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An exogenous reporter system reveals location-specific miRNA regulatory modes: translational blockade at CDS vs transcript degradation at 3'UTR

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Abstract

MicroRNA (miRNA) is a key regulator of gene expression, involved in modulating various physiological and pathological processes. Current research primarily focuses on the regulatory mechanisms of miRNA targeting the 3' untranslated region (3'UTR), while understanding of its functional mode when targeting the protein-coding sequence (CDS) remains limited. This study established an exogenous gene expression system to systematically compare the regulatory differences in gene expression between the miRNA-3'UTR and miRNA-CDS targeting modes. Western blotting and fluorescent reporter analyses confirmed that both modes effectively suppressed protein expression, but miRNA-mediated suppression targeting CDS responded more rapidly. Real-Time Polymerase Chain Reaction (RT-PCR) analysis further revealed that the miRNA-3'UTR mode led to significant downregulation of mRNA levels, whereas the miRNA-CDS mode induced only a transient and mild reduction in mRNA. Collectively, these results demonstrate that the mechanism of miRNA action is position-dependent: targeting the 3'UTR primarily induces

mRNA degradation, while targeting the CDS preferentially inhibits the translation process. This model provides an experimental framework for deciphering new mechanisms of miRNA-mediated gene expression regulation and lays a foundation for functional studies of non-coding RNAs and related disease mechanisms.

Introduction

MicroRNA (miRNA) is a class of small non-coding RNAs approximately 22 nucleotides in length, processed from precursor RNAs with stem-loop structures.[1,2] Although they do not encode proteins, they act as regulatory factors modulating gene expression within cells.[3,4] miRNA regulates gene expression at the post-transcriptional level by binding to target mRNAs and recruiting regulatory complexes;[5] miRNA can regulate various physiological activities in organisms, including growth, development, cell differentiation, aging, and apoptosis, and is also associated with numerous pathophysiological processes such as cancer development, viral infection, and drug resistance.[1,6]

Translation in eukaryotes involves numerous regulatory factors that recruit ribosomal subunits to mRNA and coordinate the initiation, elongation, and termination processes; miRNA-mediated translational repression may occur by affecting mRNA circularization or ribosomal elongation.[7,8] Additionally, miRNA can promote mRNA degradation.[9] In mammals, miRNA pairing with target sites is often imperfect; it primarily functions by recruiting regulatory factors to reduce mRNA stability, leading to 5'-3' mRNA degradation pathway.[10,11] Notably, besides their repressive function in the cytoplasm, some miRNAs can also activate gene expression. For example, transfection of miR-373 significantly increased the expression of cold-shock domain-containing protein C2 (CSDC2);[12] miR-1 can be transported to mitochondria, target mRNAs encoding mitochondrial subunits NADH dehydrogenase subunit 1 (ND1) and cytochrome c oxidase subunit I (COX1), and enhance their expression.[13]

Utilizing crosslinking immunoprecipitation coupled with high-throughput sequencing (CrossLinking ImmunoPrecipitation sequencing, CLIP-seq) technology, researchers have identified numerous miRNA binding sites in both the 3' untranslated region (3'UTR) and protein-coding sequence (CDS) of mRNAs.[14-16] Currently, the regulatory mechanisms and biological significance of miRNA-3'UTR targeting have been extensively studied, whereas research on the miRNA-CDS targeting mode remains very limited.[2] Although it is generally accepted that miRNA's primary function *in vivo* is to inhibit protein translation or promote mRNA degradation, the relative dominance of translational repression versus mRNA decay in cells remains controversial.[10,17] Some studies using quantitative mass spectrometry to investigate the impact of miRNA on protein synthesis at the proteome level found that miRNA's effect on gene expression at the protein level has certain limitations.[18,19]

This project designed and established an exogenous gene expression system to investigate the impact of miRNA targeting the CDS versus the 3'UTR on miRNA-mediated gene expression regulation. The study found that when miRNA targets the 3'UTR, its regulatory effect induces strong mRNA degradation; conversely, when miRNA targets the CDS, its primary regulatory effect likely stems from inhibition of the protein translation process. Through molecular biology techniques, we proposed and experimentally validated a miRNA binding site-dependent gene expression regulation model. This model provides new clues for studying the involvement of non-coding RNAs in gene expression regulation.

