miR-346 Up-regulates Argonaute 2 (AGO2) Protein Expression to Augment the Activity of Other MicroRNAs (miRNAs) and Contributes to Cervical Cancer Cell Malignancy

Received for publication, September 10, 2015, and in revised form, October 29, 2015 Published, JBC Papers in Press, October 30, 2015, DOI 10.1074/jbc.M115.691857

Junfei Guo, Jing Lv, Min Liu, and Hua Tang* From the Tianjin Life Science Research Center, School of Basic Medical Sciences, Tianjin Medical University, Tianjin 300070, China

MicroRNAs (miRNAs) are a class of post-transcriptional regulators of gene expression, and AGO2 is essential for miRNA activity. In this study, we focused on the regulation of AGO2 by miR-346 and the consequences in cervical cancer cells. miR-346 enhanced the expression of AGO2, resulting in the increased activity of other miRNAs and contributing to the malignancy of HeLa cells. GRSF1 participated in the regulation of AGO2 by miR-346, and the middle sequence of miR-346 was vital for the synergy effect of miR-346 and GRSF1. We determined that miR-346 promoted the migration and invasion of HeLa cells. In summary, we are the first to report that AGO2 is regulated positively by miRNA and that GRSF1 participates in the miRNA pathway.

Cervical cancer is one of the most prevalent gynecological cancers worldwide, accounting for ~12% of all cancers globally (1). The etiology of cervical cancer varies, and high-risk human papilloma virus infection is one of the most prominent features of cervical cancer (2). Recently, the role of microRNAs (miRNAs), a class of small RNAs that post-transcriptionally regulate target gene expression in a sequence-specific manner, has received widespread attention (3, 4). The dysregulation of miRNAs affects many aspects of cervical cancer, including cellular apoptosis, cell cycle progression, and cell migration and invasion (5–7).

miRNAs are transcribed as long primary transcripts that are subsequently processed by the RNase III proteins Drosha and Dicer to form mature microRNAs (8). Then, a strand of mature miRNA is incorporated into an RNA-induced silencing complex (RISC), where it exerts its effects. AGO2 is a key component of the RISC and is the only AGO2 protein with intrinsic endonuclease activity (9). The expression of AGO2 is tightly regulated at both transcriptional and post-transcriptional levels (10). Dysregulation of AGO2 has been reported in many tumors, including lung cancer (11), prostate cancer (12, 13), hepatocarcinoma (14), and melanoma (15), but the role of AGO2 and how its expression is regulated in cervical cancer have not been well studied.

In this study, we found that AGO2 was expressed in cervical cancers and that its expression was enhanced by miR-346 and GRSF1 independent of an increase in AGO2 stability. The increased expression of AGO2 augmented the activity of other miRNAs and promoted the malignant phenotype of cervical cancer cells. These findings could provide new insights into the regulation of AGO2 expression and the mechanisms by which miRNAs up-regulate the expression of their target genes.

Materials and Methods

Cell Lines and Clinical Tissue Samples—The cancer cell lines used in this study were preserved in liquid nitrogen in our laboratory, and the cells were maintained in standard culture medium as recommended by the ATCC. All transfections were performed with Lipofectamine™ 2000 reagent (Invitrogen) according to the recommendations of the manufacturer. Fourteen pairs of clinical cervical cancer tissues and respective adjacent non-cancerous tissues were obtained from the Centre Obstetrical and Gynecological Hospital of TianJin with informed consent from the patients.

Plasmid Construction—The miR-346 expression plasmid was constructed in a previous study with the following primer pair: sense, 5'-CACGGATCCCTTGTCAAGCAAGGAGTG 3'; antisense, 5'-CGGAATTCTAGGTTGGACGCCAGT 3'. The AGO2 3'UTR reporter plasmid was constructed by inserting the miR-346 target sites within the AGO2 3'UTR downstream of an EGFP open reading frame. The AGO2 3'UTR fragments were obtained by PCR with the following primer pair: sense, 5'-CAAGAATTCTGTGAATCTTCTGAGAG 3'; antisense, 5'-AGGCTCGAGCTGTAGATTAGA GTGTC 3'. Other plasmids used in this study were constructed previously in our laboratory.

