society. Recently, dysregulation of miRNAs was found to be a key regulator of many biological processes in epilepsy. In this study, rno-miR-187-3p was determined to be consistently down-regulated in stimulated groups at 4 time points compared with time-matched controls. The predicted target genes of rno-miR-187-3p were enriched in different GO terms and pathways. In addition, 7 overlapping target genes of rno-miR-187-3p were identified from 2 databases, including NFS1, PAQR4, CAND1, DCLK1, PRKAR2A, etc.

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**Table 2**

| Category | Term                                                                 | Count | P     |
|----------|-----------------------------------------------------------------------|-------|-------|
| BP       | GO:0008284—positive regulation of cell proliferation                  | 29    | 6.47E-07 |
|          | GO:0010628—positive regulation of gene expression                     | 22    | 9.06E-06 |
|          | GO:0006641—triglyceride metabolic process                              | 7     | 7.84E-05 |
|          | GO:0006970—response to osmotic stress                                  | 6     | 1.64E-04 |
|          | GO:0001666—response to hypoxia                                        | 16    | 1.97E-04 |
| CC       | GO:0005737—cytoplasm                                                  | 159   | 1.00E-06 |
|          | GO:0045121—membrane raft                                              | 19    | 3.11E-06 |
|          | GO:0030425—dendrite                                                  | 24    | 1.04E-05 |
|          | GO:0043234—protein complex                                            | 28    | 2.18E-05 |
|          | GO:0043025—neuronal cell body                                         | 26    | 2.81E-05 |
| MF       | GO:0005515—protein binding                                            | 78    | 6.33E-15 |
|          | GO:0019904—protein domain specific binding                            | 20    | 7.57E-06 |
|          | GO:0016301—kinase family                                              | 10    | 4.56E-04 |
|          | GO:0046982—protein heterodimerization activity                         | 23    | 1.27E-03 |
|          | GO:0019903—protein phosphatase binding                                 | 8     | 1.42E-03 |
| Pathways | rno04066:HIF-1 signaling pathway                                       | 10    | 7.11E-04 |
|          | rno04730:Long-term depression                                         | 7     | 3.17E-03 |
|          | rno05202:Transcriptional misregulation in cancer                      | 11    | 4.68E-03 |
|          | rno04151:PI3K-Akt signaling pathway                                   | 16    | 1.32E-02 |
|          | rno05205:Proteoglycans in cancer                                       | 11    | 1.84E-02 |
|          | rno05206:MicroRNAs in cancer                                          | 9     | 1.90E-02 |
|          | rno04610:Complement and coagulation cascades                           | 6     | 2.65E-02 |
|          | rno04910:Insulin signaling pathway                                    | 8     | 4.61E-02 |
|          | rno04923:Regulation of lipolysis in adipocytes                        | 5     | 4.91E-02 |

BP = biological process, CC = cellular component, MF = molecular function.

Count indicates the number of genes belonging to an annotation GO term or pathway. P-value is calculated based on a hypergeometric distribution algorithm.
AKAP3, and KCNK10. These 7 overlapping target genes were determined to have a different number of matched binding sites for rno-miR-187-3p.

In this study, miRNA alterations were identified in the electrically stimulated dentate gyrus of rats compared with the time-matched controls, indicating that status epilepticus led to an aberrant miRNA expression pattern, consistent with previous studies. In special, we found that rno-miR-187-3p was consistently downregulated at all time points. miR-187 has been reported to be related to certain types of cancer and other diseases. For instance, Zhang et al. showed that decreased miR-187 expression could induce retinal ganglion cell apoptosis through upregulating SMAD Family Member 7 (SMAD7) in glaucoma. In accordance with this study, we determined that the target genes of miR-187-3p could be enriched in the positive regulation of cell proliferation, suggesting that miR-187-3p may play a critical role in regulating cell proliferation and apoptosis associated with the progression of epilepsy. Moreover, a previous study reported that miR-187 expression was significantly downregulated in patients with TLE and demonstrated a critical role for miR-187 in the physiological regulation of IL-10 anti-inflammatory responses in the pathogenesis of TLE. In this study, we concluded that rno-miR-187-3p was consistently downregulated in the electrically-stimulated dentate gyrus of rats. Additionally, Wang et al. demonstrated that deregulated miR-106b-5p had significant diagnostic value for epilepsy with higher sensitivity and specificity, suggesting that miR-106b-5p may serve as a novel, noninvasive biomarker to improve the current diagnosis of epilepsy. Moreover, miRNA-based treatments could be employed as anti-epileptogenic or disease-modifying treatments. Taken together, modulation of the miR-187-3p axis may serve as a novel therapeutic approach for epilepsy. However, more investigations are necessary to verify the role of miR-187-3p in the development and progression of epilepsy.

The proteins encoded by NFS1 supply inorganic sulfur to iron-sulfur (Fe-S) clusters by removing the sulfur from cysteine, creating alanine in the process. Lipoic acid synthetase (LIAS) depends on Fe-S cluster prosthetic groups, and a deficiency in LIAS was demonstrated to cause neonatal-onset epilepsy.
CAND1 acts as a key assembly factor of SCF (SKP1-CUL1-F-box protein) E3 ubiquitin ligase complexes. Studies have shown that E3 ubiquitin ligase restricts the activity of Ca2+ and voltage-activated K+ (BK) channels, and prevents epileptogenesis. Kerjan et al. reported that mice lacking doublecortin and doublecortin-like kinase 2 displayed altered hippocampal neuronal maturation and spontaneous seizures. AKAP3 encodes a member of AKAPs, a family of functionally related proteins that target protein kinase A to discrete locations within the cell. It was demonstrated that AKAP3 synthesis was mediated by RNA binding proteins and PKA signaling. Additionally, a previous study showed that N-methyl-D-aspartate preconditioning could protect against quinolinic acid-induced seizures via PKA signaling pathways. KCNK10, also known as TREK-2, belongs to the family of potassium channel proteins containing two pore-forming P domains. Numerous studies have indicated a role in potassium channel regulation during epilepsy. On this basis, we postulate that genes NFS1, CAND1, DCLK1, AKAP3, and KCNK10 may play essential roles in the development of epilepsy. Nevertheless, there are few articles concerning the function of PAQR4 and PKR2A2. In this study, PAQR4, CAND1, DCLK1, PRKAR2A, AKAP3, and KCNK10 were identified to be overlapping target genes of two miR-187-3p using 2 databases. On the other hand, evidence demonstrates that the number of binding sites is an important factor that influence miRNA repression.

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