MicroRNA-372 Is Down-regulated and Targets Cyclin-dependent Kinase 2 (CDK2) and Cyclin A1 in Human Cervical Cancer, Which May Contribute to Tumorigenesis* §

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MicroRNAs are a class of noncoding RNAs that are ~22 nucleotides in length. MicroRNAs have been shown to play important roles in cell differentiation and in cancer. Recently, studies have shown that miR-372 is tumorigenic in human reproductive system cancers. However, we provide evidence that miR-372 acts as a tumor suppressor gene in cervical carcinoma. miR-372 was found down-regulated in cervical carcinoma tissues as compared with adjacent normal cervical tissues. Growth curve and FACS assays indicated that ectopic expression of miR-372 suppressed cell growth and induced arrest in the S/G2 phases of cell cycle in HeLa cells. We used bioinformatic predictions to determine that CDK2 and cyclin A1 were possible targets of miR-372 and confirmed this prediction using a fluorescent reporter assay. Taken together, these findings indicate that an anti-oncogenic role of miR-372 may be through control of cell growth and cell cycle progression by down-regulating the cell cycle genes CDK2 and cyclin A1.

MicroRNAs (miRNAs)3 are endogenous, noncoding, small RNAs ~22 nucleotides in length that are involved in sequence-specific negative regulation of the stability and translation of target mRNAs (1, 2). Emerging evidence suggests that miRNAs exhibit tissue- and cell-specific expression (3). They have attracted significant attention given their important roles in the cell cycle, apoptosis, and carcinogenesis (2).

Deregulation of the cell cycle machinery is considered to be a factor in tumor generation. Studies on cell cycle regulation have revealed that multiple cell cycle regulatory proteins play key roles in tumorigenesis. Recent studies have suggested that several miRNA-targeted genes are involved in cell cycle progression and cellular growth (4). These miRNAs regulate classic cell cycle control pathways by directly targeting cell cycle-related genes, such as transcription factors, cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (5). Gillies and Lorimer (6) identified the cell cycle regulator, p27kip1, as a direct target for miR-221/222 and suggested a role for these miRNAs in promoting the aggressive growth of human glioblastoma through deregulation of the cell cycle. Parallel studies using distinct experimental approaches confirmed this regulatory relationship in cell lines from prostate cancer, thyroid carcinoma, gastric cancer, and breast cancer, as well as in primary human glioblastoma samples from patients (7–10).

Previously, studies on the miR-371–373 cluster were limited to human embryonic stem cells (11, 12). Recently, studies have revealed that miR-372 may regulate cell cycle, apoptosis, invasion, and proliferation in many types of cancer. For example, aberrant expression of miR-372 has been found in hepatocellular carcinoma (13). Voorhoeve et al. (14) revealed that miR-372 and miR-373 are potentially novel tumorigenic miRNAs that are involved in the development of human testicular germ cell tumors by numbing the p53 pathway. Cho et al. (15) showed that miR-372 plays an oncogenic role through down-regulation of the tumor suppressor gene LAT2, which accelerated growth and survival of gastric cancer cells. However, in this work, we found that miR-372 has the opposite role in HeLa cells. We explored the effects of miR-372 on cell growth and cell cycle progression. Furthermore, CDK2 and cyclin A1 were identified as direct targets of miR-372. Therefore, our data indicate that miR-372 directly targets CDK2 and cyclin A1 and negatively regulates cell cycle progression and proliferation in HeLa cells.

EXPERIMENTAL PROCEDURES

Cell Culture—The human cervical cancer-derived cell lines, HeLa and C33A, were obtained from the American Type Culture Collection and cultured in RPMI 1640 (Invitrogen). The culture medium was supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 IU/ml penicillin and maintained at 37 °C in a humidified atmosphere with 5% CO2.

