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Over-expression of miR-1271 inhibits endometrial cancer cells proliferation and induces cell apoptosis by targeting CDK1

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Abstract. – OBJECTIVE: Endometrial carcinoma (EC) is one of the most common female malignancies worldwide. Growing evidence showed that microRNAs (miRNAs) are involved in the EC progression. The present study aimed to investigate the role of miR-1271 in the development and progression of EC.

PATIENTS AND METHODS: The EC tissues and adjacent normal tissues were obtained from 42 EC patients. The expression of miR-1271 in EC tissues and cells was examined using Real-time RT-PCR. Western blot was used to quantify the level of cyclin-dependent kinase 1 (CDK1) in EC tissues and cells lines. Cell proliferation, colony formation and flow cytometry were done to examine effects on cancer cell proliferation and apoptosis in vitro. Bioinformatics software was used to predict some potential target genes of miR-1271. Besides, the dual luciferase reporter gene assay was used to determine the direct targeting relationship between miR-1271 and CDK1.

RESULTS: MiR-1271 was significantly downregulated in human EC tissues and cells while CDK1 was strongly upregulated. Bioinformatics analysis indicated that CDK1 was a potential target of miR-1271. Then, luciferase reporter assay confirmed that CDK1 was a direct target gene of miR-1271. In vitro studies showed that miR-1271 overexpression reduced EC cell proliferation and promoted apoptosis, while restoration of CDK1 attenuated these effects of miR-1271 on EC cells. Moreover, we found that knockdown of miR-1271 significantly promoted EC cell growth and suppressed apoptosis.

CONCLUSIONS: Our findings showed that miR-1271 served as a tumor suppressor in EC via targeting CDK1, suggesting miR-1271 as a new potential target for therapy strategy in EC.

Key Words:
miR-1271, Endometrial carcinoma, Proliferation, Apoptosis, CDK1.

Introduction

Endometrial carcinoma (EC) is one of the three most common types of gynecologic cancer and its global incidence has increased in recent years[1]. In 2016, there were 60,050 new expected cases of EC with an estimated 10,470 deaths in USA[2]. Radical surgery might be the only hope for curing EC in the stage of precursor lesions and the patients are diagnosed at an early stage with a favorable prognosis[3]. However, the expected survival of patients with recurrent disease is only 12-15 months at the time of diagnosis[4,5]. Therefore, elucidating the potential mechanism that mediate the initiation and progression of EC is urgent and of great interest. MicroRNAs (miRNAs) are a class of small (19-24 nucleotides), non-coding RNAs that are involved in post-transcriptional gene regulation and/or degradation[6,7]. It has been demonstrated that miRNAs regulate the expression of specific target genes by binding to the 3'-untranslated regions (3'-UTRs) of messenger RNA[8]. Growing evidence shows that miRNAs play important role in various biological processes, such as tumor angiogenesis, proliferation, cell differentiation, apoptosis, metastasis[9,10]. Indeed, miRNAs serve as oncogenes or tumor suppressors depending on the types of tumors[11]. These information highlight the critical effect of miRNAs in tumor progression and provide new insight into the molecular mechanisms underlying carcinogenesis. MiR-1271 is a member that was newly discovered in the miRNA family. Previous studies indicated that miR-1271 functions as tumor suppressors in various tumors, including gastric cancer[12], oral squamous cell carcinoma[13], and pancreatic cancer[14]. However, to our best knowledge, the

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effect of miR-1271 in EC remains unknown. In the present study, we aimed to explore the role of miR-1271 in progression of EC and try to confirm a targeting gene of this miRNA.

Patients and Methods

Tissue Samples

This study was approved by the Research Ethics Committee of Maternal and Child Care Service Centre of Kuiwen District. Written informed consents were obtained from all the individuals involved in the trial. Human endometrial cancer tissue and matched adjacent normal tissues from 42 patients with EC were collected in 20014 and 2015. None of the patients received radiotherapy or chemotherapy before surgery. All samples were evaluated by two pathologists. The collected sample was snap frozen in liquid (-70°C) until use.