RNA Isolation and qRT-PCR—Total RNA was extracted with TRIzol reagent (Sigma-Aldrich). The cDNA was obtained by reverse transcription with random primers, and the levels of AGO2 and β-actin mRNA were determined by real-time PCR with the following primers: AGO2 sense, 5'-GTC TCTGAAGGCACTTTGAGCCA GTTCCAGAAGC 3'; AGO2 antisense, 5'-ATAGAGGCTCTCA-CGGATGTCG 3'; β-actin sense, 5'-CTACGTGCGGTCG GATGTGCAGGGAGCGCGC 3'; β-actin antisense, 5'-GATGGGAGCGG-CGATCCACAGGGC 3'. The -fold change in expression of each target RNA relative to U6 snRNA or β-actin mRNA was calcu-
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Fluorescence Reporter Assay—The fluorescence reporter assay was performed according to the methods described by Liu and co-workers (6). The AGO2 3′ UTR reporter plasmid and miR-346 or ASO-miR-346 were transfected simultaneously into HeLa cells, and a plasmid expression of red fluorescent protein fluorescence was added to the transfection mixture to serve as a transfection efficiency control. The intensity of GFP and red fluorescent protein fluorescence was determined by spectrophotometry 24 h post-transfection.

To investigate the influence of miR-346 on the activities of other miRNAs or siRNAs, miR-346 was transfected simultaneously with reporter plasmids for the respective miRNAs. EGFP expression was determined by Western blot analysis as described by Li et al. (16), and GAPDH served as a loading control.

Western Blot Assay—The detailed procedure for Western blotting has been described elsewhere (24). The primary antibodies used in this study included GFP, PTEN, AGO2, ERGIC3, CDK2, CHL1, and GAPDH, which were obtained from Saier Biotechnology Co. (Tianjin, China). The secondary goat anti-rabbit and goat anti-mouse antibodies were obtained from Sigma. GAPDH was used as the endogenous control to normalize the expression levels of proteins of interest.

Migration and Invasion Assays—24-well Boyden chambers with an 8-μm pore size polycarbonate membrane (Corning, Cambridge, MA) were used to analyze the migration and invasion of tumor cells. Briefly, ~5 × 10⁶ cells were resuspended in culture medium without FBS and seeded in the upper chamber. Then the chamber was placed into a 24-well plate containing 600 μl of culture media with 20% FBS. Approximately 48 h later, the cells were fixed with paraformaldehyde and stained with crystal violet, and the cells that passed through the membrane were counted.

Cell Cycle Analysis via Flow Cytometry—The cells were seeded into 6-well plates in duplicate. When the cells reached ~60% confluence, the culture medium was replaced with serum-free culture medium for 24 h before one group of cells was harvested. The other group was returned to complete culture medium for 24 h before harvest. The harvested cells were fixed in 95% (v/v) ethanol and stored at −60 °C. Before analysis, the stored cells were washed with PBS and resuspended in propidium iodide staining buffer (PBS with 50 μg/ml propidium iodide and 0.1 mg/ml DNase-free RNase) for 30 min at 4 °C. The samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (BD Biosciences).

Xenograft Tumor Formation Assay—HeLa cells were transfected with pri-miR-346 or pcDNA3 and passaged for four generations with culture media containing 500 μg/ml G418. Cells were then harvested, and about 5 × 10⁶ cells, resuspended in 100 μl of serum-free RPMI 1640 culture medium, were injected subcutaneously into the flanks of the nude mice. Tumor size was measured every day beginning on day 7 after the injection. The tumor volume was calculated as follows: length × width² × ½. All mice were sacrificed on day 17 post-injection. The tumors were isolated from the mice and stored at −80 °C. All studies were performed under American Association for the Accreditation of Laboratory Animal Care guidelines for humane treatment of animals and adhered to national and international standards.