Transfection and Stable Cell Line Selection—The HeLa cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Transfection efficiency was monitored by fluorescence microscopy 48 h after transfection with pcDNA3/EGFP and Cy5-oligonucleotides. Stably transfected cells were selected by adding 0.4 mg/ml G418 to the culture medium. Individual colonies were selected...
TABLE 1
Oligonucleotides used in this work

| Name                     | Sequence (5' → 3')* |
|--------------------------|---------------------|
| pri-miR-372 sense        | GTCAGCCAGATCTCTCTCCCTAGCCAGAACATAC |
| pri-miR-372 antisense    | GTCGACATCTCTCTCCCTAGCCAGAACATAC |
| Cyclin A1 forward        | GGTCAGCCAGATCTCTCTCCCTAGCCAGAACATAC |
| Cyclin A1 reverse        | GGTCAGCCAGATCTCTCTCCCTAGCCAGAACATAC |
| CDK2 forward             | GGTCAGCCAGATCTCTCTCCCTAGCCAGAACATAC |
| CDK2 reverse             | GGTCAGCCAGATCTCTCTCCCTAGCCAGAACATAC |
| CyclinA1-mut forward     | GGTCAGCCAGATCTCTCTCCCTAGCCAGAACATAC |
| CyclinA1-mut reverse     | GGTCAGCCAGATCTCTCTCCCTAGCCAGAACATAC |
| CDK2-mut forward         | GGTCAGCCAGATCTCTCTCCCTAGCCAGAACATAC |
| CDK2-mut reverse         | GGTCAGCCAGATCTCTCTCCCTAGCCAGAACATAC |
| U6-RT                    | GGTCAGCCAGATCTCTCTCCCTAGCCAGAACATAC |
| miR-372-RT               | GGTCAGCCAGATCTCTCTCCCTAGCCAGAACATAC |
| U6 forward               | GGTCAGCCAGATCTCTCTCCCTAGCCAGAACATAC |
| miR-372 forward          | GGTCAGCCAGATCTCTCTCCCTAGCCAGAACATAC |
| Reverse (universal)      | GGTCAGCCAGATCTCTCTCCCTAGCCAGAACATAC |
| Actin forward            | GGTCAGCCAGATCTCTCTCCCTAGCCAGAACATAC |
| Actin reverse            | GGTCAGCCAGATCTCTCTCCCTAGCCAGAACATAC |

* Restriction sites are underlined.

2 weeks after transfection, and miRNA levels were quantified using real-time PCR.

Growth Curves—Treated cells were plated in 24-well plates (4,000 cells/well) in triplicate. At the different times, the cells were detached by trypsinization and counted using a hemocytometer.

FACS Assays—Transfected HeLa cells (or stable cell lines) were seeded in 6-well plates for 24 h in complete medium. Subsequently, the cells were deprived of serum for harvesting and then returned to complete medium for an additional 24 h. All cells were collected by centrifugation, fixed in 95% ethanol, incubated at −20 °C overnight, and washed with phosphate-buffered saline (PBS). Then cells were resuspended in 1 ml of FACS solution (PBS, 0.1% Triton X-100, 60 μg/ml propidium iodide, 0.1 mg/ml DNase-free RNase, and 0.1% trisodium citrate). After a final incubation on ice for 30 min, cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). A total of 10,000 events were counted for each sample.

MTT Assay—The HeLa cells were seeded in 96-well plates at 4,000 cells/well. 48 h after transient transfection, the cells were incubated with 15 μl of MTT (at a final concentration of 0.5 mg/ml) at 37 °C for another 4 h. Then the medium was removed, and the precipitated formazan was dissolved in 100 μl of dimethyl sulfoxide (DMSO). After shaking for 15 min, the absorbance at 570 nm (A<sub>570</sub>) was detected using a μQuant universal microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT).

Plasmid Construction—To construct a plasmid expressing miR-372, a 341-bp DNA fragment carrying pri-miR-372 was inserted between the BamHI and EcoRI sites in pcDNA3. The EGFP reporter vector pcDNA3/EGFP was constructed as described previously (16). The 3'-UTRs, including the target sites, of human CDK2 and cyclin A1 were amplified by RT-PCR and cloned downstream of EGFP between the EcoRI and BamHI (or XhoI) sites. Similarly, 3’-UTR mutants, which contained mutated miR-372 binding sites, were cloned to the pcDNA3/EGFP between the same sites. The primer sequences used for RT-PCR amplification are shown in Table 1. Sequences that were inserted into the plasmid were verified by DNA sequencing.

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miR-372, a 341-bp DNA fragment carrying pri-miR-372 was inserted to the plasmid pCMV-EGFP. EGFP Reporter Assay—HeLa cells were transfected with pcDNA3/pri-miR-372, control vector, pcDNA3 antisense oligonucleotide (ASO), control ASO, or the reporter plasmids described above in 48-well plates. An expression vector expressing red fluorescent protein (RFP), pDsRedz-N1 (Clontech), was used for normalization. The cells were lysed with radio immunoprecipitation assay lysis buffer (1 mM MgCl<sub>2</sub>, 10 mM Tris–HCl, pH 7.4, 1% Triton X-100, 0.1% SDS, 1% Nonidet P-40), and 48 h later, protein was harvested. EGFP and RFP expression was detected using a fluorescence spectrophotometer F-4500 (Hitachi). All assays were performed more than three times.