Cell Culture and Transfection

Human EC cells lines, including ECC-1, RL95-2 and AN3 CA, and endometrial fibroblast cell T-HESC were purchased from American Type Culture Collection. The above cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA). The medium was supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO^2. MiR-1271 mimics (miR-1271), miR-1271 inhibitors and their negative controls were purchased from GenePharma Corporation. Overexpression CDK1 plasmid pCDNA3.1-CDK1 was granted from Doctor Ming Wang. The empty pCDNA3.1 Vector without any insert was used as a control of plasmid transfection. Transfection was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) method.

miRNA Real-time RT-PCR

Total RNA was extracted from surgical specimens or cell lines using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For analysis of miR-1271 expression, qRT-PCR was performed using Hairpin-it miRNAs qPCR Quantitation Kit (catalog number QPM-010, GenePharma, Shanghai, China). All samples were carried out in triplicate. U6 was used as an endogenous control. The primers for PCR were designed and purchased from Invitrogen (Carlsbad, CA, USA). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relevant expression values of miR-1271 in EC and corresponding normal tissues.

Protein Extraction and Western Blot

Total proteins were extracted with a Total Extraction Kit (Solarbio, Beijing, China). Cytoplasmic and nuclear proteins were extracted with a nuclear and cytoplasmic protein extraction kit (Beyotime, Shanghai, China). Western blotting was performed to determine CDK1 protein expression. The standard procedure performed as described previously[15].

Luciferase Assays

To confirm that miR-1271 can bind to the predicted site CDK1, we conducted a luciferase reporter assay in the EC cell line. Reporter constructs containing pGL3-wt-CDK1 and pGL3-mut-CDK1 (with a mutated target seed sequence) were obtained from Bio-Asia (Haiding, Beijing, China). Then, the recombinant CDK1 3'UTR-wt plasmid or CDK1 3'UTR-mut plasmid, and miR-448 mimics were co-transfected to EC cells with Lipofectamine 3000 (Invitrogen, San Diego, CA, USA). Following transfection at 37°C for 48 h, the luciferase activities were determined on an LD400 luminometer (Beckman Coulter, Inc., Brea, CA, USA).

Methylthiazolyl Tetrazolium (MTT) Assay for Cell Proliferation

Twenty-hours after transfection, a total of 2×10^3 cells were seeded into 96-well plates and cultured in medium with 10% FBS. MTT assay was used to detect cell viability at 0, 12, 24, and 48 h after seeding. Optical density (OD) was detected at a wavelength of 490 nm using an enzyme-labeled analyzer. Each experiment was repeated three times.

Clonogenic Assay

At 12 h post-transfection, $3\text{-}5\times10^3$ EC cells were seeded into 60-mm Petri dishes in triplicate and maintained in RPMI 1640 (Gibco, Rockville, MD, USA) with 10% fetal bovine serum (FBS). When there was visible colony by naked eye, the number of colonies containing more than 50 cells was counted manually. Colonies were counted and calculated in relation to the values obtained from the mock and scramble-treated controls.

Apoptosis Assay

Apoptosis was assessed using Annexin V-FITC/PI double staining kit (Beyotime, Pudong, Shanghai, China). Cells were harvested and stained with Annexin V-FITC and PI according to the manufacturer's instructions. Cells were then examined by flow cytometry (FACScan; BD Biosciences, Jiangsu, Zhejiang, China) on in instrument equipped with CellQuest software (BD Biosciences, Jiangsu, Zhejiang, China).

Statistical Analysis

Statistical analysis was performed using SPSS software (version 19.0, IBM, Chicago, IL, USA). Data were collected from at least three separate experiments and were expressed as the mean ± standard error. Paired *t*-test was used to analyze comparisons between the groups and paired data. Statistical significance was considered when $p < 0.05$.

