MicroRNAs activate gene transcription epigenetically as an enhancer trigger

Min Xia, Jian Li, Wei Li, Yu Wang, Feizhen Wu, Yanping Xi, Lan Zhang, Chao Ding, Huaibing Luo, Yan Li, Lina Peng, Liping Zhao, Shihua Dong, Jie Cao, and Wenqiang Yu

ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs that function as negative gene expression regulators. Emerging evidence shows that, except for function in the cytoplasm, miRNAs are also present in the nucleus. However, the functional significance of nuclear miRNAs remains largely undetermined. By screening miRNA database, we have identified a subset of miRNA that functions as enhancer regulators. Here, we found a set of miRNAs show gene-activation function. We focused on miR-24-1 and found that this miRNA unconventionally activates gene transcription by targeting enhancers. Consistently, the activation was completely abolished when the enhancer sequence was deleted by TALEN. Furthermore, we found that miR-24-1 activates enhancer RNA (eRNA) expression, alters histone modification, and increases the enrichment of p300 and RNA Pol II at the enhancer locus. Our results demonstrate a novel mechanism of miRNA as an enhancer trigger.

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Introduction

MicroRNAs (miRNAs) are small non-coding RNAs (ncRNAs) that primarily function through the destabilization or translational repression by targeting the 3’ untranslated region (3’UTR) of mRNA transcripts in the cytoplasm. However, a number of recent studies indicate that miRNAs have also been implicated to positively-regulate gene transcription by targeting promoter elements, a phenomenon known as RNA activation (RNAs). With the exception of altering chromatin states, but also enhancer-like long non-coding RNA (lncRNA)19,20 the other fundamental intermediate steps that permits miRNA transcriptional activation is still a mystery. Thus, due to much uncertainty surrounding the intrinsic mechanisms of positive enhancer regulation, this unconventional yet biologically significant function by miRNAs is generally unappreciated.

Enhancers are traditionally known as cis-acting DNA elements that can increase gene transcription.11 It is well established that precise regulation of gene expression in specific tissue and cell type is inseparably linked with enhancers.12 Hence, enhancers are able to enable their genomic function to determine exactly when, where and at what levels is each gene is expressed to adapt to specific physiological, pathological or environmental conditions. For instance, the super enhancer, a particular group of enhancers, is newly recognized to be essential for maintaining cell identity and the network of cell function.14 Furthermore, markers for enhancers include binding of p300, histone H3K27 acetylation/H3K4 monomethylation, overlapped with DNase I hypersensitive sites and enhancer RNAs (eRNAs) expression.12 Primary research showed that eRNAs were strong indicators of enhancer activity, but recent evidence suggests eRNAs directly contributing to enhancer functions. In fact, it is not only eRNA, but also enhancer-like long non-coding RNA (IncRNA)19,20 and the DNMT1 interacting ncRNA21 play a crucial role in gene activation. However, unlike the IncRNA, miRNA has limited anecdotal evidence22 for contributing to enhancer induced gene activation currently.

Knowingly, miRNA is identified as a negative regulating ncRNA that functions in the cytoplasm. Yet unexpectedly, small RNA deep sequencing shows that some miRNAs are also overwhelmingly present in the nucleus, and certain evidence indicates that some miRNAs exert biological function in the nucleus.24 Besides, emerging data suggests that miRNA and protein-coding genes undergoes coordinated expression through their chromosomal loci interactions.25 Recently, analysis of a large number of breast cancer samples has indicated that to some extent, miRNAs and their neighboring genes may have a positive correlative expression.26 Thus, these results drive us to explore whether miRNAs also function as positive regulators in gene expression.
In this study, miRNAs overexpression in HEK293T revealed their positive influence on the neighboring protein-coding genes. Furthermore, detailed analysis of miR-24-1 demonstrates that miR-24-1 can also function as an enhancer trigger by modifying chromatin status that are favorable for transcriptional gene activation. Thus, we anticipate that miRNA mediated transcriptional gene activation may represent a novel mechanism of miRNAs.