Results

miR-346 Up-regulates the Expression of AGO2 but Does Not Influence the Stability of AGO2—Our previous study found that the expression of miR-346 was increased significantly in both cervical cancer cell lines and clinical cervical cancer tissues compared with the respective controls. miRNAs regulate the expression of target genes, so we first analyzed the potential targets of miR-346 with publicly available algorithms: TargetScan (17) and miRanda (18). Both algorithms predicted hundreds of target genes for miR-346, including AGO2 (EIF2C2). The target sites of miR-346 in the AGO2 3′ UTR are highly conserved among mammals (Fig. 1A). To validate the target sites of miR-346 in the AGO2 3′ UTR, we constructed an EGFP reporter plasmid with the miR-346 target sites within the AGO2 3′ UTR inserted downstream of the stop codon of GFP (Fig. 1A). The reporter plasmid was then transfected into HeLa cells with a miR-346 expression plasmid or a miR-346-specific antisense oligomer (ASO). As shown in Fig. 1B, the expression of the reporter was increased significantly by miR-346 and reduced significantly by ASO-miR-346, and this was dependent on the miR-346 target sites within the AGO2 3′ UTR because mutating the target sites abolished the effects of both miR-346 and ASO-miR-346. The results were interesting because most miRNAs have been reported to negatively regulate the expression of their target genes. To further confirm the positive regulation of AGO2 by miR-346, we examined the influence of miR-346 on endogenous AGO2 protein and mRNA expression by Western blot analysis and qRT-PCR, respectively. miR-346 increased AGO2 protein and mRNA levels, whereas ASO-miR-346 reduced AGO2 expression at both levels (Fig. 1, C and D). The up-regulation of AGO2 by miR-346 occurred in the C33A cervical cancer cell line, the SW480 colorectal cancer cell line, and the BGC-823 gastric cancer cell line (Fig. 1E). All of these results demonstrate that miR-346 enhances the expression of AGO2.

A previous study by Smibert and colleagues (19) demonstrated that the stability of AGO2 is dependent on the availability of miRNAs in mammalian cells. Therefore, we aimed to ascertain whether miR-346 increased the protein level of AGO2 by enhancing the stability of AGO2. Time course analyses following cycloheximide treatment showed that miR-346 did not increase the stability of AGO2 (Fig. 1F), indicating that miR-346 enhanced the expression of AGO2 independent of its stability. Furthermore, the up-regulation of AGO2 was specific to miR-346 because overexpression of other miRNAs did not increase the expression of AGO2 (Fig. 1G). This result demonstrates that the increase in AGO2 protein level is independent of miRNA availability in HeLa cells. To further validate the positive regulation of AGO2 by miR-346, we detected the expression of miR-346 and AGO2 in 14 pairs of clinical cervical cancer and adjacent non-cancerous tissues. As shown in Fig. 1H, miR-346 was up-regulated in ten and down-regulated in four cervical cancer tissues compared with the respective adjac-
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**A**

![Diagram A]

**B**

![Diagram B]

**C**

![Diagram C]

**D**

![Diagram D]

**E**

![Diagram E]

**F**

![Diagram F]

**G**

![Diagram G]

**H**

![Diagram H]
cent non-cancerous tissues, and miR-346 and AGO2 had the same trends of expression in 11 pairs of tissues, indicating that the positive regulation of AGO2 by miR-346 was clinically significant. Together, the results demonstrate that miR-346 increases the level of AGO2 independent of its stability in cervical cancer cell lines.

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FIGURE 1. miR-346 enhances AGO2 expression in cervical cancer cell lines. A, the conservation of miR-346 target sites within the AGO2 3’UTR and a schematic of the EGFP reporter; UAA is a stop codon. B, HeLa cells were transfected simultaneously with an EGFP reporter plasmid and the indicated plasmid or oligonucleotides, and, 24 h later, EGFP fluorescence intensity was determined. The intensity of the control group was set to 1. *, p < 0.05; **, p < 0.01. C and D, the effect of miR-346 on AGO2 expression was determined by Western blot analysis and qRT-PCR, respectively. *, p < 0.05; **, p < 0.01. E and F, HeLa cells were co-transfected with a GRSF1 expression plasmid or a GRSF1 knockdown plasmid and miR-346 or miR-346 loop mut, and AGO2 protein levels were measured by Western blot analysis. G, an RNA immunoprecipitation assay was performed with an anti-GRSF1 antibody or IgG isotype control, and AGO2 mRNA and miR-346 were quantitated in the precipitated complexes by qRT-PCR. GAPDH mRNA and miR-16 in the complexes served as negative controls, and GPX4 mRNA served as a positive control. **, p < 0.01; ***, p < 0.001; ns, non-significant. H, cells were transfected with the indicated plasmids or oligonucleotides, RNA immunoprecipitation was performed, and the precipitated miR-346, AGO2 mRNA, and GAPDH mRNA were determined by qRT-PCR. *, p < 0.05.

