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MiR-1271 inhibits cell proliferation and metastasis by targeting LDHA in endometrial cancer

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Abstract. – OBJECTIVE: Endometrial cancer (EC) is one of the most frequent malignant with high incidence worldwide. The poor prognosis of EC is mainly due to the lack of efficient treatment and diagnosis. The biological role of microRNA-1271 (miR-1271) in EC is still unknown. In the current research, we mainly aimed to figure out the role of miR-1271 in EC progression.

PATIENTS AND METHODS: MiR-1271 expression in EC was evaluated by quantitative Real-time polymerase chain reaction (qRT-PCR) assay. Cell counting kit-8 (CCK-8) assay and colony formation assay were employed to examine cell proliferation ability. Transwell assay and Matrigel assay were conducted to detect cell migration and invasion. Bioinformatics analysis and dual-luciferase reporter gene assay were employed to predict and verify the target genes of miR-1271. The protein level of lactate dehydrogenase A (LDHA) was assessed by Western blotting with normalization to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

RESULTS: MiR-1271 was downregulated in EC through qRT-PCR determination. CCK-8 assay and colony formation assay indicated that over-expression of miR-1271 suppressed cell proliferation in EC. Through the transwell assay and Matrigel assay, the data indicated that miR-1271 suppressed cell migration and invasion. By bioinformatics analysis and dual-luciferase reporter gene assay, LDHA was verified to be a direct down stream target of miR-1271. In the rescue assay, it was proved that miR-1271 inhibited cell proliferation and metastasis by regulating LDHA in EC.

CONCLUSIONS: We showed that miR-1271 functioned as a tumor suppressor in EC by targeting LDHA, which may bring a novel insight for developing novel biomarkers and treatment strategies in EC.

Key Words:
MiR-1271, Proliferation, Migration, Invasion, LDHA.

Introduction

Endometrial cancer (EC) is a common type of malignancy with increasing incidence in the world[1]. Prognosis of EC is related with various factors, including tumor stage and clinical grade[2]. The overall survival time of EC patients is not favorable due to cancer recurrence and the lack of efficient treatment[3]. Hence, it is vital to figure out the underlying molecular mechanism in EC development and to seek for novel treatment strategies and biomarkers in EC. microRNAs (miRNAs) are short, non-coding RNAs with a length of 18-23 nuclear acids, which exerts post-transcriptional regulation of genes[4]. MiRNAs are reported to have multiple functions in regulation of cell progression, including cell proliferation, cell metastasis, cell cycle and cell apoptosis, cell differentiation[5-7]. MiRNAs participate in the progression of different cancer types, including EC[8]. Nevertheless, the role of miR-1271 in EC remains unknown. Herein, this study was designed to figure out the physiological functions of miR-1271 in EC progression. Based on the current study, we examined the relative expression level of miR-1271 in EC. MiR-1271 was found to be downregulated in EC. Subsequently, functional experiments indicated that miR-1271 inhibited cell proliferation and metastasis in EC. After that, bioinformatics analysis and dual-luciferase reporter assay indicated that lactate dehydrogenase A (LDHA) may serve as a direct down-stream target of miR-1271 in EC. The rescue assay was conducted and the results verified that miR-1271 inhibited cell proliferation and metastasis by regulating LDHA in EC.

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Patients and Methods

Tissue Specimens

A total of 30 paired of EC tissues and paired normal tissues were obtained during surgical procedures in Linyi Central Hospital from April 2016 to December 2017. This study was approved by the Ethics Committee of Linyi Central Hospital. Signed written informed consents were obtained from all participants before the study. All tissue specimens were stored at liquid nitrogen after resection. Tissue specimens were deposited at -80°C until use.

Cell Culture

Four EC cell lines (ECC-1, KLE, AN3CA) and 1 endometrial fibroblast cell line (T-HESC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were cultured at 37°C with 5% CO_2.

Cell Transfection

Oligonucleotides for over-expressing or down-expressing miR-1271 (mimics or inhibitor) and their negative controls were provided by GenePharma (Shanghai, China). The plasmid pcDNA-3.1 (GenePharma, Shanghai, China) was used to up-regulate the expression of LDHA and the empty pcDNA-3.1 plasmid was taken as a control. Transfection efficiency was examined by quantitative Real-time polymerase chain reaction (qRT-PCR).