Western Blot Analysis—The cells were transfected and then lysed 48 h later with radio immunoprecipitation assay lysis buffer, after which the proteins were harvested. An equal amount of protein was loaded onto a 12% SDS denaturing polyacrylamide gel, separated by electrophoresis, transferred to a nitrocellulose membrane, and incubated with the specific primary antibody overnight at 4 °C. The membranes were then washed and subsequently incubated with the secondary antibody conjugated to horseradish peroxidase (HRP). Protein was visualized using enhanced chemiluminescence. Anti-cyclin A1, anti-CDK2, and anti-GAPDH antibodies were purchased from Saierbio, Inc. The resulting Western blot bands were quantified using the LabWorks<sup>TM</sup> 4.0 software.

RNA Extraction and qRT-PCR Analyses—Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. For miRNA detection, 2 μg of small RNA was reverse transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase (Promega). Quantitative real-time PCR (qRT-PCR) analysis for miR-372 was performed in triplicate with the SYBR Premix Ex Taq<sup>TM</sup> kit (TaKaRa) according to the manufacturer’s instructions. U6 RNA was used to normalize expression.

To detect the target genes, 5 μg of large RNA was reverse transcribed to cDNA using oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase (Promega). qRT-PCR was used to determine the expression levels of cyclin A1 and CDK2 using the primers described in Table 1. β-Actin levels were used to normalize expression.
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**Statistical Analysis**—The data are expressed as the mean ± S.D. from at least three separate experiments. The differences between groups were analyzed using the double-sided Student’s *t* test, and statistical significance was determined by a *p* value of less than 0.05.

**RESULTS**

**miR-372 Is Down-regulated in Human Cervical Cancer**—Recent evidence suggests that miR-372 is tumorigenic; however, we found that it may play a different role in cervical cancer. We measured the expression levels of miR-372 in 18 pairs of human cervical cancer tissues and adjacent normal tissues using real-time PCR. We found that miR-372 expression levels were generally lower in cervical cancer tissues than in the matched normal cervical tissues, with the exception of one sample (Fig. 1), suggesting that miR-372 expression is down-regulated in cervical cancer.

**miR-372 Suppresses Cell Growth Activity in HeLa Cells**—Gain-of-function experiments are widely used for functional studies of miRNAs. To investigate the role of miR-372 in tumor cell growth, a miR-372 overexpression vector, pcDNA/pri-miR-372, was constructed. miR-372 overexpression from the vector was confirmed by qRT-PCR, which indicated a 4.3-fold increase of miR-372 expression in pri-miR-372-transfected HeLa cells (Fig. 2A, panel 1). To characterize the effects of miR-372 on cell growth, HeLa cells were transfected with pri-miR-372, and growth was monitored using a cell growth curve, which indicated that overexpression of miR-372 inhibited cell growth (Fig. 2B, panel 1). HeLa cell lines stably expressing miR-372 were then selected, and miRNA activity was confirmed using qRT-PCR (Fig. 2A, panel 2). As seen in the transiently transfected cells, the growth of HeLa cells stably expressing miR-372 was about 50% slower than control cells (Fig. 2B, panel 2).

To further explore the role of miR-372, experiments with loss-of-function analyses were performed. A miR-372 ASO was used to block miR-372 and resulted in an ~70% reduction of miR-372 in HeLa cells (Fig. 2A, panel 3). To determine whether inhibiting miR-372 could reverse the inhibition of cellular proliferation observed by overexpression, we transfected the HeLa cells stably expressing miR-372 with miR-372 ASO or a negative control ASO. An increase in proliferation was observed in cells transfected with miR-372 ASO as compared with cells transfected with the control (Fig. 2B, panel 3). These studies indicate that HeLa cell growth can be negatively modulated by miR-372.