GRSF1 Participates in the Up-regulation of AGO2 in a miR-346 Middle Sequence-dependent Manner—In this study, we found that miR-346 enhanced the expression of AGO2, and we sought to determine the possible mechanism. We first analyzed the secondary structure that forms between miR-346 and the AGO2 3’UTR. B, miR-346 or miR-346 loop mut was transfected into HeLa cells along with the AGO2 reporter plasmid, and the intensity of EGFP was measured 24 h later. The value in the control group was set to 1. *, p < 0.05; **, p < 0.01. C and D, the effect of the miR-346 middle sequence on AGO2 protein (C) and mRNA (D) levels was determined by Western blot analysis and qRT-PCR, respectively. *, p < 0.05; **, p < 0.01. E and F, HeLa cells were co-transfected with a GRSF1 expression plasmid or a GRSF1 knockdown plasmid and miR-346 or miR-346 loop mut, and AGO2 protein levels were measured by Western blot analysis.
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(20). As shown in Fig. 2A, the 5’ and 3’ ends of the two RNA molecules interact with each other with almost perfect base pairing, forming a bubble-like structure in the middle of miR-346 (hereafter termed the miR-346 loop). We speculated that this loop structure participates in the up-regulation of AGO2 by miR-346. Indeed, when we mutated the middle sequence of miR-346, the ability of miR-346 to activate the AGO2 3’UTR EGFP reporter plasmid (Fig. 2B) and up-regulate endogenous AGO2 protein (Fig. 2C) and mRNA (Fig. 2D) was abolished, indicating that the middle sequence of miR-346 is essential for its ability to promote AGO2 expression.

RNA binding proteins are a class of proteins participating in many aspects of RNA biology, from RNA biogenesis to its cellular localization and function. Recent studies have indicated that RNA binding proteins may also participate in miRNA function (21) and that some RNA binding proteins may endow new and unusual functions to miRNAs, such as the ability to up-regulate the expression of target genes (22). According to the above results and the loop structure of miR-346, we inferred that some RNA binding proteins may be involved in the regulation of AGO2 by miR-346. In an attempt to identify possible candidates, we focused on GRSF1 because of its pivotal role in enhancing the expression of many transcripts, although its role in the function of miRNAs remains unclear. In HeLa cells, the exogenous expression of GRSF1 resulted in increased AGO2 protein levels (Fig. 2E, third and fourth lanes), whereas knockdown of endogenous GRSF1 reduced AGO2 protein levels (Fig. 2E, seventh and eighth lanes), indicating the participation of GRSF1 in the expression of AGO2. When we simultaneously transfected HeLa cells with miR-346 and GRSF1, we obtained stronger AGO2 expression (Fig. 2E, fourth lane), whereas the knockdown of endogenous GRSF1 abolished the ability of miR-346 to promote AGO2 expression (Fig. 2E, eighth lane). These results indicate that GRSF1 participates in the regulation of AGO2 and is essential for miR-346 to enhance the expression of AGO2.