QRT-PCR

Total RNA in cells or tissues was extracted *via* TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to standard protocol. Reverse Transcription Kit (TaKaRa, Dalian, China) was used to synthesize complementary DNAs (cDNAs). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or U6 was taken as normalization in qRT-PCR. Primer sequences used in this study were as follows: LDHA, F: 5'-TTCACCCCCAGGAACTC-3', R: 5'-ATCCCGTGTCCGAAGGA-3'; miR-1271, F: 5'-GTTATGTAAACCCTCGACTG-3', R: 5'-AGGCTTGCGTTGGAGTCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Cell-Counting Kit-8 Assay

Cell counting Kit-8 (CCK-8) assay was recruited to examine the effect of miR-1271 on cell proliferation ability. Transfected cells were inoculated in 96-wells plates (7×10^3/well) and then CCK-8 solution (Beyotime, Shanghai, China) was used to incubate cells for 2 h at 37°C. The optical density (OD) value (450 nm) was evaluated at a spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, USA).

Colony Formation

Cells (1×10^3) were inoculated into the culture plates (60 mm) for 2 weeks. Cells were washed by phosphate-buffered saline (PBS) twice and fixed in ice-cold 70% methanol for 15 min. Crystal Violet Staining Solution (Beyotime, Shanghai, China) was used to stain the cell colonies. All the colonies were captured using a microscope.

Transwell Assay and Matrigel Assay

We carried out transwell assay to figure out the invasion ability of transfected EC cells. Transwell chambers and 24-wells plates were obtained from Corning (Corning, NY, USA). 1×10^5 cells suspended in serum-free medium (100 μL) were applied on the upper chamber. In the Matrigel assay, the upper chamber was pre-coated with Matrigel (BD Bio-Sciences, Franklin Lakes, NJ, USA). After 36 h, the invasive cells were counted from images of five random fields using an inverted microscope (Olympus, Tokyo, Japan).

Bioinformatics Analysis

To seek for the potential targets of miR-1271, we predicted target genes in 4 public available databases: RNA22, TargetScan, miRWalk and MiRanda. The results suggested that 3'-UTR of LDHA had potential binding sites with miR-1271.

Dual-Luciferase Reporter Gene Assay

Wild LDHA 3'-UTR sequence or the mutant LDHA 3'-UTR sequence was inserted to pGL3 promoter vector (Genscript, Nanjing, China). Luciferase activity was determined following the standard protocol.

Western Blot

Total protein was isolated by radioimmunoprecipitation assay (RIPA) buffer (Thermo-Fisher Scientific, Waltham, MA, USA) and phenylmethanesulfonyl fluoride (PMSF). Protein lysates separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Membrane was immunostained at 4°C by rabbit anti-LDHA (1:1000, CST, Danvers, MA, USA) overnight. Protein expression was evaluated by Image J software (Bethesda, MD, USA).

Statistics Analysis

All experiments in this study were independently performed for at least three times. Pearson correlation was performed for correlation analysis. All data recorded were exhibited as mean ± standard deviation (SD). Student's unpaired *t*-test was performed for analyzing differences between two groups. $p<0.05$ indicated significant difference.

Results

MiR-1271 Expression Level was Decreased in EC

MiR-1271 expression in EC was examined by qRT-PCR. As Figure 1A exhibited, miR-1271 expression in EC tissues was exactly downregulated compared with para-tumor tissues. Consistently, miR-1271 was also downregulated in EC cell lines (Figure 1B). In particular, ECC-1 cell line was chosen for over-expression of miR-1271 and KLE cell line was selected for down-expression of miR-1271. Transfection efficiency was accessed by qRT-PCR (Figure 1C).

MiR-1271 Inhibited EC Cell Proliferation In Vitro

To elucidate the effect of miR-1271 on proliferation of EC cells, CCK-8 assay and colony formation assay were conducted. In CCK-8 assay, over-expressed miR-1271 significantly inhibited cell proliferation in comparison with control group (Figure 2A). As Figure 2B showed,