Materials and methods

Cell culture and transfection

HeLa cells (China Center for Type Culture Collection, CCTCC Shanghai, China; Cat# TCHu187) were cultured in H-DMEM (Sigma, St. Louis, USA, #D5796) supplemented with 10% (v/v) fetal bovine serum (FBS, Thermo Fisher Scientific, MA, USA, Cat. #A5670701), 100 U/mL penicillin, and 100 μg/mL streptomycin (penicillin–streptomycin solution,100×, Thermo Fisher Scientific, MA, USA, Cat. #15140122). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. Plasmid transfection was performed using polyethylenimine HCl MAX transfection reagent

(Polysciences, Warrington, USA, #24765) according to the manufacturer's protocol.

Established recombinant plasmids

The recombinant plasmids were constructed using the pcDNA3.1 vector, which contains a cytomegalovirus (CMV) promoter, an ampicillin-resistance gene, and a neomycin selection marker. Coding sequences for either Flag-tagged mWasabi (Flag-wsb) or Flag-tagged Renilla luciferase (Flag-Rluc) were inserted into the vector; mWasabi and Renilla luciferase sequences were cloned from mWasabi-LaminB1 (Addgene, Watertown, USA, #56507) and pLenti.PGK.blast-Renilla_Luciferase (Addgene, Watertown, USA, #74444). Binding-site sequences for different miRNAs were then introduced immediately upstream of the stop codon of the Flag-wsb/Flag-Rluc coding region.

To position the miRNA binding site within either the 3′UTR or the coding sequence (CDS), we further inserted an additional stop codon (TGA) or a glycine codon (GGA) between the Flag-wsb/Flag-Rluc coding region and the miRNA binding site. In addition, a 2A peptide sequence and the β-globin intron were incorporated into the reporter system to eliminate potential interference from amino acids encoded by the miRNA binding site and to prevent genomic DNA contamination from affecting reporter RNA measurements. Importantly, the mRNAs generated from the two reporter plasmids differ by only a single nucleotide (U versus G).

Western blotting

Following HeLa cell lysis, total proteins were extracted. Subsequently, 10 μg of protein lysates were separated by 10% Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to Nitrocellulose (NC, Millipore, Burlington, USA, #HATF00010) membranes. Membranes were blocked with 5% non-fat milk in Tris-Buffered Saline with Tween 20 (TBST, Epizyme Biotech, Shanghai, China, #PS103) for 1 hour at room temperature, then incubated overnight at 4°C with primary antibodies against hemagglutinin (HA) (Cell Signaling Technology, Danvers, USA, #3724T) and FLAG (Cell Signaling Technology, Danvers, USA, # 8146T). All antibodies were diluted at a ratio of 1:3,000, according to manufacturer specifications. Protein

bands were visualized by chemiluminescent detection.

RNA isolation and quantitative real-time PCR

We extracted total HeLa RNA using RNAiso Plus (Thermo Fisher Scientific, MA, USA, # 15596026CN) following the manufacturer's protocol. To determine the mRNA level, 0.5 μg of each RNA sample was reverse transcribed to cDNA using a PrimeScript RT Master Mix Kit (TaKaRa Biotechnology, Dalian, China, #RR036A) in a 20 μL reaction system. For Real-Time Polymerase Chain Reaction (RT-PCR), we used TB Green® Premix Ex Taq™ II kit (TaKaRa Biotechnology, Dalian, China, #RR820A).

Statistical analysis

Data are presented as mean ± Standard Error of the Mean (SEM). We performed statistical comparisons between groups using a two-tailed unpaired Student's t-test, unless stated otherwise. In all analyses, statistical significance was defined as $p < 0.05$.

Results

Establishment and validation of the exogenous gene expression system

To investigate the impact of miRNA targeting different binding sites (CDS or 3'UTR) on its gene regulatory function, we first established an exogenous reporter gene system for three different miRNAs (miR-181, miR-378, and miR-483) targeting distinct binding sites (Figure 1A). To prevent the peptides encoded by different miRNA binding sites from affecting the reporter protein signal and to ensure consistency in subsequent protein level detection, we inserted the coding sequence for the 2A peptide between the reporter protein coding sequence and the miRNA binding site (Figure 1A). The 2A peptide is a self-cleaving peptide that separates the miRNA binding site-encoded peptide from the reporter protein during translation.[20] This design allowed us to investigate the

effect of different miRNA binding sites on reporter protein levels by detecting proteins of identical size and sequence.