**miR-372 Influences the Cell Cycle Distribution of HeLa Cells in Vitro**—To follow up the finding that miR-372 overexpression decreased the growth of HeLa cells, FACS was used to analyze the cell cycle of pri-miR-372-treated HeLa cells. Overexpression of miR-372 resulted in an increase of cells in S phase (from 25.3 to 38.4%), a decrease of cells in G2/M phase (from 21.8 to 7.6%), and no change in the percentage of cells in G1 phase, as

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**FIGURE 1. Quantitative analysis of miR-372 expression in human cervical cancer.** miR-372 expression levels in 18 pairs of cervical cancer tissues (Ca) and matched adjacent normal tissues (N) were measured by quantitative RT-PCR. U6 snRNA was used as an endogenous normalizer, and the relative expression levels of miR-372 from the eight pairs of gastric tissues as well as the combined result are shown (*, *p* < 0.05).

**FIGURE 2. miR-372 regulates HeLa cell growth.** A, pcDNA3/pri-miR-372 (miR-372), which expresses mature miR-372, was transfected into HeLa cells. Antisense oligonucleotide (372-ASO) was used to block miR-372 in HeLa cells. Quantitative real-time PCR was performed to determine miR-372 expression in transiently transfected cells (panels 1 and 3) and in stable cell lines expressing miR-372 or a control vector (panel 2) (*, *p* < 0.05). NC, negative control. B, HeLa cells were transfected with plasmids expressing miR-372 or a control, and cell growth activity was detected using a growth curve assay (panel 1). The same result was shown as for the stable cell lines (panel 2). HeLa cells stably expressing miR-372 were transfected with anti-miR-372 or NC-ASO (panel 3). *, *p* < 0.05.
compared with the negative control (Fig. 3A). Additionally, in HeLa cell lines stably overexpressing miR-372, a larger percentage of cells was found in S phase as compared with cells without miR-372 overexpression (Fig. 3B).

Therefore, miR-372 overexpression increases accumulation of cells in S phase and decreases entrance into G2/M in HeLa cells. We also used ASO to block miR-372 in HeLa-372 cells. As shown in Fig. 3C, blocking miR-372 rescued the cell cycle arrest, with about 10% more cells progressing from S phase into G2. This observation is consistent with the result of our gain-of-function analysis.

All above results indicated that miR-372 caused cell cycle arrest at the S/G2 phase in HeLa cells but is not known whether miR-372 interferes with DNA synthesis in the S phase. The EdU incorporation assay showed that although miR-372 suppresses cell proliferation, the percentage of EdU-positive cells was
increased (supplemental Fig. S1). It demonstrates miR-372 has an effect on the S/G2 checkpoint.

To further validate miR-372-mediated cell cycle arrest in cervical cancer cells, another cell line, C33A, was used to study the effect of miR-372 on cell proliferation and cell cycle regulation. These results were consistent with previous results in HeLa cells (supplemental Fig. S2), indicating that miR-372 may negatively affect growth at the S-to-G2 transition in cervical cancer cells.

miR-372 Regulates CDK2 and Cyclin A1 at Both the mRNA and the Protein Levels—As the function of miR-372 is determined, it is important to understand which targets are directly responsible for the observed downstream phenotypes. Four algorithm programs (TargetScan, PicTar, miRCosm, and mirnaviewer) were used to predict candidate target genes. Matchminer was used to identify the genes predicted by all four different algorithms. Given the cell cycle phenotype, gene ontology (GO) analysis (AmiGO) was used to search human genes associated with the cell cycle. We then selected CDK2 and cyclin A1, whose 3′-UTR contained the potential binding sites of miR-372, to be the candidate target genes (see Fig. 6A). We used bioinformatic predictions to determine CDK2 and cyclin A1 expression levels in 18 pairs of cervical cancers and the adjacent normal tissues by qRT-PCR. We found that CDK2 and cyclin A1 generally had higher expression levels in the cervical cancer tissue as compared with normal tissue (Fig. 4A). High expression of CDK2 and cyclin A1 inversely correlated with lower expression of miR-372 in human cervical cancer (Fig. 4B).

To determine whether miR-372 has a direct effect on CDK2 and cyclin A1 expression, a miR-372 expression vector or ASO was transfected into HeLa cells to alter the level of miR-372. When miR-372 was overexpressed, mRNA and protein levels of CDK2 and cyclin A1 were markedly reduced (Fig. 5, A and B). In HeLa cell lines stably overexpressing miR-372, CDK2 and cyclin A1 protein levels were also found down-regulated (Fig. 5C). Conversely, inhibiting miR-372 expression resulted in up-regulation of CDK2 and cyclin A1 protein levels (Fig. 5D). These observations indicate that CDK2 and cyclin A1 are negatively regulated by miR-372 at the post-transcriptional level.