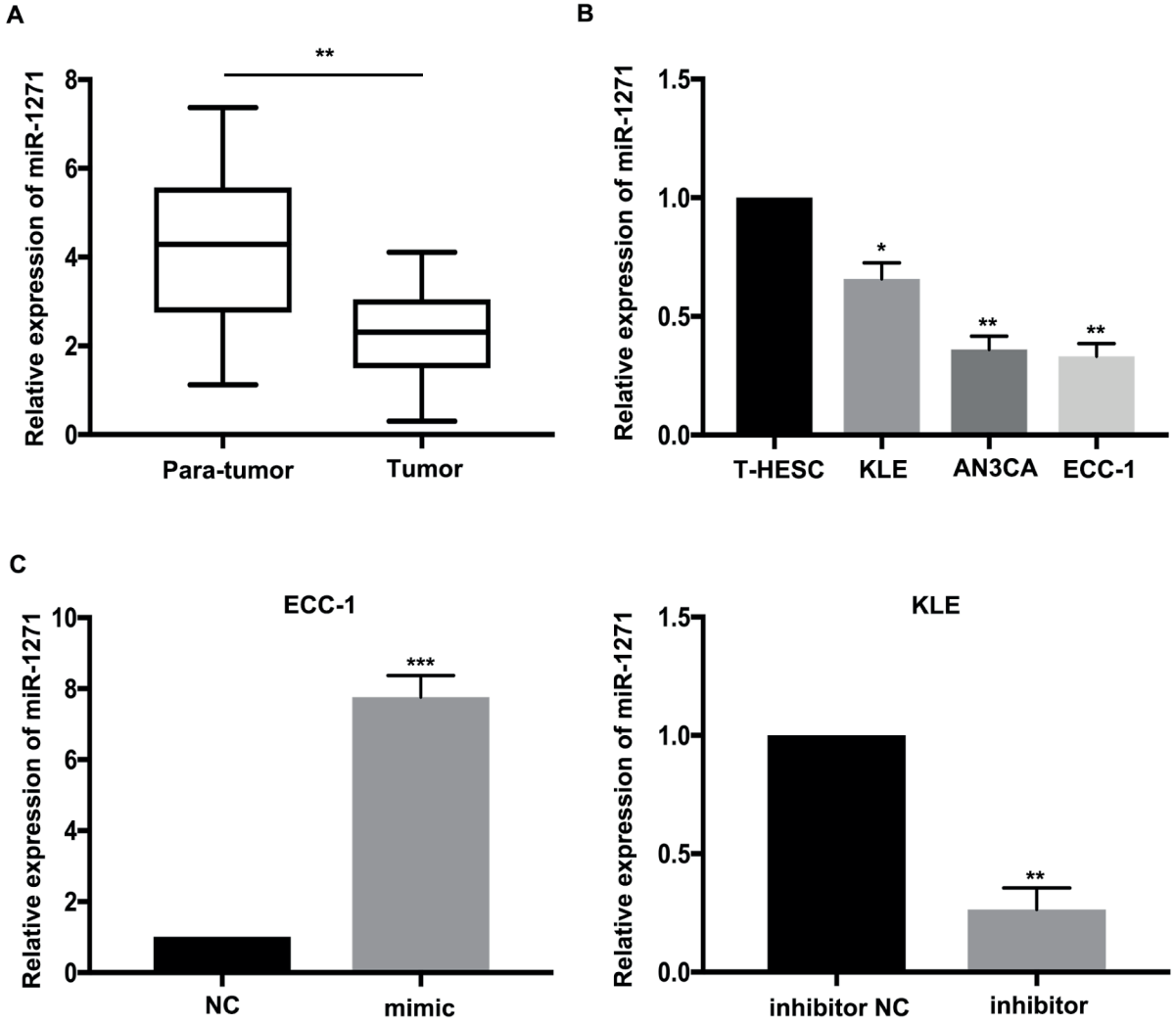

Figure 1. The expression level of miR-1271 in EC tissues and cell lines. ***A,*** Relative expression level of miR-1271 was detected in 30-paired EC tumor and para-tumor tissues. ***B,*** QRT-PCR was used to verify the relative expression level of miR-1271 in EC cell lines. ***C,*** Relative expression of miR-1271 in the ECC-1 or KLE cell line after transfected with mimic or inhibitor. $^{**}p<0.01$, $^{***}p<0.001$, compared to control group. The data expressed as the mean ± SD.