To enable miRNA targeting of either the CDS or the 3'UTR within the same system, we introduced a glycine codon (GGA) or a stop codon (TGA) into two parallel plasmid systems, respectively (Figure 1A). For the miRNA-3'UTR mode, a stop codon (TGA) was inserted between the reporter protein coding sequence and the miRNA binding site. Here, the ribosome terminates translation before reaching the miRNA binding site, resulting in miRNA targeting the 3'UTR of the reporter gene mRNA. For the miRNA-CDS mode, a glycine codon (GGA) was inserted at the same position. The ribosome translates through the glycine codon to continue translating the miRNA binding site sequence, recognizing a downstream ochre stop codon (TAA) to terminate translation; thus, miRNA targets the protein-coding sequence. The mRNA sequences of these two reporter gene plasmids differ by only one nucleotide (G or U). Furthermore, to exclude interference from genomic DNA in subsequent RT-PCR mRNA level detection, we inserted an intron sequence from β-globin upstream of the reporter gene coding sequence (Figure 1A). During post-transcriptional mRNA processing, this intron sequence is recognized and spliced out by small nuclear ribonucleoproteins (snRNPs). Therefore, we designed RT-PCR primers spanning the intron sequence. Due to the RT-PCR extension time limitation, only cDNA derived from mature mRNA (without the intron) can be fully amplified and detected with SYBR Green.

We validated the reporter gene system in HeLa cells. Transfection of the reporter plasmids alone, using an empty vector plasmid as a negative control and a plasmid expressing only Flag-wsb as a positive control, showed that both reporter plasmids expressed normally (Figure 1B). Moreover, under the self-cleavage action of the 2A peptide, the peptide sequence encoded by the miR-378a binding site did not affect the reporter fluorescent protein signal. Western blotting detection of Flag-wsb protein levels revealed that, regardless of the targeting mode, the reporter protein level was suppressed in the miR-378-containing experimental group compared to the blank control group (Figure 1C). These results demonstrate that the designed and constructed reporter gene system functions normally.

Effect of miRNA targeting different sites on protein level

To investigate the impact of miRNA-CDS and miRNA-3'UTR targeting modes on protein expression, we co-transfected HeLa cells with the reporter plasmid, miRNA overexpression plasmid, and a transfection control plasmid. The control plasmid contained an HA-tagged Green Fluorescent Protein (HA-GFP) sequence lacking any miRNA binding sites; its transcribed mRNA served as an internal reference for RT-PCR, and its encoded protein served as an internal reference for Western blotting.

To exclude miRNA-specific functional consequences and ensure the generality of results, we used three different miRNAs (miR-181, miR-378, and miR-483). Since both Flag-wsb and HA-GFP could be excited by blue light to emit green fluorescence, we replaced the reporter protein with Flag-tagged Renilla luciferase (Flag-Rluc) to eliminate interference between the two fluorescent proteins. In the experiments, a scrambled target sequence served as the negative control for the miRNA target sequence, and an empty vector plasmid served as the negative control for the miRNA overexpression plasmid. Results showed that in both targeting modes, different miRNAs suppressed reporter protein expression (Figure 2); however, when no miRNA was present or when miRNA could not specifically target the reporter mRNA, reporter protein expression remained unchanged. Grayscale analysis of Western blotting bands revealed no significant difference in the extent of protein level suppression by miRNA between the two targeting modes (Figure 2).

miRNA-CDS mode responds faster to miRNA signal than miRNA-3'UTR mode

By detecting cellular green fluorescence intensity at different time points, we investigated differences in the response speed of the reporter gene to miRNA-CDS versus miRNA-3'UTR targeting. Results showed that 24 hours post-transfection, miRNA suppression of reporter protein was stronger in the miRNA-CDS mode compared to the miRNA-3'UTR mode; however, at 48 hours post-transfection, the suppression level was comparable between the two modes (Figure 3). The faster response observed with the miRNA-CDS mode suggests that miRNA targeting CDS and 3'UTR might regulate target gene expression by eliciting different cellular responses, such as recruiting distinct regulatory factors.

