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MiRNA-584 suppresses the progression of ovarian cancer by negatively regulating LPIN1

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Abstract. – OBJECTIVE: The aim of the study was to detect the expression level of microRNA-584 (miRNA-584) in ovarian cancer (OCa) and to elucidate its regulatory effect on OCa progression by regulating LPIN1.

PATIENTS AND METHODS: Expression levels of miRNA-584 and LPIN1 in 31 matched OCa tissues and paracancerous tissues were detected. The relationship of miRNA-584 level with clinical indicators and prognosis of OCa patients was analyzed. Influences of miRNA-584/LPIN1 regulatory loop on malignant phenotypes of OVCAR3 and PEO1 cells were assessed. In addition, the interaction between miRNA-584 and LPIN1 was confirmed by Dual-Luciferase reporter gene assay and rescue experiments.

RESULTS: MiRNA-584 was lowly expressed in OCa tissues, while LPIN1 was highly expressed. OCa patients expressing a low level of miRNA-584 suffered from higher rates of lymphatic metastasis and distant metastasis, as well as worse survival. The overexpression of miRNA-584 in OVCAR3 cells attenuated proliferative and migratory abilities, while the knockdown of miRNA-584 in PEO1 cells yielded the opposite results. LPIN1 was verified to be the target binding to miRNA-584 and its level was negatively regulated by miRNA-584. The overexpression of LPIN1 accelerated OCa cells to proliferate and migrate. Importantly, LPIN1 was responsible for OCa progression regulated by miRNA-584.

CONCLUSIONS: MiRNA-584 is downregulated in OCa tissues and cell lines. MiRNA-584 level is correlated with lymphatic metastasis, distant metastasis, and poor prognosis in OCa patients. By negatively regulating LPIN1, miRNA-584 suppresses the malignant progression of OCa.

Key Words:
MiRNA-584, LPIN1, Ovarian cancer, Malignant progression.

Introduction

Ovarian cancer (OCa) is a prevalent malignancy in female reproductive organs. Epithelial ovarian cancer is the most common histopathological type in OCa, and its mortality ranks first in gynecologic malignancies[1-3]. Due to the insidious onset of OCa, there have been about 2/3 patients diagnosed at advanced stage[4,5]. Tumor cytoreductive surgery and platinum-based chemotherapy are the main treatment strategies for advanced OCa, which can temporarily relieve the disease. However, 70% of OCa patients experience a relapse within 3 years[6]. Recurrent OCa is highly resistant to anti-tumor drugs, which severely limits the 5-year survival[7,8]. It is reported that the 5-year survival in patients with stage III and IV OCa is 29% and 13%, respectively[7,8]. Although current research has extensively explored the pathological and clinical prognostic factors (staging and grading) of OCa, and its molecular pathogenesis is still unclear[9-11]. Therefore, novel hallmarks for improving chemotherapy sensitivity and reversing drug-resistance are urgently required[10].

In recent years, extensive research[10] has been conducted on targeted therapy of tumors, with considerable advances. MicroRNA (miRNA) is a non-coding, small RNA about 22 nucleotides long, which is discovered in eukaryotes. MiRNAs are important in the development of ontogenesis and tumors[12]. Dysregulated miRNAs in OCa are well concerned[13,14]. By binding to 3'UTR of the target mRNA, a miRNA directly degrades the mRNA or inhibits its translation, thus silencing its expression at post-transcriptional level[15,16]. Critical functions of miRNAs have been discovered in regulating various aspects of tumor cell phenotypes[17-19]. MiRNA-584 is abnormally expressed in many types of malignancies, exerting a potential influence on tumor progression[20,21].

Through bioinformatics prediction, LPIN1 was considered to be the target of miRNA-584. In this experiment, we uncovered the role of miRNA-584/LPIN1 regulatory loop in the malignant progression of OCa.

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Patients and Methods

Patients and OCa Samples

A total of 31 matched OCa tissues and paracancerous tissues were surgically resected. None of OCa patients received preoperative anti-tumor treatment. Clinical data and follow-up data of enrolled patients were recorded. Tumor staging was assessed based on the guideline proposed by UICC. Patients and their families in this study have been fully informed. This investigation was approved by the Ethics Committee of the Zhoukou Central Hospital of Henan Province.

Cell Culture

OCa cell lines (PEO1, A2780, 3AO, CAOV3, SKOV3, and OVCAR3) and normal human ovarian surface epithelial cell line (HOSEPiCs) were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and maintained in a 5% CO_2 incubator at 37°C.

