European Review for Medical and Pharmacological Sciences 2020; 24: 65-73

MiRNA-96 accelerates the malignant progression of ovarian cancer *via* targeting FOXO3a

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Abstract. – OBJECTIVE: To clarify the potential function of miRNA-96 in accelerating the malignant progression of ovarian cancer (OC) and its underlying mechanism.

PATIENTS AND METHODS: The expression patterns of miRNA-96 in 36 matched OC tissues and adjacent normal tissues were determined by qRT-PCR. We analyzed the correlation between the miRNA-96 level and the clinical parameters of OC patients. Subsequently, the cellular levels of miRNA-96 in OC cell lines were determined as well. To construct miRNA-96 inhibitor and NC, the regulatory effects of miRNA-96 on the proliferative and migratory abilities in OC cells were examined. The target gene of miRNA-96 was verified by Dual-Luciferase Reporter Gene Assay. Finally, the rescue experiments were conducted to clarify the regulatory role of miRNA-96/FOXO3a axis in the malignant progression of OC.

RESULTS: MiRNA-96 was upregulated in OC tissues relative to adjacent normal ones. Compared with OC patients presenting high-level of miRNA-96, those with low-level miRNA-96 suffered more advanced tumor staging and a worse overall survival. The transfection of miRNA-96 inhibitor markedly attenuated proliferative and migratory abilities in SKOV3 and CAOV3 cells. In addition, FOXO3a was identified to be the target gene of miRNA-96, which was negatively regulated by miRNA-96. FOXO3a exerted a lower abundance in OC tissues relative to adjacent normal ones. Finally, the rescue experiments revealed that FOXO3a knockdown could abolish the inhibitory role of miRNA-96 knockdown in the proliferative, migratory, and invasive abilities in OC cells.

CONCLUSIONS: The knockdown of miRNA-96 attenuated the proliferative and migratory abilities in OC cells by targeting FOXO3a. We believed that miRNA-96 accelerates the malignant progression of OC, which could be utilized as a therapeutic target in clinical application.

Key Words:
MiRNA-96, FOXO3a, Ovarian cancer, Metastasis.

Introduction

Ovarian cancer (OC) is a prevalent gynecological malignancy. It is estimated that in 2018, there are 22,240 new cases and 14,030 death cases of OC in the United States[1,2]. The pathogenesis of OC is complex, manifesting with obscure early-stage symptoms, high rates of metastasis, and extensive infiltration. Over 70% of OC patients are diagnosed in advanced stage with a relatively poor prognosis. More seriously, the 5-year survival of OC is at 35% although the active treatments of surgery and chemotherapy[3,4]. The molecular targeted therapy[4,5] has been considered as an effective strategy for tumor treatment. It is of great value to clarify the molecular tumorigenic mechanism of OC, thus developing therapeutic targets for improving the outcomes of OC patients[5,6].

The transcriptional and post-transcriptional regulations of the genes are mediated by epigenetic mechanisms. DNA methylation and miRNA regulation are the two hot topics in epigenetics[7,8]. It is believed that the abnormalities in DNA methylation and miRNA expressions are involved in the occurrence and progression of tumors[8]. MiRNA is widely expressed in different types of cells and exerts various biological functions. It is capable of mediating the target gene expressions and the corresponding protein synthesis through binding to 3'UTR of target mRNAs[9-12]. Tumorigenesis is a complex process involving different malignant behaviors of cells and angiogenesis[12-14].

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The inactivation of the tumor-suppressor genes and the activation of the oncogenes are the leading causes of tumorigenesis[15]. Paul et al[10] have identified the involvement of miRNAs in the occurrence and progression of tumors. MiRNA participates in different aspects of tumors, and influences the gene expressions and signaling pathways, thereafter, regulating tumor progression[16]. Shao et al[17] suggested that STXBP5-AS1 suppress cervical cancer progression *via* targeting miR-96-5p/PTEN axis. Besides, by targeting PTEN, miR-96-5p regulates radio-sensitivity and chemo-sensitivity in HNSCC cells[18]. In addition, miR-96 functions as an oncogene in diverse diseases[19,20]. However, the role and mechanism of miR-96 in OC still remain unclear.

In this paper, we clarified the expression patterns and biological functions of miRNA-96 and FOXO3a in OC through bioinformatics and molecular biology methods. Our study aims to provide theoretical references for clinical prevention and treatment of OC.