Results

miR-1271 Expression is Reduced While CDK1 Expression is Increased in EC Tissues and Cell Lines

To investigate the possible role of miR-1271 in EC development, we first examined the expression of miR-1271 in specimens and cells in EC by RT-PCR. As shown in Figure 1A, the results showed that expression of miR-1271 was decreased in EC samples ($p < 0.01$), compared to normal matched tissues. Similarly, Figure 1B revealed that miR-1271 was also significantly decreased in three EC cell lines compared with that of T-HESC (all $p < 0.01$). On the other hand, we further detected the expression levels of CDK1 in EC tissues and cell lines. The results of Western blot indicated that CDK1 was significantly upregulated in EC tissues or cell lines compared to the

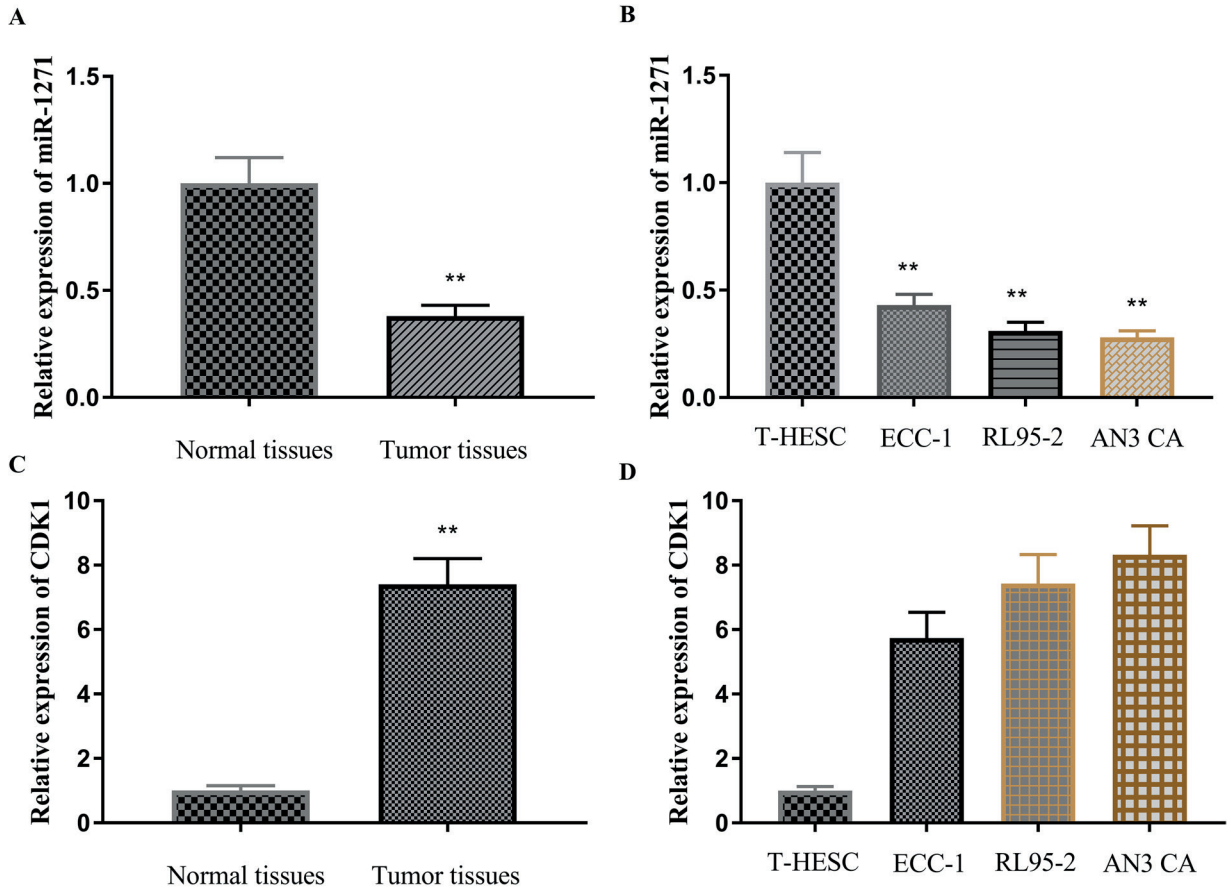

Figure 1. Analysis of miR-1271 and CDK1 expression in human EC tissues and cell lines. ***(A)*** qPCR was performed to determine the relative expression of miR-1271 in EC tissues and matched noncancerous tissues. ***(B)*** qPCR was performed to determine the relative expression of miR-1271 in EC cell lines (ECC-1, RL95-2, and AN3 CA) and normal T-HESC cells. ***(C)*** Western blot was performed to determine the relative expression of CDK1 in EC tissues and matched noncancerous tissues. ***(D)*** Western blot was performed to determine the relative expression of CDK1 in EC cell lines (ECC-1, RL95-2, and AN3 CA) and normal T-HESC cells. Data were expressed as mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$.

adjacent normal tissues or T-HESC cells. These results suggested that miR-1271 and CDK1 may play an important role in EC.