Transfection

The cells were inoculated in a 6-well plate. Transfection was conducted at 40% confluence using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). After transfection for 48 hours, the cells were harvested for functional experiments. Transfection plasmids were constructed by GenePharma (Shanghai, China).

Cell Counting Kit-8 (CCK-8)

The cells were inoculated in a 96-well plate ($2×10^3$ cells/well). At the appointed time points, absorbance value at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Molecular Technologies, Kumamoto, Japan) for plotting the viability curves.

Colony Formation Aassay

The cells were seeded in a 6-well plate with 200 cells per well and incubated for 14 days. Medium was replaced once in the first week and twice in the second week. Subsequently, visible colonies were fixed in 100% methanol and dyed with 0.5% crystal violet for 20 min. The colonies were captured and calculated in a good light environment.

Transwell Assay

The cells were inoculated in a 24-well plate at $5.0×10^5$ cells/mL. 200 μL of suspension was applied in the upper side of the transwell chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate. In the bottom side, 500 μL of medium containing 10% FBS was applied. After 48 h of incubation, the penetrated cells in the bottom side were fixed in methanol for 15 min, dyed with crystal violet for 20 min and counted using a microscope. Numbers of penetrating cells were counted in 5 randomly selected fields per sample (magnification 20×).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNAs were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNA underwent qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal references. The experiment was performed in triplicate for each sample, and the relative level was calculated by $2^{-\Delta\Delta Ct}$. The primer sequences are listed in Table I.

Table I. Primer sequence.

Gene	Primer sequence
LPIN1	Forward: 5'-CATGATCCGAGATGTGGAACTGGC-3' Reverse: 5'-CTGGCTCAGCCACTCCA-3'
GAPDH	Forward: 5'-CGCTCTCTGCTCCTCCTGTTC-3' Reverse: 5'-ATCCGTTGACTCCGACCTTCAC-3'
MiRNA-584	Forward: 5'-TTATGGTTTGCCTGGGACTGAG-3' Reverse: 5'-GCGAGCACAGAATTAATACGAC-3'
U6	Forward: 5'-CTCGCTTCGGCAGCACA-3' Reverse: 5'-AACGCTTCACGAATTTGCGT-3'

Western Blot

Cellular protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and blocked in 5% skim milk for 1 hour. The specific primary antibody was incubated with the membrane overnight at 4°C, followed by secondary antibody incubation for 2 hours at room temperature. After Tris-Buffered Saline and Tween 20 (TBST) washing for 1 min, the chemiluminescent substrate kit was used for exposure of the protein bands.

Dual-Luciferase Reporter Gene Assay

According to the binding sequences, Luciferase vectors pmirGLO-miRNA-584-wt, pmirGLO-miRNA-584-mut, and pmirGLO were constructed. OVCAR3 and PEO1 cells were co-transfected with NC mimics/miRNA-584 mimics and pmirGLO-miRNA-584-wt/pmirGLO-miRNA-584-mut/pmirGLO, respectively. After 48 h, the cells were lysed for determining the relative Luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used for data analyses. Data were expressed as mean ± standard deviation. The differences between the two groups were analyzed by the *t*-test. Kaplan-Meier curves were introduced for survival analysis, followed by Log-rank test for analyzing differences between the two curves. Spearman correlation test was performed to assess the relationship between the levels of miRNA-584 and LPIN1 in OCa tissues. $p<0.05$ was considered statistically significant.

Results

Low Expression of MiRNA-584 in OCa

MiRNA-584 levels in 31 matched OCa tissues and paracancerous tissues were determined. Its level remained lower in OCa tissues (Figure 1A, 1B). Similarly, *in vitro* level of miRNA-584 was downregulated in OCa cell lines as well (Figure 1C).