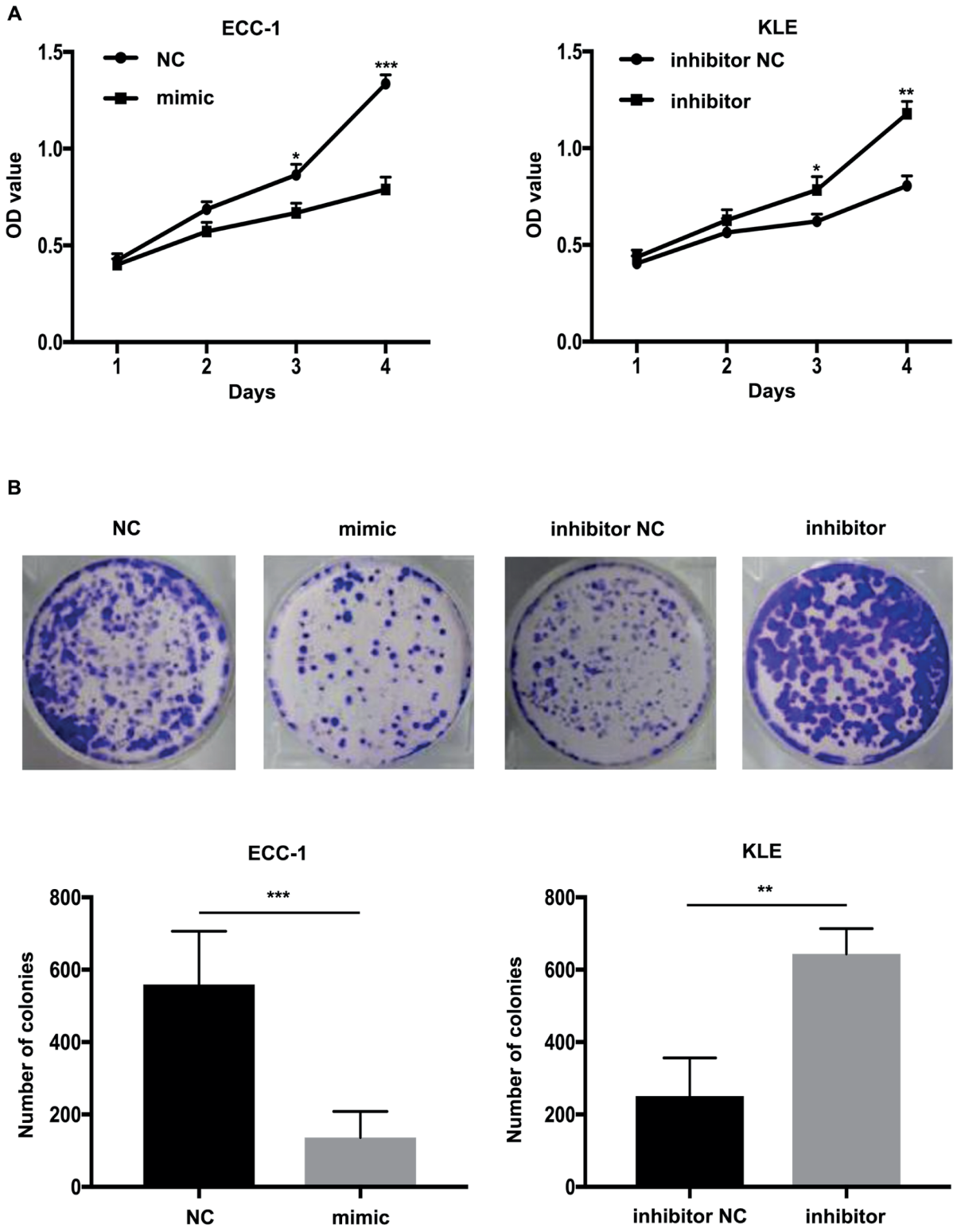

Figure 2. MiR-1271 suppressed cell proliferation in EC cell lines. ***A,*** CCK-8 assay was performed in transfected cells. ***B,*** Colony formation assay was recruited in transfected cells. *p<0.05, **p<0.01, ***p<0.001, compared with control group. The data expressed as the mean ± SD.

over-expressed miR-1271 group formed relatively fewer colonies when compared with control group. Conversely, miR-1271 down-expression formed more colonies. Hence, we considered that miR-1271 could inhibit cell proliferation in EC.

MiR-1271 Suppressed Cell Migration and Invasion in EC Cell Lines

For examination of the ability of cell migration and invasion, transwell and matrigel assay were involved. As Figure 3A showed, over-expression of miR-1271 increased migratory and invasive abilities of EC cells. However, downregulation of miR-1271 reduced the number of migratory or invasive cells than controls (Figure 3B). In sum, miR-1271 suppressed migration and invasion of EC cells.