CDK1 was a Direct Target of miR-1271

To understand the regulating mechanisms of miR-1271, we examined its potential targets by Target Scan. As shown in Figure 2A, the data predicted CDK1 as a putative target for miR-1271. To determine whether CDK1 was a target of miR-1271, we cloned the predicted CDK1 3′UTR binding site, as well as its mutant form to firefly luciferase reporter gene, respectively. Co-transfection with miR-1271 inhibited the luciferase activity of the reporter containing the mutant 3'UTR but not the wild type in EC cell (Figure 2B). In addition, endogenous CDK1 expression in RL95-2 cells transfected with miR-1271 mimic was examined. As shown in Figure 2C, the results showed that enhanced expression of miR-1271 by miR-1271 mimics in the EC cells leads to decreased CDK1 levels. Taken together, our findings suggested that miR-1271 directly targets CDK1 expression by binding to the 3′UTR region of CDK1.

Over-expression of miR-1271 Suppressed EC Progression in vitro

To analyze the effect of miR-1271 on proliferation of EC cells, we transfected miR-1271 mimic and negative control (NC) in RL95-2 cells. As shown in Figure 3A, miR-1271 mimic was able significantly increased the expression of miR-1271. Next, we performed MTT and colony formation assays. The results indicated that a significant reduction in cell viability was observed in the RL95-2 cells transfected with miR-1271 compared with those transfected with the miR-NC ($p < 0.01$, Figure 3B). Also, miR-1271 over-expression decreased the clonogenicity of RL95-2 cells compared with NC cells ($p = 0.01$, Figure 3B). Moreover, flow cytometry was performed to assess whether this effect was mediated through the induction of apoptosis. As shown in Figure 3C, our results showed that up-reguation of miR-1271 increased RL95-2 cell apoptosis. More importantly, we found that restoration of CDK1 partially attenuated the effects of miR-1271 on RL95-2 cells (Figure 3B, 3C and 3D). These together, our results suggested that miR-1271 served as a tumor suppressor via targeting CDK1 in EC.