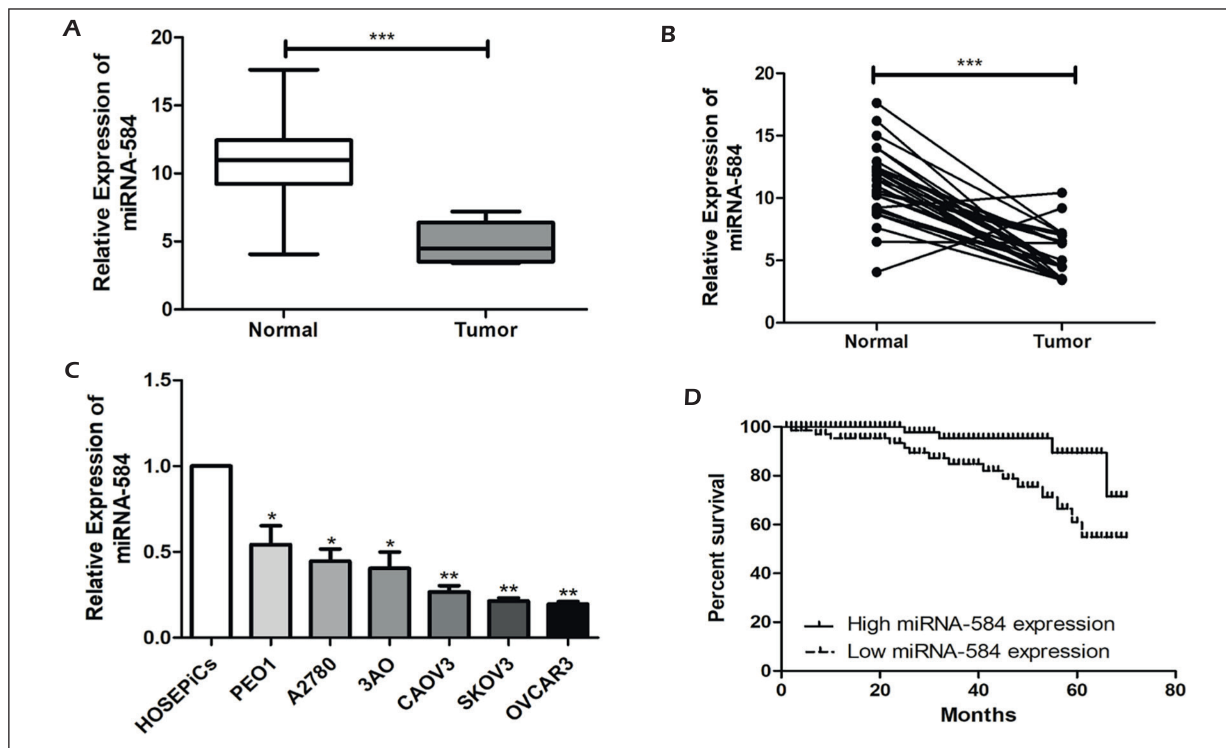

Figure 1. Low expression of miRNA-584 in OCa. **A, B,** MiRNA-584 levels in OCa tissues and adjacent normal ones. **C,** MiRNA-584 levels in OCa cell lines (PEO1, A2780, 3AO, CAOV3, SKOV3 and OVCAR3) and normal human ovarian surface epithelial cells (HOSEPiCs). **D,** Survival in OCa patients in high miRNA-584 expression group and low miRNA-584 expression group.

MiRNA-584 Expression Was Correlated with Metastasis and Overall Survival in OCa Patients

According to the average level of miRNA-584, OCa patients were classified into high expression and low expression groups. Their clinical indicators were collected and analyzed. It is shown that miRNA-584 level was negatively correlated to distant metastasis and lymphatic metastasis rates, rather than age and tumor staging in OCa patients (Table II). Moreover, Kaplan-Meier curves predicted worse prognosis in OCa patients expressing a low level of miRNA-584 (Figure 1D).

MiRNA-584 Suppressed Proliferative and Migratory Abilities in OCa

To elucidate the biological functions of miRNA-584 in OCa, the overexpression and knockdown models of miRNA-584 were constructed in OVCAR3 and PEO1 cells, respectively (Figure 2A). In OVCAR3 cells overexpressing miRNA-584, cell viability, clonality, and migratory ability were markedly attenuated. Conversely, these indicators were stimulated in PEO1 cells transfected with miRNA-584 inhibitor (Figure 2B-2D).

Interaction Between MiRNA-584 and LPIN1

Binding sequences in the promoter regions of miRNA-584 and LPIN1 were predicted by bioinformatics (Figure 3A). According to the binding sequences, Luciferase vectors pmirGLO-miRNA-584-wt, pmirGLO-miRNA-584-mut, and pmirGLO were constructed. Dual-Luciferase reporter gene assay uncovered that the overexpression of LPIN1 remarkably weakened the Luciferase activity in wild-type miRNA-584 vector, which was not affected in the mutant-type miRNA-584 vector (Figure 3A). It is indicated that LPIN1 was the target binding to miRNA-584. Both protein and mRNA levels of LPIN1 were downregulated in OVCAR3 cells overexpressing miRNA-584 (Figure 3B, 3C). In PEO1 cells with miRNA-584 knockdown, LPIN1 level was upregulated. Besides, miRNA-584 level was negatively influenced by LPIN1 as well (Figure 3D). Compared with that in adjacent tissues, LPIN1 was upregulated in OCa tissues (Figure 3E). In addition, a negative correlation was discovered between the expression levels of LPIN1 and miRNA-584 in OCa tissues (Figure 3F).