LDHA Was a Direct Down-Stream Target of miR-1271

The bioinformatics analysis in this study was performed to seek for the underlying target of miR-1271, and the result showed that LDHA might be a target gene of miR-1271. As shown in Figure 4A, dual-luciferase reporter gene assay was recruited to examine the combination of miR-1271 and LDHA. The result suggested that LDHA was a target of miR-1271. MiR-1271 expression in EC was accessed by qRT-PCR, and the data showed that LDHA was upregulated in EC (Figure 4B). Pearson correlation analysis was subsequently carried out. As shown in Figure 4C, expression of miR-1271 in EC was negatively correlated with LDHA. After that, the expression level of LDHA was detected in transfected cells *via* Western blotting. As Figure 4D exhibited, LDHA expression was downregulated in miR-1271 over-expressed group. Taken together, all results verified that LDHA was a direct down-stream target of miR-1271 in EC.

MiR-1271 Inhibited Cell Proliferation and Metastasis by Regulating LDHA in EC

To confirm if miR-1271 functioned as a tumor suppressor in EC by targeting LDHA, rescue assay was performed. As shown in Figure 5A, the expression of LDHA was up-regulated in cells co-transfected with miR-1271 mimics and LDHA overexpression plasmid compared with control group. Subsequently, cell proliferation ability increased in LDHA over-expressed group (Figure 5B). In transwell assay, cell migration ability was upregulated in LDHA over-expressed group (Figure 5C). Consistently, invasive cells in LDHA up-regulated group significantly increased in comparison with control group (Figure 5C). Hence, the results of rescue assay suggested that miR-1271 inhibited cell proliferation and metastasis by regulating LDHA in EC.

Discussion

The prognosis of EC is mainly related with the tumor stage and clinical grade[2]. The prognosis of EC at advanced stage is still poor due to the lack of efficient treatment and diagnosis[9]. Hence, it is important to seek for novel treatment strategies and biomarkers for EC. Herein, in current study, we aimed to figure out the role of miR-1271 in EC and hope to give a new insight for clarifying EC development. MiRNAs are reported to have multiple functions in cell progression, including cell proliferation, metastasis, cell cycle, cell apoptosis[10-12]. Hence, miRNAs exert vital roles in tumor progression and development. MiRNAs are reported to have diverse functions in different tumor types. It is reported[13] that miR-145-5p regulated cell differentiation by targeting KLF5 in gastric cancer. MiR-27a is proved to promote cell migration by regulating EMT *via* targeting FBXW7 in breast cancer[11]. MicroRNA-876-5p is reported to function as a tumor suppressor in glioblastoma multiforme by regulating Forkhead boxM1[14]. MicroRNA-296 exerts a tumor suppressor role by regulating SGLT2 in lung cancer[15]. However, the physiological role of miR-1271 in EC is still not known. In current study, the results suggested that miR-1271 was down-expressed in EC. MiR-1271 could inhibit cell proliferation and metastasis in EC.

Usually, miRNAs exert their functions by degrading mRNAs expression[16]. Hence, we used the public available databases to seek for the potential targets of miR-1271. LDHA was merged to be an underlying target of miR-1271 in EC. Subsequently, dual-luciferase reporter gene assay confirmed their binding relationship. The result verified that LDHA was a down-stream target of miR-1271. LDHA participates in glycolysis and serves as a vital role in glycolysis by catalyzing the conversion of pyruvate to lactate[17]. LDHA is regulated by miRNAs and further regulate tumor progression. MiR-33b inhibits cell proliferation *via* regulating Lactate Dehydrogenase A (LDHA) in osteosarcoma[18]. It is also reported that miR-323a-3p can suppress glycolysis in osteosarcoma through targeting LDHA[19]. Nevertheless, the correlation