Patients and Methods

OC Samples

A total of 36 paired OC tissues and adjacent normal tissues were surgically resected from OC patients. The samples were immediately transferred into liquid nitrogen and preserved at -80°C. Tumor staging was evaluated in accordance with the guidelines proposed by UICC (the Union for International Cancer Control). Patients and their families in this study have been fully informed. This study was approved by the Ethics Committee of The Central Hospital of Weinan.

Cell Culture

OC cell lines (SKOV3, CAOV3, PEO1, A2780, 3AO, OVCAR3) and the normal human ovarian surface epithelial cell line (HOSEPiCs) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS (Life Technologies, Austin, TX, USA) and maintained at 37°C, in a 5% CO_2 incubator.

Transfection

MiRNA-96 inhibitor, negative control, sh-FOXO3a, sh-NC or FOXO3a overexpression vector (GenePharma, Shanghai) were transfected into OC cells inoculated in a 6-well plate at 70% confluence using Lipofectamine 3000 (Invitrogen, CA, USA). 48 hours later, the transfected cells were harvested for functional experiments.

Cell Proliferation Assay

The cells were seeded in the 96-well plate with 5.0×10^3 cells per well. The viability was determined at the appointed time points (24 h, 48 h, 72 h, and 96 h) using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan). Absorbance at 490 nm was recorded for plotting the viability curve.

Transwell Assays

The transfected cells for 48 h were digested and adjusted to 3.0×10^5/mL. 200 μL/well suspension was applied in the upper side of the transwell chamber (Millipore, MA, USA). In the bottom side, 600 μL of medium containing 20% FBS was applied. After 48 h of incubation, the migratory cells were fixed in methanol for 15 min, dyed with 0.2% crystal violet for 20 min, and counted using a microscope. The penetrating cells were counted in 5 randomly selected fields per sample.

Wound Healing Assay

The cells were seeded in a 6-well plate with 5.0×10^5/well. Until 90% confluence, an artificial wound was created in the confluent cell monolayer using a 1 mL pipette tip. The images were taken at 0 and 24 h using an inverted microscope, respectively. The percentage of the wound closure was calculated.

Dual-Luciferase Reporter Gene Assay

Based on the predicted binding sequences between miRNA-96 and FOXO3a, pmirGLO-miRNA-96-wt, pmirGLO-miRNA-96-mut, and the negative control pmirGLO were constructed. OC cells were co-transfected with pmirGLO-miRNA-96-wt/pmirGLO-miRNA-96-mut/pmirGLO and FOXO3a overexpression vector/NC, respectively. The cells were lysed and subjected to luciferase activity determination 48 h later.

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

The cells were lysed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the isolated RNAs were purified by DNase I treatment. The purified RNA was subjected to reverse transcription into cDNA using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The cDNA was amplified by Real Time quantitative-PCR using

SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). The primer sequences were as follows: MiRNA-96: forward: 5'-TTAGCTCAGGATCATCATCATTTACATAGATAGGG-3'; reverse: 5'-AACACTCGAGTGAGAGAAGAGAGTGCCTAGA-3'; U6: forward: 5'-CTCGCTTCGGCAGCACA-3'; reverse: 5'-AACGCTTCACGAATTTGCGT-3'; FOXO3a: forward: 5'-TACCATCAACTCCAACGG-3'; reverse: 5'-GAACCCAAGGCATCTCCA-3'; β-actin: forward: 5'-CCTGGCACCCAGCACAAT-3'; reverse: 5'-GCTGATCCACATCTGCTGGAA-3'. Each sample was performed in triplicate, calculated by the $2^{-\Delta\Delta Ct}$ method and analyzed by iQ5 2.0.

Western Blot

The total protein from cells was extracted using radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) and quantified by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). 50 μg protein sample was loaded for electrophoresis at 80 V for 40 min and then, 120 V for 60-80 min. After being transferred on a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), they were blocked in 5% skim milk for 2 hours, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. The bands were exposed by enhanced chemiluminescence (ECL; Millipore, Billerica, MA, USA) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Statistical Analysis

The Statistical Product and Service Solution SPSS 22.0 software (IBM Corp., Armonk, NY, USA) was used for data analyses. The data were expressed as mean ± standard deviation. The student's *t*-test was applied to analyze the differences between the two groups. The comparison among multiple groups was done using the One-way ANOVA test, followed by the post-hoc test (Least Significant Difference). The Kaplan-Meier curve was introduced for survival analysis. $p < 0.05$ was considered statistically significant.