Effect of miRNA targeting different sites on mRNA level

To investigate the impact of miRNA-CDS and miRNA-3'UTR modes on mRNA levels, we extracted total RNA from HeLa cells at 24, 48, and 72 hours post co-transfection. RT-PCR analysis revealed that under the miRNA-CDS mode, reporter mRNA levels showed only a noticeable downward trend at 24 hours (Figure 4A), while under the miRNA-3'UTR mode, reporter mRNA levels were significantly downregulated by miRNA (Figure 4B). However, at 48 or 72 hours post-transfection, mRNA abundance showed no significant downregulation in the miRNA-CDS mode (Figure 4A). These results indicate that when the miRNA binding site was located in the 3'UTR, its regulatory effect induces strong mRNA degradation; whereas when miRNA targets the CDS, its primary regulatory effect likely arises from inhibition of the protein translation process.

Discussion

This study utilized molecular biology techniques to establish an exogenous gene expression system to investigate the impact of miRNA-CDS and miRNA-3'UTR targeting modes on gene expression regulation. Testing the reporter system in HeLa cells showed stable expression and precise response to miRNA signals. Subsequently, transient co-transfection of reporter plasmids and miRNA overexpression plasmids in HeLa cells, followed by Western blotting, revealed that miRNA significantly suppressed reporter protein levels in both targeting modes. Detection of reporter protein fluorescence signals at different time points post-transfection showed that at early time points (24 hours), suppression was stronger in the miRNA-CDS mode; however, by 48 hours, protein suppression levels were comparable between the two modes. This suggests that the two modes may mediate protein level suppression through distinct molecular mechanisms, and that suppression of protein synthesis is faster and more direct when miRNA binds the CDS. At the mRNA level, RT-PCR results showed that under the miRNA-CDS mode, reporter mRNA levels were only mildly downregulated and recovered to control levels within 72 hours. In contrast, under the miRNA-3'UTR mode, mRNA levels decreased substantially and did not recover within 72

hours. This indicates that miRNA targeting the mRNA 3'UTR significantly and stably promotes mRNA degradation.

In agreement with our findings, growing evidence suggests that miRNA binding within the CDS often represses translation without inducing substantial mRNA degradation. A study published in 2013 by Hausser and colleagues demonstrated that CDS-located miRNA-Recognition Elements (MREs) effectively inhibit translation by modulating ribosome elongation dynamics, which is consistent with our observation that miRNA-CDS targeting produces a rapid suppression of protein expression.[9] Similarly, another study described a class of MREs that function exclusively within coding regions, further supporting the idea that CDS targeting utilizes regulatory factors distinct from classical 3'UTR-mediated mechanisms.[21] These reports align with our data showing only a mild and transient reduction in mRNA levels under the miRNA-CDS mode.

In contrast, our results showing strong and persistent mRNA degradation in the miRNA-3'UTR targeting mode are highly consistent with transcriptome-wide studies demonstrating that miRNA-mediated gene silencing in mammals is predominantly achieved through mRNA decay.[10,17] This pathway is mediated through the recruitment of GW182/TNRC6 proteins, which engage the carbon catabolite repression 4–negative on TATA-less (CCR4–NOT) and mRNA-decapping enzyme 1/2 (DCP1/2) complexes to induce deadenylation and decapping (11). The substantial and sustained reduction in mRNA abundance in our miRNA-3'UTR experiments is therefore fully in line with established molecular models of miRNA-mediated transcript destabilization.

Additionally, recent CLIP-seq and Sequencing of Hybrids (CLASH) studies have revealed that CDS-associated miRNA binding is far more common than previously recognized and may engage different protein cofactors compared with 3'UTR sites (14, 15). These findings provide a mechanistic foundation for our observation that CDS-targeting elicits a fast but largely mRNA-independent mode of repression. Such noncanonical regulation may be particularly relevant for endogenous transcripts with short 3'UTRs or alternative UTR isoforms, a phenomenon frequently observed in proliferative or cancerous cells.