LPIN1 Promoted Proliferative and Migratory Abilities in OCa

Subsequently, the biological functions of LPIN1 were explored in OCa. Transfection efficiencies of pcDNA-LPIN1 in OVCAR3 cells and si-LPIN1 in PEO1 cells were tested (Figure 4A). After overexpression of LPIN1 in OVCAR3 cells, cell viability, clonality, and migratory ability were all enhanced. Conversely, transfection of si-LPIN1 in PEO1 cells achieved the opposite results (Figure 4B-4D).

MiRNA-584 Regulated OCa Progression by Negatively Regulating LPIN1 Expression

Based on the above findings, we speculated that LPIN1 was involved in OCa progression

Table II. Association of miRNA-584 expression with clinicopathologic characteristics of ovarian cancer.

Parameters	Number of cases	miRNA-584 expression		*p*-value
		High (%)	Low (%)	
Age (years)				0.925
< 60	14	8	6	
≥ 60	17	10	7	
T stage				0.768
T1-T2	20	12	8	
T3-T4	11	6	5	
Lymph node metastasis				0.027
No	19	14	5	
Yes	12	4	8	
Distance metastasis				0.010
No	22	16	6	
Yes	9	2	7	

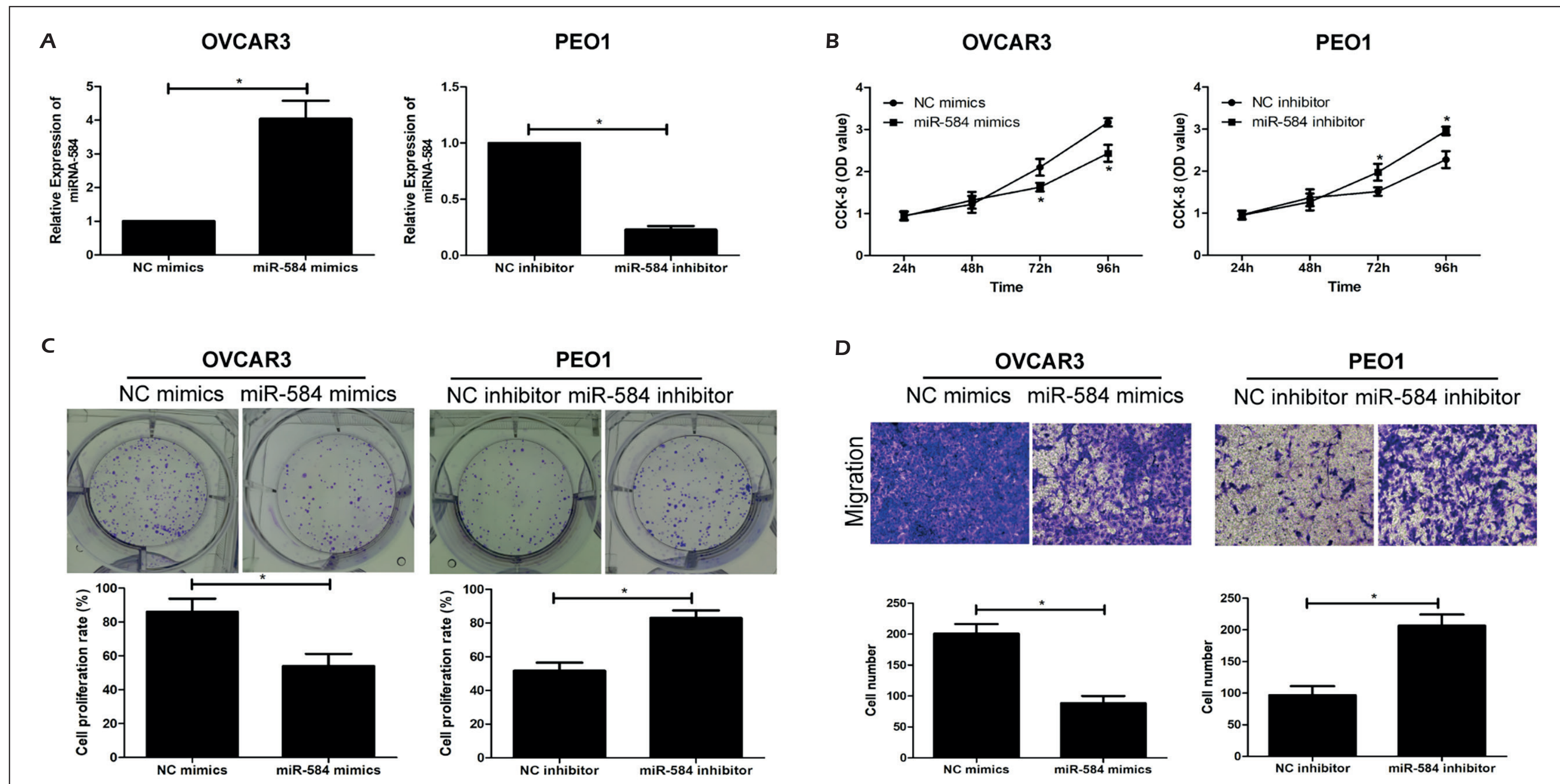

Figure 2. MiRNA-584 suppresses proliferative and migratory abilities in OCa. **A,** Transfection efficiencies of miRNA-584 mimics and inhibitor. OVCAR3 cells are transfected with NC mimics or miRNA-584 mimics and PEO1 cells with NC inhibitor or miRNA-584 inhibitor. **B,** Viability. **C,** Clonality (magnification 10×). **D,** Migratory cell number (magnification 20×).