Results
Characterization of miRNAs in enhancer loci from human annotated miRBase

We compared the 1594 annotated human miRNA precursor loci and the regions with enrichment of the active enhancer marker H3K27ac among commonly used cell types, a total of 303 miRNAs loci were identified within the peaks of H3K27ac modification (Fig. 1A and Table S1). These above selected miRNAs are significantly differential conserved in sequence (p < 0.0001) (Fig. S1A). We then took one of the cell lines as examples, and found that the enrichment of H3K4me1, p300/CBP, and DNase-I-hypersensitive sites on miRNAs loci, all exhibited a similar pattern to H3K27ac (Fig. 1B and Fig. S1E), these evidence showed that these miRNAs are related to the enhancer. Interestingly, when we integrated miRNAs expression (Fig. 1B, line 7), protein-coding gene expression (Fig. 1B, line 8), and histone modification (Fig. 1B, line 1–4), we found that our collection of H3K27ac-enriched miRNA tend to be present in the nuclear subcompartment (p = 2.533 × 10^-10, one-side Wilcoxon rank sum test) (Fig. 1B, Fig. S1B, C); in addition, we also found that there is a positive correlation between expression of nuclear miRNAs and their neighboring genes (p < 2.2 × 10^-16, one-sided Wilcoxon rank-sum test) (Fig. 1B, Fig. S1C, D).

MicroRNAs in enhancer loci display a transcriptional activator function

We reasoned that similar to miRNAs function at the promoter loci, our collection of miRNAs may act to regulate their neighboring genes. To test this hypothesis, we initially chose to investigate the well studied miR-26a-1, which is surrounded by protein-coding genes ITGA9, CTDSPL, VILL and PLCD1 in a 400kb window (Fig. 2A, upper panel) and
the miR-26a-1 gene derived DNA region was experimentally validated its enhancer activity by luciferase (Fig. S2A). We then constructed a miR-26a-1 plasmid and obtained a reproducible ectopic high-level expression of miRNA. Contrary to the repressive action of miRNA in the cytoplasm, overexpression of miR-26a-1 in HEK293T cells resulted in a concurrent increase in the transcription of neighboring ITGA9 and VILL genes (Fig. 2A, upper panel). To further confirm the activation potential of our miRNAs, we next investigated miR-339 (Fig. S2A), and found its expression also activated the neighboring gene GPER by 4-fold (Fig. 2A, lower panel).

Next we analyzed miR-3179 and miR-3180, two miRNAs with obviously different enrichment levels of H3K27ac. These two miRNAs are located at the same intergenic region between NOMO and PKD1P1 gene on chromosome 16, which are ideal pairs of miRNA to investigate regulatory capacity by simultaneously detecting the expression of both neighboring genes (Fig. 2B, upper panel). Firstly, we confirmed that the DNA fragments harboring miR-3179 possess enhancer activity in
luciferase reporter assays (bar 4) (Fig. S2A), while miR-3180 itself does not possess any enhancer activating capacity (bar 3) (Fig. S2A), indicating that the H3K27ac enrichment is a reliable marker for potential enhancer elements. Secondly, upon transient transfection of miR-3179 into HEK293T cells, we found that the neighboring genes ABCC6 and PKD1P1 were up-regulated by 3-fold and 5-fold, respectively (Fig. 2B, lower panel, left). In contrast, miR-3180 did not activate either local gene (Fig. 2B, lower panel, right), implying that miRNA associated with enhancer features may be a prerequisite for enhancer-associated miRNA function.

To further investigate the function of those miRNAs, we focused on miR-24-1 and miR-24-2. Coincidentally, the mature sequences of them are identical, yet their pre-miRNA-NAs are respectively derived from chromosome 9 and chromosome 19 with different neighboring genes (Fig. S2B, C, upper panels). As predicted, miR-24-1 and miR-24-2 precursors can indeed simultaneously activate their neighboring genes from both loci (Fig. 2B–E). In addition, in these experiments, a nuclear fraction with high purity was isolated from HEK293T cells (Fig. S2H) and Northern blotting showed that mature miR-24 can be detected both in cytoplasm and nucleus in the pre-miR-24 overexpressed HEK293T cells (Fig. S2I). As shown in Fig. S2J–O, pre-miRNA transfection can not only increase the miRNAs expression in the cytoplasm but also increase the expression in the nucleus.

Next, we decided to choose miR-24-1 as an example to do further research, and firstly we observed that the protein levels of miR-24-1's neighboring genes, FBP1 and FANCC, consequently increased as well (Fig. 2C). Meanwhile, as expected, genes whose 3'UTRs are targeted by miR-24-1 in the cytoplasm showed a down-regulation pattern (Fig. S2F), implying that this miRNA can also function as a canonical miRNA in the cytoplasm.