Conclusions

Our data support a model in which the regulatory effect of miRNA is highly dependent on target-site location: miRNA–3'UTR binding tends to promote robust mRNA degradation, whereas miRNA–CDS binding primarily induces translational inhibition with minimal long-term impact on mRNA stability. This positional specificity expands our understanding of miRNA regulatory diversity and highlights the importance of considering target-site architecture when interpreting miRNA function. Future studies combining proteomics, RNA-induced silencing complex (RISC)-interactome profiling, and endogenous gene validation will be valuable for uncovering the molecular determinants that distinguish CDS- and 3'UTR-specific miRNA regulatory pathways.

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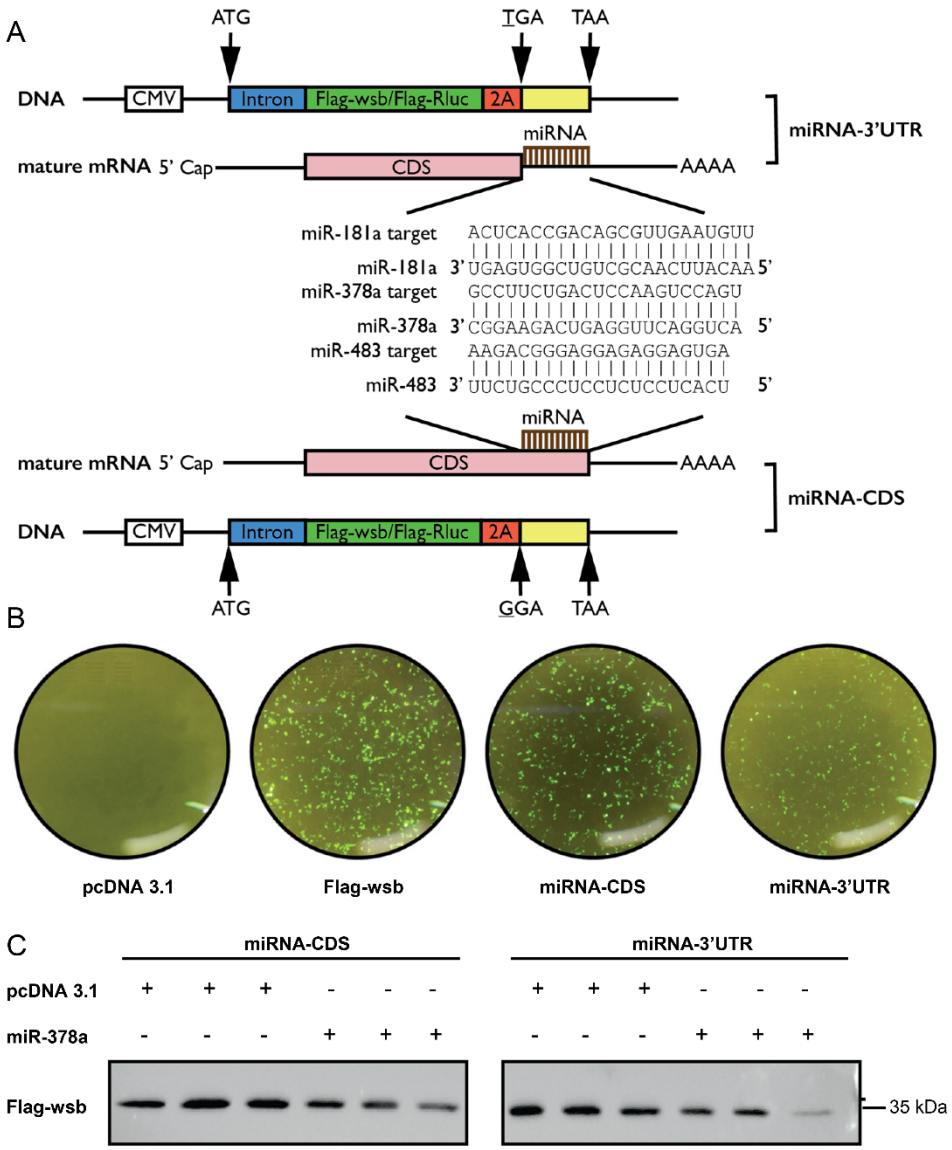

Figure 1. Establishment and Validation of the Exogenous Gene Expression System. A) Schematic diagram of the reporter gene system for studying the action modes of miRNA-3'UTR and miRNA-CDS; B) The green fluorescent protein produced can emit green fluorescence under excitation by blue light; C) Immunoblotting assays of Flag-wsb in HeLa cells. CMV: cytomegalovirus, 3'UTR: 3' untranslated region, CDS: protein-coding sequence, Flag-wsb: Flag-tagged mWasabi.