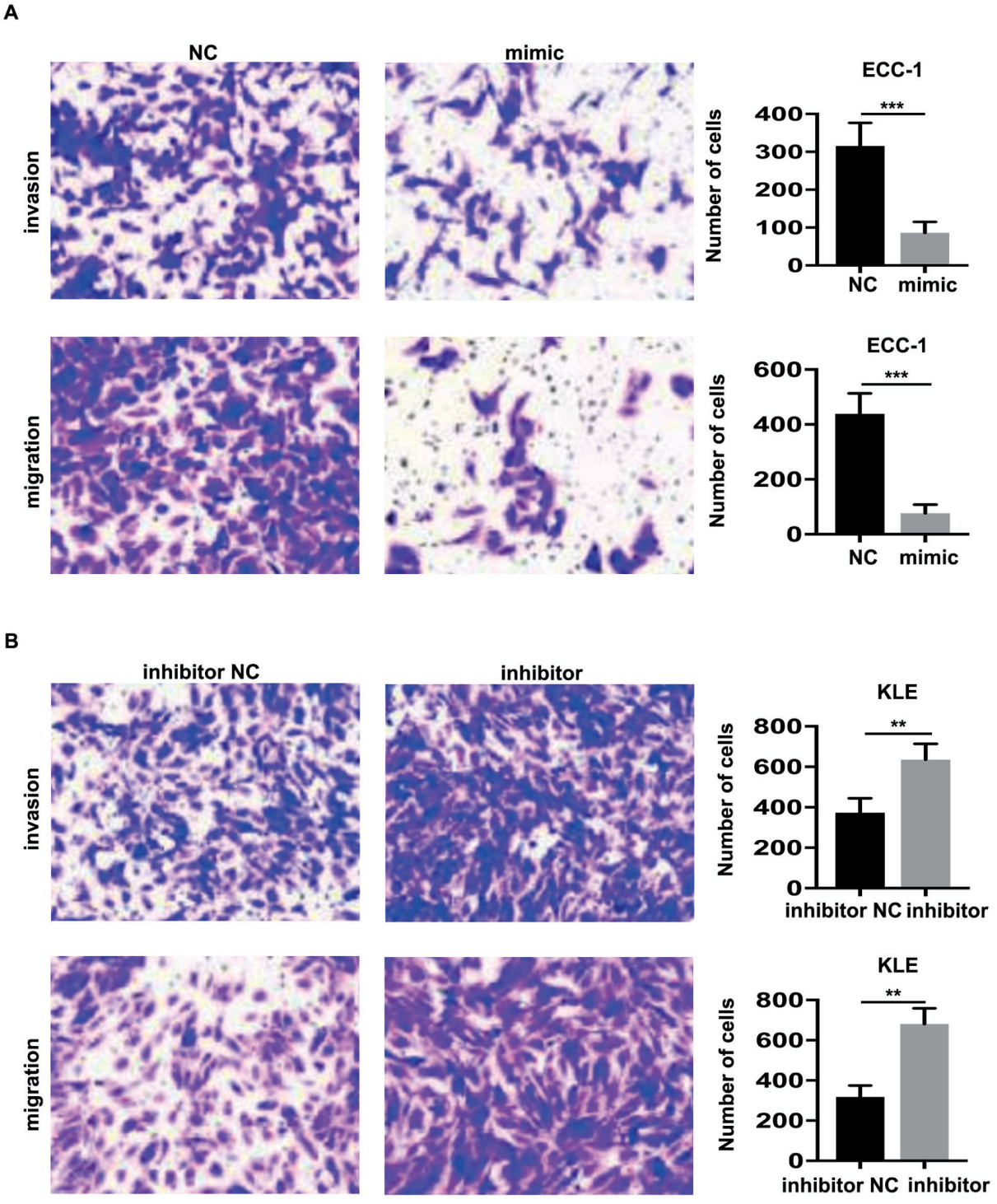

Figure 3. MiR-1271 suppressed cell metastasis in EC cell lines. ***A,*** Transwell assay and matrigel assay were performed in transfected ECC-1 cells. ***B,*** Transwell assay and matrigel assay were performed in transfected KLE cells. $^{**}p<0.01$, $^{***}p<0.001$, compared with control group. The data expressed as the mean ± SD.