To validate that mature miRNA participated in triggering activation, we conducted transfection of miR-24 mimics and found that mature miRNA can elevate its respective neighboring gene's mRNA level like precursor miRNAs, as did the miR-3179 mimics (Fig. 2D and Fig. S2G). To further evaluate the miRNA function in vivo, we used the antisense oligonucleotide (antagomiR) to exclusively and effectively knock down the endogenous mature miR-24. As shown in Fig. 2E, qPCR revealed that miR-24 antagomiRs led to significant mRNA reduction of FBP1 and FANCC in HEK293T cells.

### miR-24-1 facilitated gene transcription dependent on the presence of the intact enhancer and miRNA

To explore the importance of base-pairing rule for miRNA targeting to enhancer, we either deleted or mutated the seed region of miR-24-1 (Fig. 3A) and found that both the deletion and mutations abolished miRNA-induced transcriptional activation (Fig. 3B).
It is important to explore whether the miRNA targeted enhancer genome locus is essential for activating gene expression. We then undertook a TALE nuclease genome-editing approach \(^{28}\) to delete the miR-24-1 enhancer locus in the HEK293T cell line (Fig. 3C and Fig. S3A, B). Specific primers designed around the mutation sites allowed detection of the allelic mutable editing events (Table S3). Two clones (miR-24-1 deletion1# and deletion2#) were identified in the miR-24-1 enhancer locus (Fig. 3C, and Fig. S3C, D), in which the enhancer region were disrupted and the miRNA expression was significantly reduced (Fig. S3E). In these 2 TALEN-deletion cell strains, miR-24-1 neighboring genes were not able to be activated any further (Fig. 3D, E), even by high levels of miR-24-1 ectopic overexpression (Fig. S3E, F), whereas the expression level of the neighboring gene of miR-24-2, which was the homologous originating locus of miR-24-1 mentioned before, was upregulated (Fig. S3G). The above results indicate that the transcriptional activation of miR-24-1 neighboring genes is dependent on the presence of the intact enhancer.

**miR-24-1 functions through increased endogenous miRNA expression, chromatin state alteration of the enhancer**

To explore the underlying mechanism by which our miRNA modulates the activity of the enhancer, we first ascertained that miR-24-1 can in fact enter the nucleus by observing the nuclear translocation of Cy5 labeled miR-24-1 (Fig. S4A, B). In order to determine whether endogenous miRNA is involved in miRNA function, we quantified the endogenous miRNA after ectopic expression of miR-24-1. To this end, we transfected the miR-24-1 mimics and then specifically detected endogenous

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**Figure 4.** Epigenetic events involved in miRNAs’ transcriptional activation. (A) miRNA mimic (green lines) increases the endogenous miRNA expression (red rectangle, mature miRNA; blue rectangle, pre-miRNA). Arrows indicate primer locations for qPCR analysis of endogenous miRNA. (B) Depletion of AGO2 blocks transcriptional activation of neighboring genes. (C) Pol II, p300 and H3K27ac ChIP assays on the miR-24-1 locus. (D) The schematic diagram (upper panel) represents the position of eRNA primers. qPCR data from the lower panel shows that eRNAs are produced from the miR-24-1 locus upon miRNA overexpression. Fig. A-D, data shown are mean ± SEM from 3 independent transfections. \(^{**}P < 0.01, \^{***}P < 0.001\) by 2-tailed Student’s t-test. (E) The occupancy pattern of chromatin status of miR-24-1 enhancer locus by NOMe-seq. Nuclei were extracted from HEK293T cells and treated with M.CviPi GpC methyltransferase and subjected to bisulfite conversion and cloning. Circles represent GpC dinucleotides (Blue: the reference GpC sites; Gray: occupied, unmethylated and inaccessible to M.CviPi; Orange: unoccupied, methylated and accessible to M.CviPi). Horizontal lines represent randomly selected clones. Y-axis indicates the clone number and X-axis marks the sequence length of DNA. The changed occupancy in the enhancer and promoter region is around 70bp, unable to accommodate a singular nucleosome, hinting that those regions may be occupied by regulation factors. Both regions gained occupancy by miR-24-1 overexpression as compared with the control.
miR-24-1 precursor expression (Fig. 4A, upper panel). Surprisingly, we found that the exogenous miRNA can increase endogenous miR-24 expression in the activated enhancer region (Fig. 4A, lower panel).