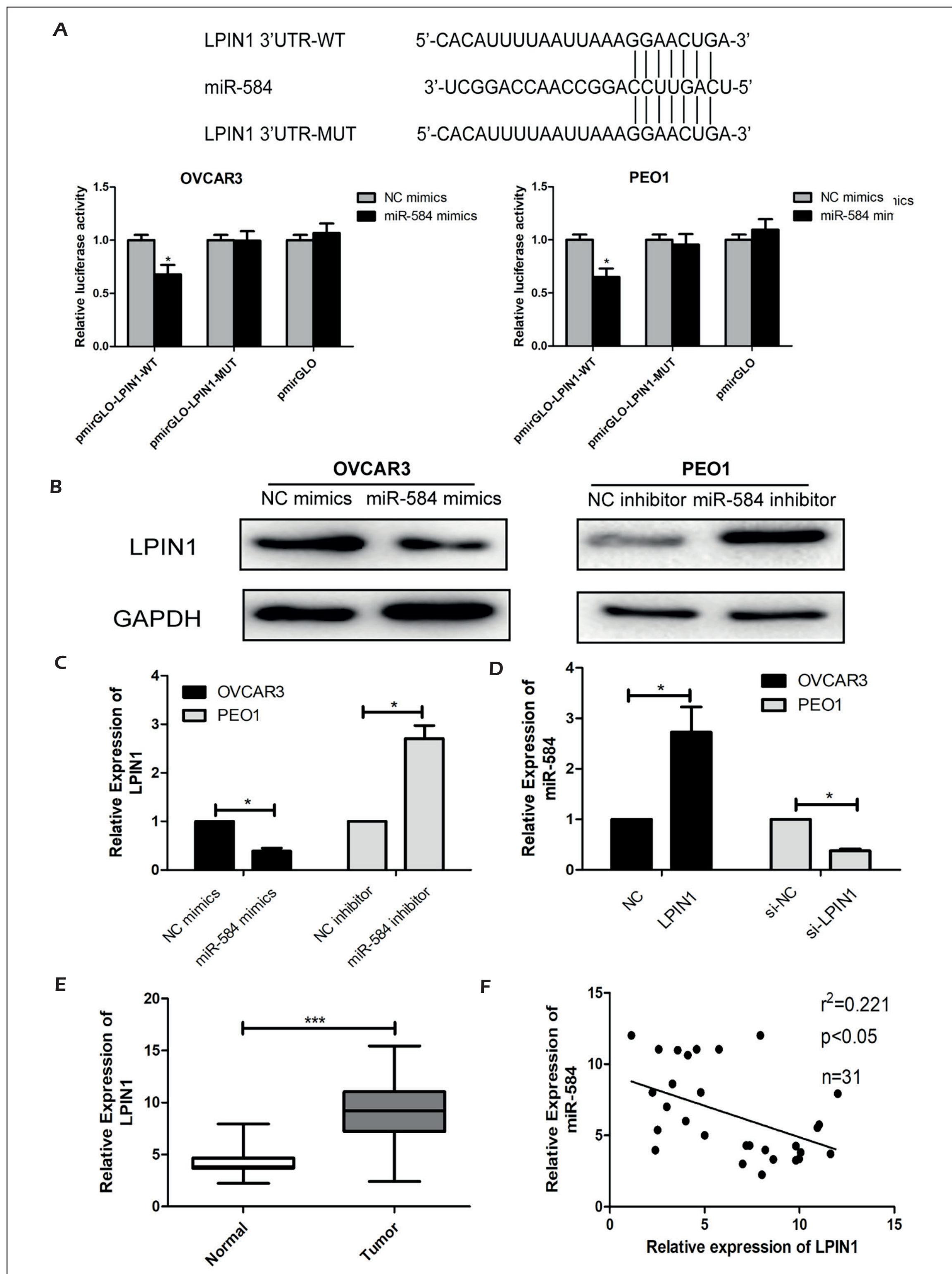

Figure 3. Interaction between miRNA-584 and LPIN1. **A,** Luciferase activity in OVCAR3 and PEO1 cells co-transfected with NC mimics/miRNA-584 mimics and pmirGLO-miRNA-584-wt/pmirGLO-miRNA-584-mut/pmirGLO. **B, C,** Protein (**B**) and mRNA levels (**C**) of LPIN1 in OVCAR3 cells transfected with NC mimics or miRNA-584 mimics, and PEO1 cells transfected with NC inhibitor or miRNA-584 inhibitor. **D,** MiRNA-584 levels in OVCAR3 cells transfected with NC or pcDNA-LPIN1, and PEO1 cells transfected with si-NC or si-LPIN1. **E,** LPIN1 levels in OCa tissues and adjacent normal ones. **F,** A negative correlation between expression levels of miRNA-584 and LPIN1 in OCa tissues.