On the basis of the above findings, we speculated that GRSF1 may enhance the expression of AGO2 in a manner dependent on the miR-346 loop structure. AGO2 protein levels were increased by miR-346 but decreased by the miR-346 loop middle sequence mutation (mut) (Fig. 2F, first through third lanes), and the synergy effect between miR-346 and GRSF1 was abolished by the miR-346 loop mut (Fig. 2F, fourth through sixth lanes), indicating that GRSF1 increases the expression of AGO2 in a manner that depends on the miR-346 middle sequence. To further validate the role of GRSF1 in the regulation of AGO2 by miR-346, we performed RNA immunoprecipitation assay. The results of RNA immunoprecipitation demonstrated that GRSF1 interacts with endogenous AGO2 mRNA and miR-346 (Fig. 2G). GPX4 mRNA, which has been demonstrated to interact with GRSF1 (23), was also enriched significantly in GRSF1-precipitating complexes, indicating the effectiveness of the RNA immunoprecipitation assay. miR-16 and GAPDH mRNA served as negative controls and were not enriched significantly in this complexes, demonstrating that the interaction between miR-346 or AGO2 mRNA with GRSF1 was specific. In a previous study, we demonstrated that GRSF1 interacts with human telomerase reverse transcriptase (hTERT) mRNA in a miR-346 dependent manner. We tried to find out whether GRSF1 interacts with AGO2 mRNA in a miR-346-dependent manner. We found that the precipitated AGO2 mRNA was correlated positively with the miR-346 level in the cell, whereas that of GPX4 mRNA was not affected by the miR-346 level (Fig. 2H), demonstrating that the interaction between AGO2 mRNA and GRSF1 was miR-346-dependent. These results indicate that GRSF1 is necessary for miR-346 to up-regulate the expression of AGO2.

miR-346 Modulates the Activity of Other miRNAs—AGO2 is a critical component of the RISC and is essential for miRNA and siRNA function. Our results indicate that miR-346 enhances the expression of AGO2. Therefore, we anticipated that miR-346 might affect the activities of other miRNAs or siRNAs. To address this question, HeLa/CXCR4 cells that stably express a CXCR4 reporter plasmid were transfected simultaneously with si-CXCR4 and miR-346, and the expression of EGFP was determined by microscopy. As shown in Fig. 3A, the expression of EGFP was not affected by miR-346 in normal control (si-NC)-transfected cells, whereas miR-346 significantly reduced the expression of the CXCR4 reporter plasmid in si-CXCR4-transfected cells. These results indicate that miR-346 may modulate the activity of siRNAs. Next, to determine the role of miR-346 in regulating the activity of other miRNAs, we analyzed the effects of miR-346 on the activities of up-regulated miRNAs (miR-10a, miR-21, and miR-150) and a down-regulated miRNA (miR-372) in cervical cancer. miR-346 reduced the expression of reporter genes for miR-10a, miR-21, and miR-150, but miR-372 reporter expression was barely affected (Fig. 3B). Endogenous targets of the given miRNAs had the same changes as their respective reporter (Fig. 3C), demonstrating that miR-346 regulates the activity of other miRNAs by increasing AGO2 expression. To further validate the effect of miR-346 on the activity of other miRNAs, we investigated the influence of miR-346 on the activity of miR-490–3p, which has been reported to enhance the expression of ERGIC3 in an AGO2-dependent manner (24). As shown in Fig. 3, B and C, miR-346 significantly increased the expression of the ERGIC3 reporter gene and endogenous ERGIC3. Together, these results indicated that miR-346 enhances the activity of other miRNAs or siRNAs by increasing the expression of AGO2.

miR-346 Promotes the Malignant Phenotype of Cervical Cancer Cells by Up-regulating AGO2—AGO2 is a crucial component of the RISC, and the dysregulation of AGO2 plays an important role in tumor onset and progression (12, 25). Therefore, we investigated the role of miR-346 in the malignant phenotypes of cervical cancer cell lines and the participation of AGO2. miR-346 promoted the S/G2 transition in HeLa cells (Fig. 4A). AGO2 was necessary for miR-346 to promote the S/G2 transition (Fig. 4B). Metastasis is the most prominent cause of death from malignant tumors. Therefore, we aimed to determine whether miR-346 influences the migration and invasion of HeLa cells. As shown in Fig. 4C, the migration and invasion abilities of HeLa cells were increased significantly by miR-346 and decreased significantly by ASO-miR-346. When si-AGO2 was transfected into HeLa cells with miR-346, the promoting effects of miR-346 on AGO2 expression as well as on the migration and invasion abilities.
of HeLa cells were abrogated (Fig. 4D), suggesting that miR-346 enhanced cell migration and invasion in an AGO2-dependent manner.