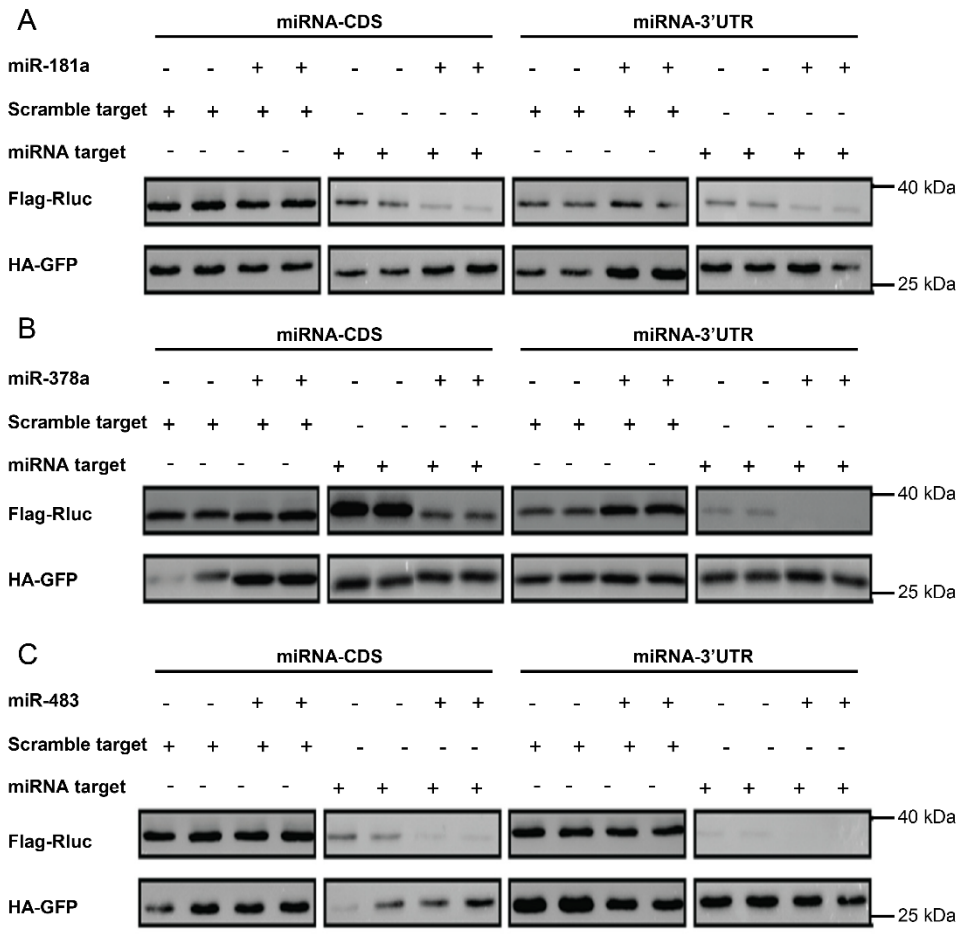

Figure 2. Effect of miRNA targeting different sites on protein level. A) Immunoblotting assays of Flag-Rluc and HA-GFP in HeLa cells transfected with scramble target, miR-181a or miRNA target. HA-GFP was used as loading control; B) Immunoblotting assays of Flag-Rluc and HA-GFP in HeLa cells transfected with scramble target, miR-378a or miRNA target. HA-GFP was used as loading control; C) Immunoblotting assays of Flag-Rluc and HA-GFP in HeLa cells transfected with scramble target, miR-483a or miRNA target. HA-GFP was used as loading control. Flag-Rluc: Flag-tagged Renilla luciferase, Flag-wsb: Flag-tagged mWasabi. HA-GFP: HA-tagged green fluorescent protein.