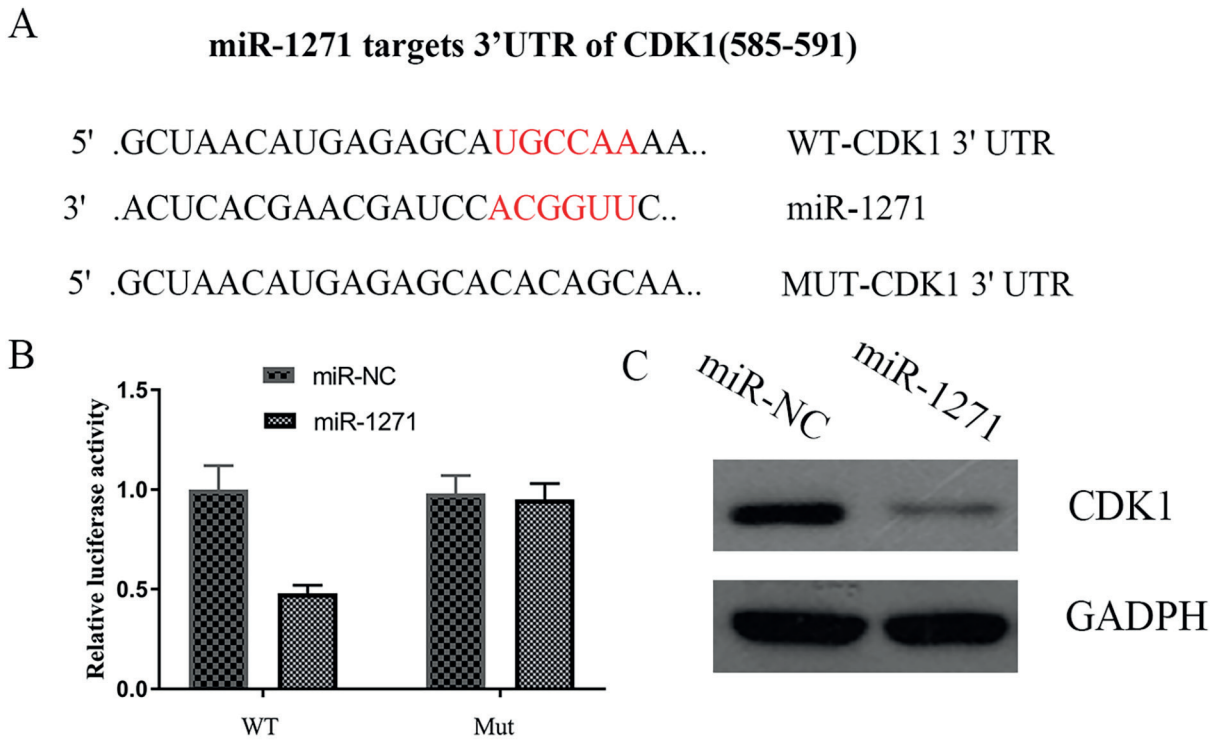

Figure 2. miR-1271 directly targets the CDK1 3'UTR. ***(A)*** the predictive miR-1271 binding site of AKT1 3'UTR and the corresponding mutant binding site were shown. ***(B)*** miR-1271 significantly suppressed the luciferase activity that carried wild-type CDK1 but not the mutant CDK1. ***(C)*** levels of CDK1 in RL95-2 cells transfected with miR-1271 mimics or negative control. *$p < 0.05$, **$p < 0.01$.