So far, we demonstrated that miR-346 could enhance the expression of AGO2 and, therefore, the activities of other miRNAs. Furthermore, miR-346 increased the migration and invasion abilities of HeLa cells in an AGO2-dependent manner. Does miR-346 enhance the migration and invasion abilities of HeLa cells via increasing the activities of oncomiRNA, such as miR-10a? To address this, we transfected HeLa cells with miR-10a alone or in combination with miR-346- or AGO2-expressing plasmids or inhibitors. As reported previously (5), miR-10a promoted the migration and invasion of HeLa cells. Simultaneous transfection with miR-346 or AGO2 expression plasmids further increased, whereas ASO-miR-346 or si-AGO2 reduced, the promoting effects of miR-10a (Fig. 4, E and F). The expression of AGO2 as well as CHL1, the functional target of miR-10a in cervical cancer cells, was determined by Western blot analysis. The expression of AGO2 was increased by AGO2- or miR-346-expression plasmids and decreased by ASO-miR-346 or si-AGO2. miR-10a decreased the CHL1 level, and the CHL1 level in AGO2 or miR-346 expression plasmid co-transfected cells decreased further. ASO-miR-346 and si-AGO2 could reverse the inhibitory effects of miR-10a on CHL1 (Fig. 4, E and F). These results show that miR-346 could enhance the activities of miR-10a, therefore promoting the malignant phenotype of HeLa cells.

To further validate the effect of miR-346 on the malignant phenotype of cervical cancer cells, we conducted a xenograft tumor formation assay. As shown in Fig. 4G, miR-346 significantly promoted the growth of xenograft tumors in nude mice, suggesting that miR-346 could also promote the malignant phenotype of cervical cancer cells in vivo. In conclusion, our results demonstrate that miR-346 modulates the activity of other miRNAs by up-regulating the expression of AGO2 in a GRSF1-dependent manner in cervical cancer cell lines and that this process affects the malignant phenotype of cervical cancer cells both in vitro and in vivo.

FIGURE 3. miR-346 modulates the activity of other miRNAs. A, miR-346 was transfected simultaneously with si-CXCR4 into HeLa cells stably expressing a CXCR4 reporter plasmid, and the expression of EGFP was determined by microscopy. The image capture parameters were identical for all images shown. B, an EGFP reporter plasmid for the indicated miRNA for a given target gene was transfected simultaneously into HeLa cells with miR-346, and the expression of EGFP was measured by Western blot analysis. C, the influence of miR-346 on the expression of the indicated genes (targets of the miRNAs indicated in B) was assessed by Western blot analysis.
Discussion

In this study, we found that miR-346 directly increased the expression of AGO2 in a GRSF1-dependent manner in cervical cancer cell lines and that this event regulated the activity of other miRNAs and the malignant phenotype of cervical cancer cell lines. Generally, miRNAs negatively regulate the expression of their target genes at the post-transcriptional level. However, accumulating studies have indicated that miRNAs can enhance the expression of their target genes (26–29). We found that miR-346 increased the expression of AGO2 in cervical cancer HeLa cells. In fact, miR-346 has been reported to enhance the expression of its target gene RIP140 in mouse brain and p19 cells in an AGO2-independent manner (30). In previous studies, we found that miR-346 enhanced the expression of hTERT in an AGO2-independent but GRSF1-dependent manner. Our findings presented here further imply a distinctive characteris-
tic of miR-346 in target gene regulation. Except for AGO2, there are three or more AGO proteins that also participate in miRNA functions. Whether these AGO proteins are involved in the up-regulation of AGO2 by miR-346 is unclear now and needs further investigation. Furthermore, we found that the up-regulation of AGO2 by miR-346 also occurred in other types of cancer cells, including SW480 colorectal cancer cells and OVCAR3 ovarian cancer cells. The positive regulation of AGO2 by miR-346 existed in clinical cervical cancer tissues as well. All of these data suggest that the up-regulation of AGO2 by miR-346 might be widespread.