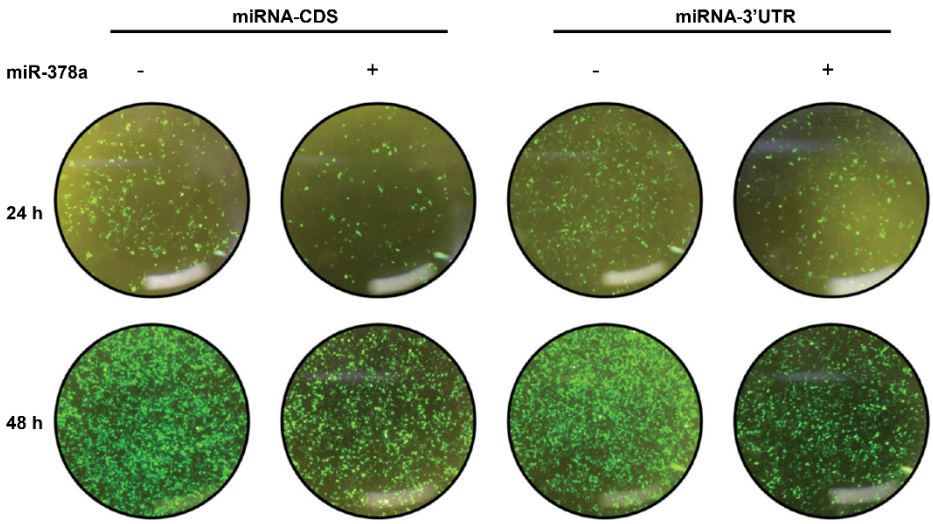

Figure 3. miRNA-CDS mode responds faster to miRNA signal than miRNA-3'UTR mode. Green fluorescence intensity of HeLa cells transfected with scramble or miR-378a at different time points observed using a fluorescence microscope. 3'UTR: 3' untranslated region, CDS: protein-coding sequence.

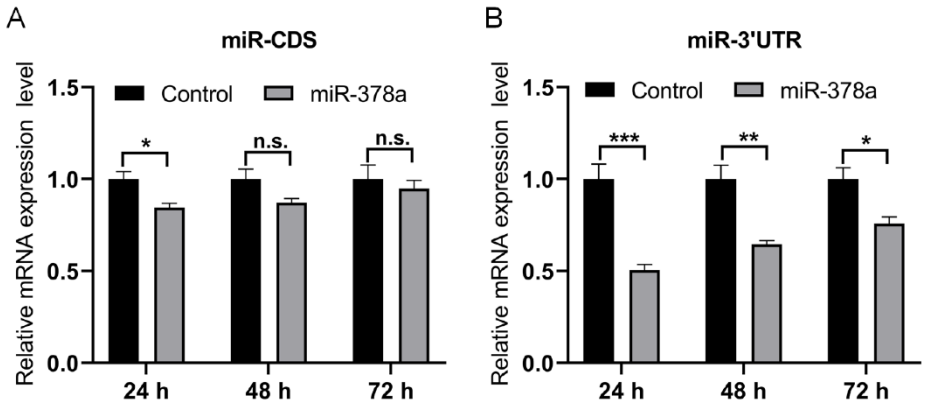

Figure 4. Effect of miRNA targeting different sites on mRNA level. A) The mRNA levels of *wsb* in miRNA-CDS mode were analyzed by RT-PCR; B) The mRNA levels of *wsb* in miRNA-3'UTR mode were analyzed by RT-PCR. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, n.s., not significant. 3'UTR: 3' untranslated region, CDS: protein-coding sequence, RT-PCR: Real-time polymerase chain reaction, wsb: mWasabi.

Contributions

Dandan Wang conceived and designed the methodology and data analysis, and reviewed and edited the manuscript.; Le Bai performed the experiments, analyzed the data and prepared figures; Le Bai and Dandan Wang analyzed the data and wrote the manuscript.

Conflict of interest

We declare that there is no any conflict of interest in this paper.

Ethics approval: the Ethics Committee of INSTITUTION approved this study (APPROVAL CODE). The study is conformed with the Helsinki Declaration of 1964, as revised in 2013, concerning human and animal rights.

Data Availability Statement

The experimental or analytical data from this study are available upon request from the corresponding author.

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