CDK2 and Cyclin A1 Are Direct Targets of miR-372—To determine whether the interaction between miR-372 and the mRNA of CDK2 and cyclin A1 is direct, we used an EGFP reporter system in which we cloned the CDK2 or cyclin A1 3′-UTR fragments containing presumed target sites to downstream of an EGFP. Subsequently, we constructed an additional EGFP reporter vector containing the 3′-UTR of either CDK2 or cyclin A1 with the potential mutant target sites of miR-372 seed sequence (Fig. 6A). We used bioinformatic predictions to determine CDK2 and cyclin A1 3′-UTR fragments containing presumed target sites to downstream of an EGFP. Subsequently, we constructed an additional EGFP reporter vector containing the 3′-UTR of either CDK2 or cyclin A1 with the potential mutant target sites of miR-372 seed sequence (Fig. 6A). Co-transfection was performed with an RFP reporter and with either a miR-372 vector or ASO in HeLa cells. As shown in Fig. 6, B and C, the intensity of EGFP fluorescence in HeLa cells transfected with pcDNA3/EGFP-CDK2 3′-UTR or pcDNA3/EGFP-cyclin A1 3′-UTR and pcDNA3/pri-miR-
372 was decreased as compared with the control group by 20–30%. Furthermore, inhibition of endogenous miR-372 by ASO increased EGFP expression from the reporter construct containing the 3’-UTR of CDK2 or cyclin A1 by 20%. Importantly, expression of the reporter constructs mutated at the miR-372 target site of CDK2 or cyclin A1 3’-UTR was not

FIGURE 6. Validation of CDK2 and cyclin A1 as the direct targets of miR-372. A, a schematic of the Targetscan predicted seed region in the 3’-UTRs of CDK2 and cyclin A1 is shown above, as well as mutations (mut) used in this study. HeLa cells were co-transfected with miR-372, miR-372 ASO, or controls, together with the indicated fluorescent reporter vector containing the 3’-UTR (or mutant) of CDK2 or cyclin A1, and an RFP expression vector was used as the loading control. Fluorescent activity was measured 48 h after transfection. CDK2 and cyclin A1 reporter results are shown in B and C, respectively (*, p < 0.05). NC, negative control.
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FIGURE 7. Cell cycle repression by miR-372 can be reversed by CDK2 and cyclin A1 overexpression. A, HeLa cells were transfected with a control vector or miR-372 overexpression vector, together with pcDNA3/CDK2 or pcDNA3/cyclin A1 respectively. Cell growth was monitored using a MTT assay. Each data point represents the mean ± S.D. from three experiments (*, p < 0.05). B, FACS analysis was performed to determine the cell cycle progression of HeLa cells transiently transfected with the same vectors as in panel A of this figure.

affected by either miR-372 overexpression or inhibition, highlighting the importance of the miR-372 binding site for regulation by miR-372. These results show that miR-372 regulates gene expression through direct binding of the 3’-UTR of CDK2 and cyclin A1 in HeLa cells.

Knocking Down CDK2 or Cyclin A1 Inhibits Cell Cycle—Next, we constructed CDK2 and cyclin A1 siRNA expression vector to explore the role of CDK2 and cyclin A1 in cell cycle regulation (see supplemental Experimental Procedures). The results indicated that the CDK2 and cyclin A1 siRNA could significantly reduce endogenous protein levels (supplemental Fig. S3A). CDK2 and cyclin A1 knockdown resulted in an increase of cells in S phase and a decrease of cells in G2/M phase (supplemental Fig. S3B), which is consistent with the results of miR-372 overexpression.

CDK2 and Cyclin A1 Overexpression Counteracts miR-372—To further investigate the relationship between CDK2 and cyclin A1 with miR-372-mediated cell cycle inhibition, we overexpressed miR-372 with CDK2 or cyclin A1 together in HeLa cells. Cell growth was measured using a MTT assay, and cell cycle progression was analyzed using FACS. We found that overexpression of CDK2 or cyclin A1 with miR-372 reversed the inhibition of cell growth and the arrested cell cycle at S/G2 phase caused by miR-372 (Fig. 7, A and B). Therefore, we conclude that overexpression of CDK2 and cyclin A1 counteracts the repressive effects of miR-372 on cell growth and cell cycle progression.