GRSF1 is a potent translation-promoting factor participating in enhancing the expression of various transcripts, such as GPX4 (23) and influenza virus proteins (31), through recruiting the transcripts to the ribosomes for translation. In our previous study, we found that GRSF1 recruits hTERT mRNA to the ribosomes in an miR-346-dependent manner, enhancing the expression of hTERT (39). In this study, we demonstrated that GRSF1 interacts with AGO2 mRNA in a miR-346-dependent manner similar to that of hTERT mRNA. GRSF1 might enhance the expression of AGO2 in the same manner. Although it has a prominent role in enhancing the translation of many transcripts, the details of the mechanism by which GRSF1 recruits the transcripts to the ribosome are still obscure, and more efforts need to be made in this field.

Previous studies have demonstrated that the stability of AGO2 is coupled post-transcriptionally to miRNA abundance (10, 19). Therefore, we examined the effect of miR-346 on AGO2 stability to ascertain whether miR-346 increased AGO2 stability or not. As demonstrated by the cycloheximide assay, miR-346 barely affected the protein levels by increasing the stability of AGO2. As demonstrated by the cycloheximide assay, miR-346 barely affected the stability of AGO2. Ectopic expression of other miRNAs failed to increase AGO2 protein levels, implying that the up-regulation of AGO2 did not result from increased AGO2 stability or miRNA abundance. Moreover, the results of the AGO2 3’UTR reporter assays demonstrated that miR-346 directly enhances the expression of AGO2 via its target sites.

Sequences within miRNAs may endow specific characteristics, such as the decoy activity provided by the C-rich element within miR-328 (32). We found that the middle sequence of miR-346 is GC-rich and forms a loop structure when miR-346 binds to the AGO2 3’UTR. Mutating the middle sequence of miR-346 abolished its ability to promote AGO2 expression. Several studies have found that RNA binding proteins participate in the activity of miRNAs (33, 34). In this study, knockdown of GRSF1, a RNA binding protein with GC binding activity, abrogated the ability of miR-346 to promote AGO2 expression. Wild-type miR-346 synergized with GRSF1 to enhance AGO2 expression, but this effect was abolished by mutating the miR-346 middle sequence. GRSF1 participates in many aspects of RNA biology, from mitochondrial RNA processing (35, 36) to enhancing specific mRNA translation (23). Our finding demonstrated that GRSF1 may be involved in the miRNA pathway.

AGO2 is a critical component of the RISC and the only AGO protein with endonuclease activity (37). The expression of AGO2 is dysregulated in many types of cancer (13, 15, 38), and the dysregulation of AGO2 results in altered miRNA activity. We found that miR-346 increases the activity of other miRNAs or siRNAs in HeLa cells. We anticipate that miR-346 might have a greater effect on the activity of miRNAs with higher abundance (so-called oncomiRs) than on those with lower abundance. Indeed, the activities of miR-21, miR-10a, and miR-150, which have been reported to be increased in cervical cancer (5, 7), were up-regulated significantly by miR-346, whereas the activity of miR-372, which is down-regulated in cervical cancer, was barely affected by miR-346. Simultaneously increasing AGO2 protein levels and oncomiR abundance in cervical cancer cells would result in strong activities of oncomiRs. Increasing the activity of oncomiRs, such as miR-21 and miR-150, results in the down-regulation of their target genes with tumor suppressor activities, such as PTEN and p53. miR-346 significantly reduces PTEN and p53 protein levels. In this study, we also found that miR-346 increased the migration and invasion of HeLa cells in an AGO2-dependent manner. We concluded that miR-346 functions as an oncomiR that modulates the activities of other microRNAs, especially oncomiRs, by enhancing the expression of AGO2 in cervical cancer cell lines.

In conclusion, we demonstrated that miR-346 increases the expression of AGO2 in a GRSF1-dependent manner, thereby participating in the modulation of the activity of other miRNAs. This finding implies that miR-346 may be a potential therapeutically target for cervical cancer prophylaxis and treatment.

Author Contributions—G. F. G. and J. L. performed and analyzed the experiments. M. L. drafted the manuscript. H. T. conceived and supervised experiments, revised the manuscript, and approved the final version.

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