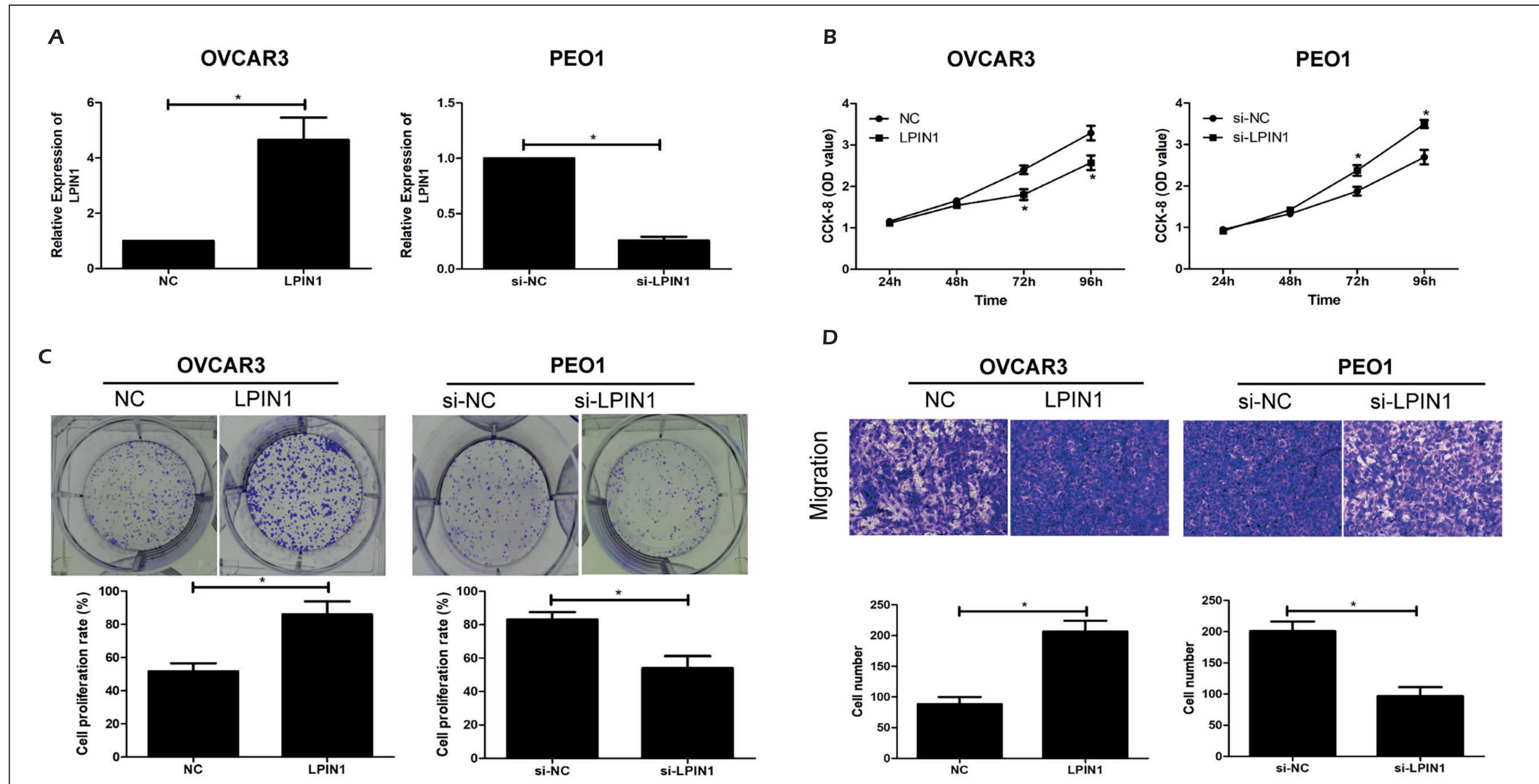

Figure 4. LPIN1 promotes proliferative and migratory abilities in OCa. **A,** Transfection efficiencies of pcDNA-LPIN1 and si-LPIN1. OVCAR3 cells are transfected with NC or pcDNA-LPIN1 while PEO1 cells with si-NC or si-LPIN1. **B,** Viability. **C,** Clonality (magnification 10×). **D,** Migratory cell number (magnification 20×).

regulated by miRNA-584. First, the downregulated LPIN1 level in OVCAR3 cells overexpressing miRNA-584 was elevated by co-overexpression of LPIN1. Meanwhile, the knockdown of miRNA-584 upregulated LPIN1 level in PEO1 cells, which was further reduced by co-silence of LPIN1 (Figure 5A). Rescue experiments illustrated that LPIN1 was able to abolish the regulatory effects of miRNA-584 on the proliferative and migratory abilities in OCa (Figure 5B, 5C).

Discussion

The mortality of OCa is the highest in gynecological malignancies[1-3]. Although great strides have been achieved in therapeutic strategies for OCa[5-7], tumor cytoreductive surgery combined with postoperative chemotherapy is the standard strategy for OCa[4,5]. Unfortunately, clinical outcomes of OCa are still unsatisfactory, which may be linked to chemotherapy-resistance and