Growing evidence suggests that AGO2 can also exist and functions in the nucleus. To inquire whether AGO2 is involved in our miRNA function, pre-hsa-mir-24-1 was transfected, and then AGO2 was knocked down. As shown in Fig. 4B, the expression levels of miR-24-1 neighboring genes were significantly decreased in cells transfected with AGO2 shRNA (Fig. S4C, D). This observation implies that AGO2 is important for miR-24 activity.

It is known that enhancer activation is associated with the initiation of RNA polymerase II (RNAPII) transcription, producing functional transcripts from the enhancer. As shown in Fig. 4C (left panel), RNAPII was enriched in the miR-24-1 enhancer region. Furthermore, we can detect increased eRNA transcripts from the active enhancers provoked by the exogenous expression of miR-24-1, miR-26a-1, miR-339, or miR-3179, respectively (Fig. 4D and Fig. S4E-G). Consistently, in response to miR-24-1 overexpression, ChIP-qPCR showed that the AGO2 and p300/CBP binding was enriched (Fig. 4C, middle panel, Fig. 4H, left panel). Meanwhile, the active enhancer marker H3K27ac was increased and the repressive marker H3K9me3 was depleted (Fig. 4C, right panel, and Fig. S4H, left panel). Taking advantage of NOMe-Seq, which can monitor in vivo protein occupancy on the DNA sequence, we found significant changes of protein occupancy in the miR-24-1 enhancer locus and promoter region of FBP1 (Fig. 4E, Fig. S4I-K). These results suggest that miR-24-1 overexpression can adjust the chromatin occupancy of the enhancer locus and its corresponding gene promoter. Overall, our data demonstrate that miRNA overexpression leads to an increased endogenous miR-24 expression and direct chromatin state alteration of the enhancer, which are all involved in gene activation (Fig. S4L).

**miR-24-1 promotes global gene transcription through the binding and activating of its targeted enhancers**

miRNA as a small molecule can target the 3’UTR of many genes. As miR-24-1 and miR-24-2 can activate each of their neighboring genes, we wondered whether miRNA may also be able to activate global gene expression. From microarray data, we noticed that miR-24-1 ectopic expression could increase as well as decrease gene expression (Fig. 5A and Fig. S5A). Bioinformatics analysis by miRanda showed that down-regulated genes caused by miR-24-1 overexpression has more potential binding sites in their 3’UTR (Fig. S5B), suggesting that miRNAs generally function in the cytoplasm. Meanwhile, we propose that some miRNAs may activate gene expression through the enhancer-associated miRNA mechanism. To further investigate whether there are some genes activation caused by miR-24-1, we performed ChIP-seq assay of active enhancer markers H3K27ac and identified a prominent enrichment of H3K27ac in 3282 enhancer regions responding to miR-24-1 overexpression. In addition, a motif similar to the seed sequence of miR-24-3p was found in the elevated H3K27ac enhancer region (Fig. S5C), where a statistically significant high frequency of DNA sequence that perfectly matches the miR-24-1 seed sequence was also discovered (Fig. S5D). Importantly, miR-24-1 targets predicted by the miRanda algorithm were significantly more prevalent in the activated enhancer regions than randomly selected sequences (Fig. 5B). Moreover, the up-regulated genes revealed by microarray assay tend to flank the activated enhancers (Fig. 5C), and taking one as an example, we can see that in the KDM6B gene locus, the enrichment of enhancer related markers changed accordingly (Fig. 5D). Thus, we speculate that miR-24-1 can bind to loci beyond their original sequence and activate genes transcription, which is additionally supported by the findings that the long-range target genes DUSP16 and ENO3 mRNA levels were activated by miR-24-1 (Fig. S5E-G), but conversely decreased in miR-24-1 antagomiR transfection (Fig. S5H). To further confirm the importance of the integrity of the miRNA targeted enhancer, we disrupted the miRanda predicted enhancer in the KDM6B gene locus using FastTALE™ TALEN Assembly Kit (SIDANSA1) (Fig. 5E, F and Fig. S5I-K) and found that this deletion can abolish the neighboring genes up-regulation of KDM6B, LSMD1 and CYB5D1 when we transfected miR-24-1 (Fig. 5G, H).