DISCUSSION

Over the past few years, hundreds of miRNAs have been described that play important roles in regulating gene expression by mRNA cleavage or translational repression in a variety of model systems (2, 17, 18). Documented evidence has demonstrated that miRNAs may function as a novel class of both tumor-promoting and tumor-suppressing genes (19). For example, miR-17–92 is significantly increased in both small cell lung cancer and human B-cell lymphomas and plays a key role in tumorigenesis (20, 21). Let-7 could directly regulate multiple cell cycle-associated tumorigenesis proteins (CDK6, CDC25a, CCND2) and thus potentially act as a tumor suppressor gene (22, 23). Although it has been reported that miR-372 and miR-373 are overexpressed in some cancers (14, 24, 25) and may play an oncogenic role by targeting the tumor suppressor LATS2 (14, 15), our studies showed that miR-372 was down-regulated in human cervical cancer tissues. Overexpression of miR-372 in human cervical cancer cell lines suppresses cell growth and arrests the cell cycle at S/G2 phase. miRNA and their specific targets are dependent on the specific cellular environment (26). For example, miRNA-155 is significantly up-regulated in diffuse large B cell lymphoma (27) and is down-regulated in human breast cancer (27, 28). Depending on which factors are driving tumorigenesis in the specific cellular milieu, the same miRNA may act as a tumor suppressor in some cancers and as a tumorigenic agent in others. Therefore, we speculate that cell-specific environments may account for the differences observed between the functions of miR-372 in cervical cancer as compared with other cancers.

Cell cycle progression is highly complex and is controlled by many factors. Deregulation of the cell cycle leads to abnormal cell growth and tumorigenesis (30–32). Cyclins are regarded as the major regulators of the cell cycle (33–35). All kinds of cyclin expression present periodic variations in cell cycle (36). Cyclin A1 is an alternative, A-type cyclin that is present at very low levels in cells during G0. It increases throughout the progression of the cell cycle and reaches the highest levels in S and G2/M (37). In addition, CDKs are another class of cell cycle regulators that act as the catalytic subunit of the active cyclin-CDK complex, which is key to coordination of the cell cycle (38, 39). CDK2 is thought to be essential in the mammalian cell cycle and functions by driving cells through S phase in conjunction with A-type cyclins (40). CDK2 is also essential for spermatogenesis and contributes to leukemogenesis (41, 42). The cyclin A1-CDK2 complex is crucial for successful DNA replication and normal cell cycle progression.

Our phenotypic analyses revealed that miR-372 inhibits HeLa cell growth and induces cell cycle arrest, which increases the percentage of cells in S phase and blocks entry into G2. We then identified cyclin A1 and CDK2 as target genes of miR-372.
Our results can be summarized by six major findings. (a) We combined bioinformatic prediction software including TargetScan, PicTar, miRCoSmo, and mirnaviewer, with human gene associations of the cell cycle and compiled the resulting data using the AmiGO website. The cell cycle regulators, cyclin A1 and CDK2, were predicted to be candidate targets for further study. (b) We found that miR-372 is down-regulated, whereas CDK2 and cyclin A1 are up-regulated in cervical cancer tissue as compared with adjacent normal tissue. (c) miR-372 negatively regulates cyclin A1 and CDK2 at both mRNA and protein levels. (d) Expression of the EGF reporter containing the 3'-UTR of cyclin A1 and CDK2 was inhibited when miR-372 was overexpressed. (e) It was also demonstrated that overexpression of CDK2 and cyclin A1 accelerates DNA replication in S-phase, entry into G2, and vice versa (43, 44). (f) Expression of CDK2 and cyclin A1 is sufficient to rescue miR-372-mediated cell cycle repression and to promote cell growth. Accordingly, we conclude that miR-372 targets cyclin A1 and CDK2.

LATS2 is a tumor suppressor gene that induces G2/M arrest through the inhibition of Cdc2 kinase activity in HeLa cells (29). Recently, it was suggested that LATS2 is also a functional target of miR-372 in gastric cancer cells and testicular germ cell tumors (14, 15). Because miR-372 is down-regulated in human cervical cancer cells, the previously identified tumor suppressor gene LATS2 could not help us understand the effect of miR-372 on cell cycle regulation in cervical cancer cells, but characterizing the detailed mechanism remains to be understood.

Taken together, our results demonstrate that miR-372 suppresses the growth of HeLa cells by directly targeting cyclin A1 and CDK2, which represses cell cycle progression. Additionally, miR-372 functions as a tumor suppressor in cervical cancer.

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