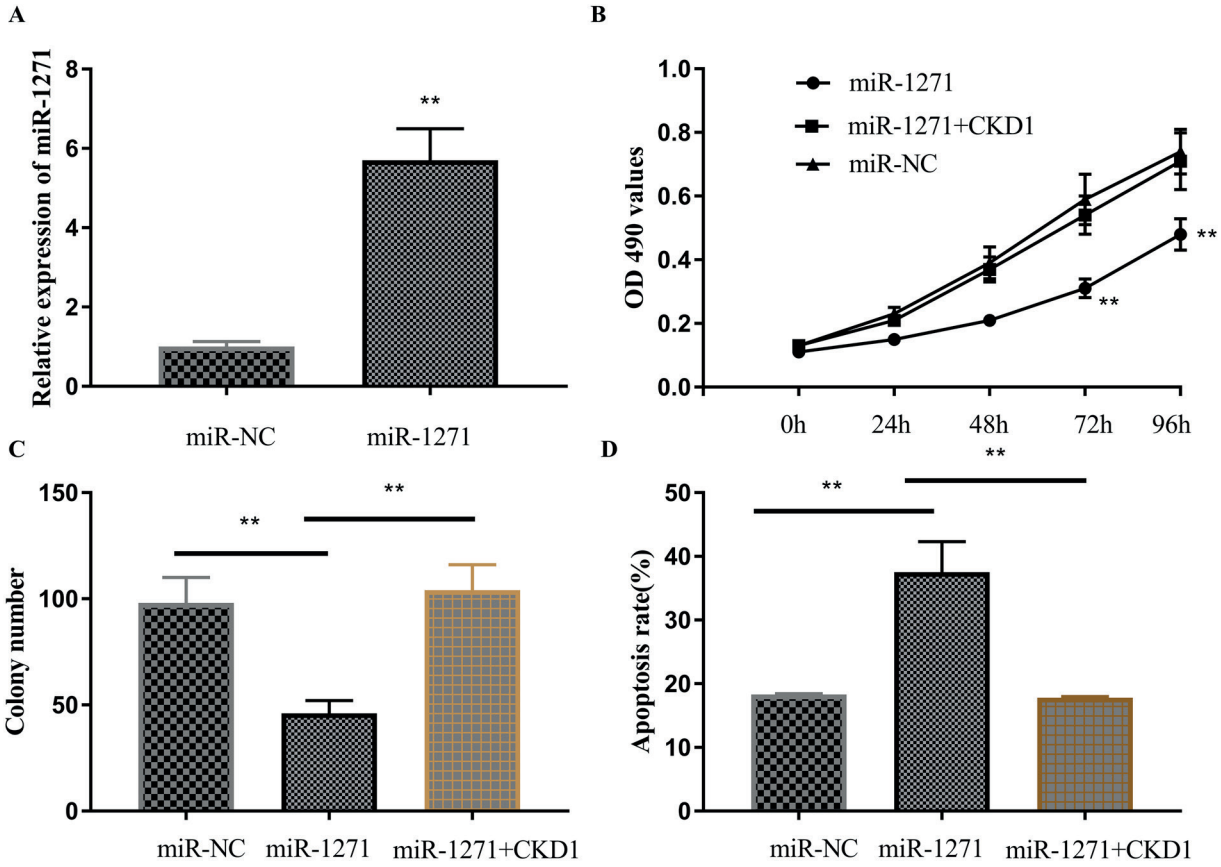

Figure 3. miR-1271 suppresses proliferation and colony formation, and induces apoptosis via targeting CDK1 in EC. ***(A)*** overexpression of miR-1271 in EC cells was confirmed by qRT-PCR. ***(B)*** Cell viability was assessed by MTT assay. ***(C)*** representative quantification of crystal violet-stained cell colonies. ***(D)*** apoptosis rate of transfected EC cells measured by flow cytometry. Data were expressed as mean ± SD of three independent experiments. *$p < 0.05$, ** $p < 0.01$.

Knockdown of miR-1271 Promoted EC Progression in vitro

To explore the effect of miR-1271 in progression of EC, we transfected miR-1271 inhibitor and negative control (NC) in RL95-2 cells. RT-PCR confirmed the down-regulated expression of miR-1271 when EC cells was transfected with miR-1271 inhibitor (Figure 4A). As shown in Figure 4B and 4C, knockdown of miR-1271 significantly promoted EC cell growth by MTT and colony formation assays. In addition, the results of flow cytometry revealed that knockdown of miR-1271 significantly suppressed apoptosis (Figure 4D). These results showed that miR-1271 served as a tumor suppressor in EC.

Discussion

The development of EC is a multistep process with accumulation of genetic and epigenetic alterations. Great efforts have been made to understand the principles of molecular changes during the progression of this tumor[16]. CDK1 is a 34 kD protein encoded by cell division cycle gene 2 (cdc2) and is a member of the Ser/Thr protein kinase family[17]. It has been reported that CDK1 may play a negative regulator in various tumors, such as ovarian epithelial carcinoma[18], lung adenocarcinoma[19], and endometrial cancer[20]. Some previous reports[21,22] also showed that some miRNAs were involved in the progression of tumors by through the modulation of CDK1. In the present study, we firstly found that miR-1271 served as a tumor suppressor in EC. Then, we searched the bioinformatics approach and found that miR-1271 could bind to the CKD1-3'UTR. Thus, we wondered whether miR-1271 could regulate the expression of CKD1. MiR-1271 has been found to participate in the carcinogenesis of some types of cancers. For instance, Zhong et al[23] observed that the overexpression of miR-1271