**Discussion**

MicroRNA induced translation repression results in gene silencing, and mainly occurs in the cytoplasm. However, miRNAs also exist in the nucleus, leaving us to question the precise function of nuclear miRNAs. Recent reports have demonstrated that miRNA or small RNA may be able to up-regulate translation through the interaction between miRNAs and promoters, as well as enhance translation. Our work on the function of enhancer-associated miRNA presents a somewhat novel concept.

In our research, we found that a subset of miRNAs deriving from the enhancer loci is capable of activating transcription, and these miRNAs are associated with active enhancers. Choosing miR-24-1 for further investigation, we found it function as unconventional mediators for transcriptional gene activation through chromatin remodeling at enhancer regions. Meanwhile, when it is within the cytoplasm, miR-24-1 would still be able to function canonically as a repressor for its target genes (Fig. S2F and Fig. S5B). In knowing this, one would visualize that miRNA carries a dual function ability, activation in the nucleus as well as repression in the cytoplasm.

This model, was later predominantly applied to the understanding of the miRNA-mRNA regulation network among diverse organismal systems. Our work has identified that miRNAs can function as an unconventional mediator for transcriptional gene activation through chromatin remodeling at enhancer regions. The positive and negative modulating effects of miRNAs in gene regulation may both contribute to gene
Figure 5. miRNAs activate genome-wide gene transcription. (A) Microarray shows that 1074 genes were upregulated after miR-24-1 transfection to HEK293T cells. Up- and downregulated genes are shaded in red and blue, respectively. (B) The miRanda algorithm analysis shows that 60.42% of total 3282 H3K27ac enriched regions were predicted as miR-24-1 targeted sites, while only 13.24% of total 2070 mock regions were predicted (P = 9.756e-254, χ² Test). Correlation between miR-24-1 target enhancers and adjacent up-regulated gene number calculated in ±100k of miR-24-1 targeted H3K27ac peaks. Difference in upregulated gene number is significant (P = 0.000878) relative to mock. The x-axis is the up-regulated genes number, and y-axis is the occurrence of every number in total 1 million times. In detail, we identified 1984 potential miR-24-1 targeted enhancers through H3K27ac histone ChiP-seq data and miRanda prediction. A total number of 179 upregulated genes revealed by microarray assay are located within ±100kb of these enhancers. As the control, we created a background by randomly sampling sets of mock regions which has same length with targeted enhancers for 1 million times and computing the up-regulated genes number within ±100kb of every mock region. Distribution of the upregulated genes number in the background is plotted as the black curve. Up-regulated genes number for potential miR-24-1 targeted enhancers is significant (P-value = 0.000878, red line) relative to our background model. (D) As shown by ChiP-seq boxplots, H3K27ac, H3K4me1 and Pol II was enriched while H3K9me3 decreased in the predicted miR-24-1 target region when miR-24 overexpressed. (E) An example of Fig. SD. Genomic locus of the predicted miR-24-1 target region overlapped with the strong enhancer (blue, left). After miR-24-1 overexpressed, H3K27ac, H3K4me1 and Pol II was enriched while H3K9me3 decreased. L5MD1 (pink shadow) and CYB5D1 (yellow shadow) became upregulated as RNA-seq analysis shown. Also by microarray data, L5MD1 appears to be up-regulated. (F) Schematic of TALENs design for miR-24-1 targeted locus predicted by miRanda overlapped with an enhancer (blue). (G–H) miR-24-1 target site deletion abolished neighboring gene activation. FBP1 in red box as a positive control. All values are normalized to GAPDH and error bars show mean ± SEM for 3 biological replicates **P < 0.01, ***P < 0.001 by 2-tailed student's t-test.
expression, our findings demonstrate that miRNAs can function as an activator. Thus, we expect that miRNA activated gene transcription may represent a novel mechanism.

Author contributions
W.Y. and M.X. conceived the project and planned experiments; M.X. performed most of them; J.L. W.L and Y.X performed computational analyses of ChIP-seq, RNA-seq and miRNA target prediction. F.W. assisted in computational analyses; Plasmid construction, Transfection and qPCR were contributed by C.D. and Y.W.; L.Z. and Y.X. performed western blot; H.L. assisted in NOME-seq; Y.L. assisted in ChIP-seq library preparations and sequencing; L.P. assisted in RNA-seq library preparations; the project was supervised by W.Y.; W.Y. and M.X. wrote the manuscript with contributions from other authors.

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

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