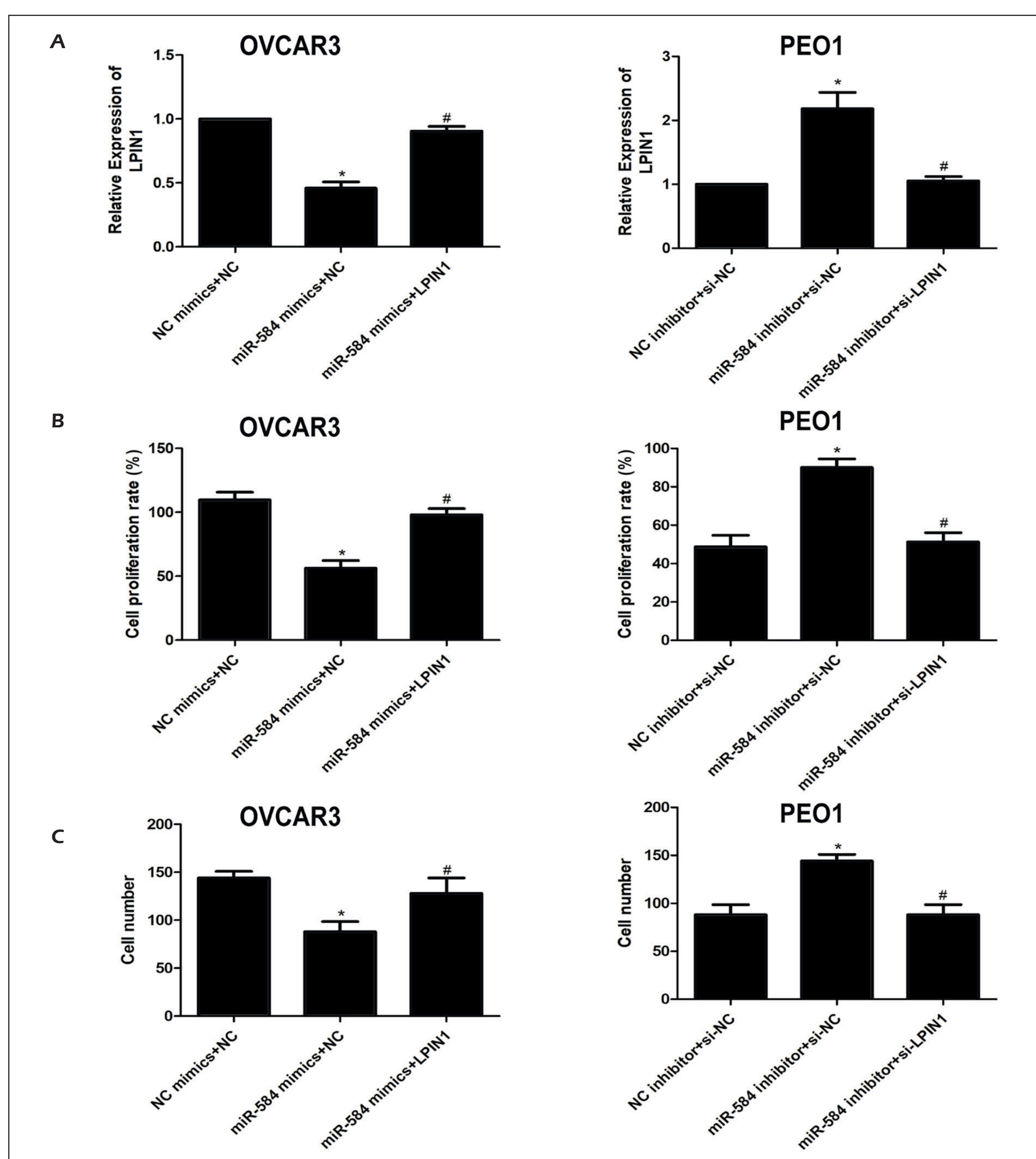

Figure 5. MiRNA-584 regulates OCa progression by negatively regulating LPIN1 expression. OVCAR3 cells are transfected with NC mimics+NC, miRNA-584 mimics+NC or miRNA-584 mimics+pcDNA-LPIN1, whereas PEO1 cells are transfected with NC inhibitor+si-NC, miRNA-584 inhibitor+si-NC or miRNA-584 inhibitor+si-LPIN1. **A,** LPIN1 level. **B,** Clonality. **C,** Migratory cell number.

metastasis[5]. Tumor-targeted gene therapy aims at distinguishing tumor tissues from normal ones. Targeted activation of genes in designated tissues or cells contributes to repairing defective genes or inhibiting tumorigenesis without influencing normal cell behaviors[7-9]. Compared with traditional chemotherapy, tumor-targeted gene therapy has the advantages of high specificity, clear curative effect, and low occurrence of adverse effects, presenting broad application prospects[10].

MiRNAs have emerged with diverse functions, including regulation of cell development, immune response, inflammatory response, and tumorigenesis[12,15-17]. By targeting downstream genes, miRNAs serve as oncogenes or tumor-suppressor genes[12,13]. These tumor-associated miRNAs could be utilized as biological hallmarks for developing tumor-targeted drugs[16,17]. Previous researches[20,21] have demonstrated that miRNA-584 participates in the progression of liver cancer by downregulating KCNE2. In this study, miRNA-584 was lowly expressed in OCa tissues. OCa patients expressing a low level of miRNA-584 had higher rates of lymphatic metastasis and distant metastasis, as well as worse survival. Overexpression of miRNA-584 in OVCAR3 cells attenuated proliferative and migratory abilities, while the knockdown of miRNA-584 in PEO1 cells yielded the opposite results.

Binding sequences between miRNA-584 and LPIN1 were predicted on the online website. Their binding relationship was further verified by Dual-Luciferase reporter gene assay. In addition, miRNA-584 was able to negatively regulate LPIN1 in OCa. Converse to those effects of miRNA-584, LPIN1 accelerated the proliferative and migratory abilities in OCa. More importantly, rescue experiments demonstrated that LPIN1 was responsible for OCa progression regulated by miRNA-584. Therefore, miRNA-584/LPIN1 regulatory loop was identified in the regulation of OCa progression.

Conclusions

Altogether, miRNA-584 is downregulated in OCa tissues and cell lines. It is correlated with lymphatic metastasis, distant metastasis, and poor prognosis in OCa patients. By negatively regulating LPIN1, miRNA-584 suppresses the malignant progression of OCa.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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