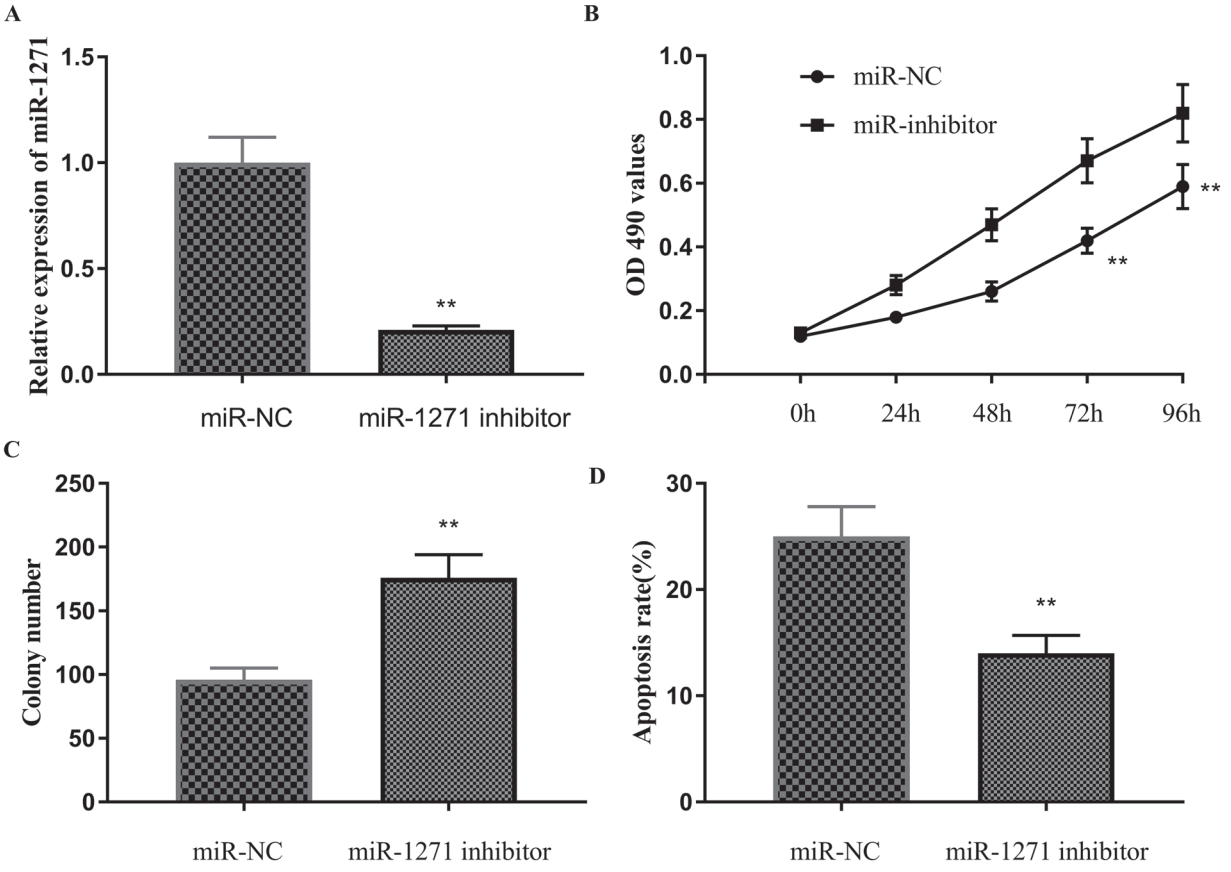

Figure 4. Down-regulation of miR-1271 promotes proliferation and colony formation, and suppresses apoptosis in EC cells. ***(A)*** down-regulation of miR-1271 in EC cells was confirmed by qRT-PCR. ***(B)*** cell viability was assessed by MTT assay. ***(C)*** representative quantification of crystal violet-stained cell colonies. ***(D)*** apoptosis rate of transfected EC cells measured by flow cytometry. Data were expressed as mean ± SD of three independent experiments. *$p < 0.05$, ** $p < 0.01$.

significantly inhibited proliferation and invasion in prostate cancer cells by targeting DIXDC1. Zhou et al[24] found that overexpression of miR-1271 suppressed the NSCLC growth *in vitro* and *in vivo*, while the inhibition of miR-1271 significantly increased NSCLC growth. Liu et al[25] indicated that the overexpression of miR-1271 inhibited the proliferation of ovarian cancer cells by targeting Cyclin G1. Furthermore, in their clinical investigation, they found that high miR-1271 expression was correlated with a high rate of patient survival. These findings have provided hints that miR-1271 may be associated with the survival of cancer cells. In the present work, we firstly showed that miR-1271 was significantly downregulated in human EC tissues and cells by TR-PCR. Our gain- and loss-of-function experiments demonstrated that up-regulation of miR-1271 inhibited cell proliferation and induced apoptosis in EC cells. Down-regulation of miR-1271 exerted the contrary effect in EC cells. In order to explore the mechanism underlying cell proliferation, our attention focused on the association between miR-1271 and CDK1. Our data showed that downregulation of CDK1 by miR-1271 resulted in the inhibition of prostate cancer cell proliferation.

Conclusions

We for the first time found that miR-1271 was downregulated in EC cells and tissues, and suppressed EC cell proliferation and induced apoptosis by targeting CDK1. These findings might provide some new insights into the potential novel treatment targets for EC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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