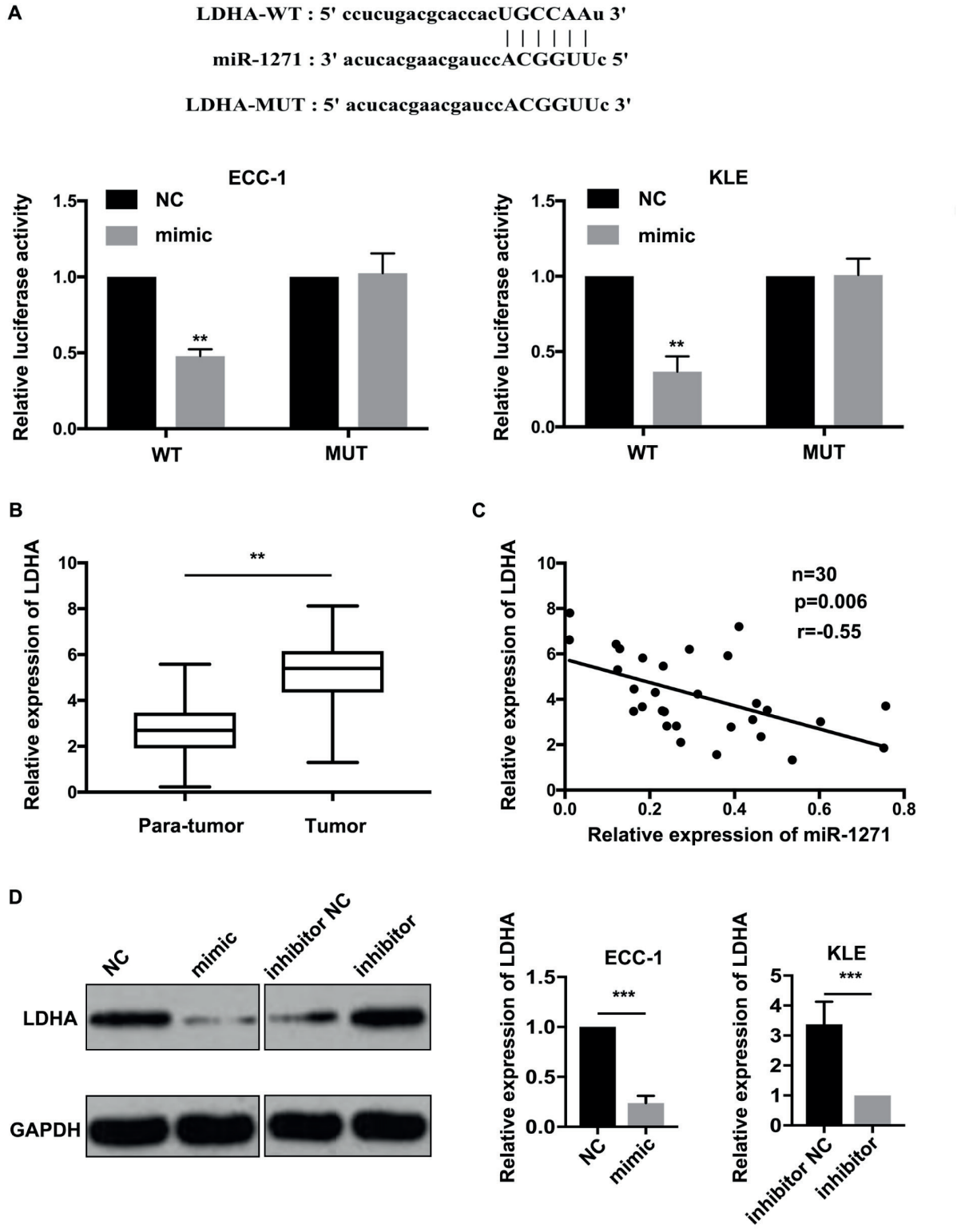

Figure 4. MiR-1271 negatively regulated the expression of LDHA in EC. ***A,*** Dual-luciferase reporter gene assay was performed for validation. ***B,*** The expression of LDHA in EC tumor tissues and para-tumor tissues. ***C,*** Pearson correlation analysis was used to analyze the correlation between the mRNA level of LDHA and the expression of miR-1271 (p=0.006). ***D,*** Western Blot was used to test the protein level of LDHA in transfected cell lines. **p<0.01, ***p<0.001, compared with control group. The data expressed as the mean ± SD.

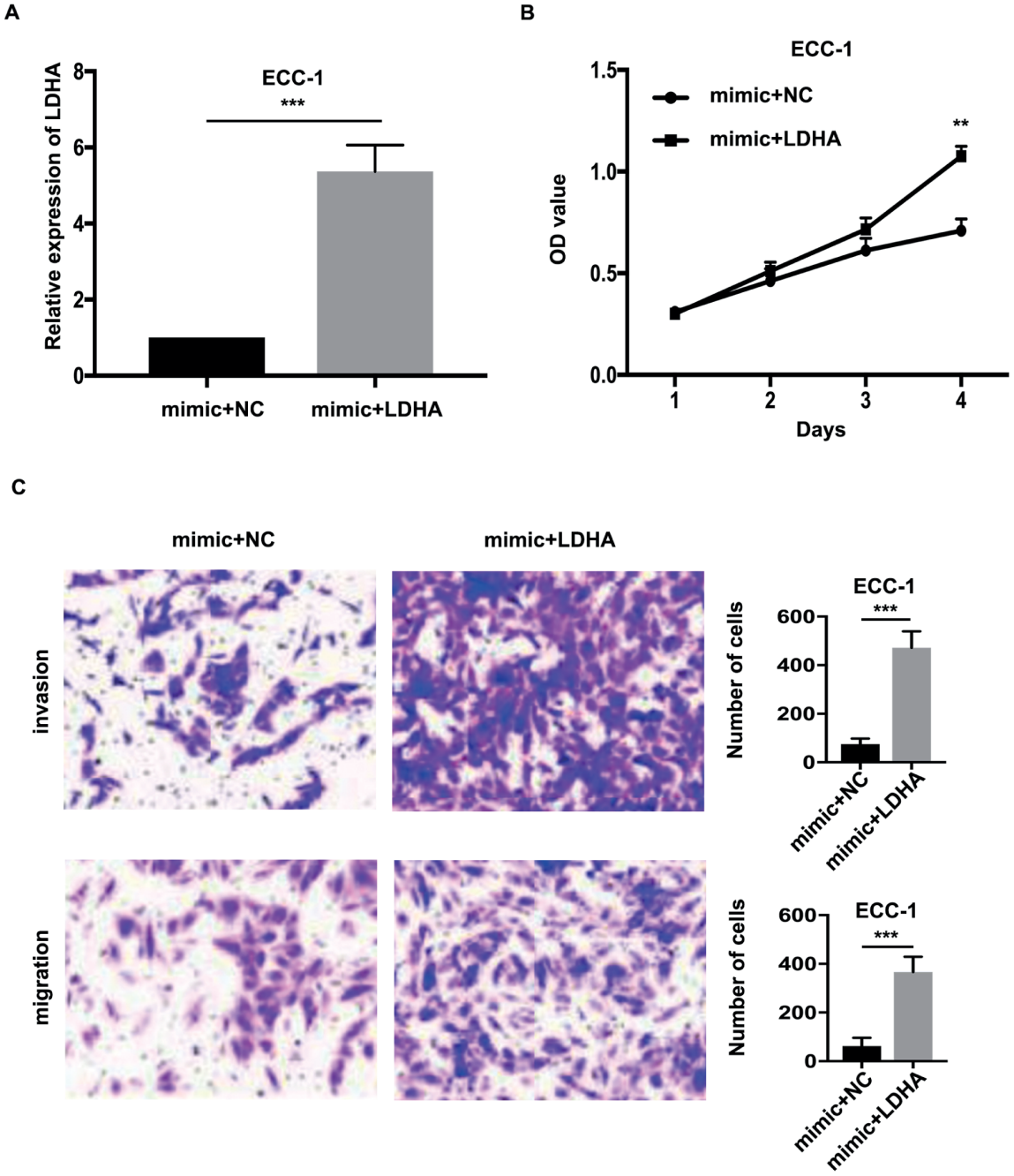

Figure 5. MiR-1271 suppressed cell proliferation and metastasis by negatively regulating LDHA. ***A,*** QRT-PCR was used to examine the expression level of LDHA in co-transfected cells. ***B,*** CCK-8 assay was employed to examine the migration ability of co-transfected cells. ***C,*** Cell migration and Invasive co-transfected cells were detected by transwell assay and matrigel assay. **$p<0.01$, ***$p<0.001$, compared to control group. The data expressed as the mean ± SD.

between miR-1271 and LDHA in EC remains unknown. Hence, we validated that LDHA was a direct down-stream target of miR-1271. Besides, through the rescue assay, the results indicated that miR-1271 functioned as a tumor suppressor in EC by regulating LDHA.

Conclusions

We showed that miR-1271 was down-expressed in EC through qRT-PCR. CCK-8 assay and colony formation assay showed that over-expression of miR-1271 can inhibit cell proliferation in EC.

Transwell assay and matrigel assay showed that miR-1271 can suppress cell migration and invasion. By bioinformatics analysis and dual-luciferase reporter gene assay, LDHA was a direct down-stream target gene of miR-1271. In rescue assay, it was proved that miR-1271 can inhibit cell proliferation and metastasis by regulating LDHA in EC. Our study provides a novel insight of treatment and biomarkers for EC.

Conflict of interest

The authors declare no conflicts of interest.

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