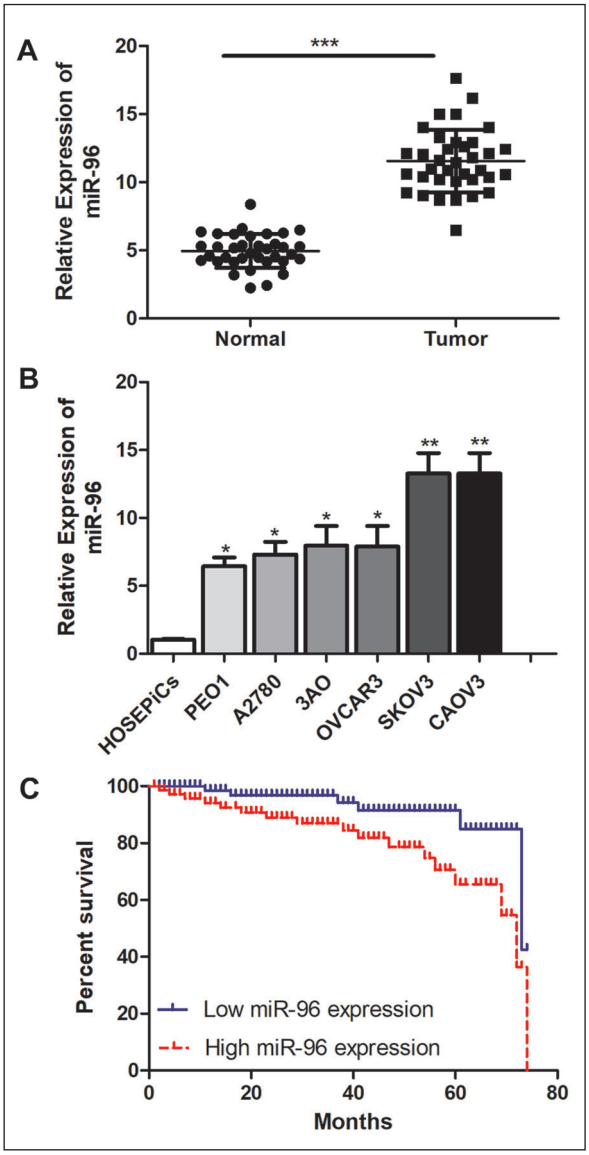

Figure 1. Expression pattern of miR-96 and its correlation with the prognosis of OC. **A,** Relative level of miR-96 in ovarian cancer tissues and adjacent normal tissues determined by qRT-PCR. **B,** Relative level of miR-96 in ovarian cancer cell lines and controls determined by qRT-PCR. **C,** Kaplan-Meier curve introduced based on miR-96 level in ovarian cancer patients. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Results

Expression Pattern of MiRNA-96 and its Correlation With the Prognosis of OC

Compared with adjacent normal tissues, qRT-PCR data revealed a higher abundance of miRNA-96 in OC tissues determined by qRT-PCR (Figure 1A). Identically, miRNA-96 was highly expressed in OC cell lines than that of HOSEPiCs cell line (Figure 1B). In particular, SKOV3 and CAOV3 cells expressed a relatively high level of miRNA-96, which were chosen for subsequent experiments. Based on miRNA-96 level, enrolled OC patients were divided into the high-level group and low-level group. The Kaplan-Meier curve was introduced for survival analysis. The

data showed that OC patients in the high-level group had a worse prognosis than those in the low-level group (Figure 1C).

We further analyzed the correlation between the miRNA-96 level and the clinical parameters of OC patients. As depicted in Table I, the miRNA-96 level was positively correlated to distant metastasis of OC, rather than age and tumor staging. It is indicated that miRNA-96 may serve as a novel biomarker for predicting the malignant progression of OC.

Knockdown of MiRNA-96 Inhibited OC Cells to Proliferate and Migrate

To evaluate the biological function of miRNA-96 in OC, we first constructed miRNA-96 inhibitor and NC. The transfection of miRNA-96 inhibitor markedly downregulated miRNA-96 level in SKOV3 and CAOV3 cells (Figure 2A). CCK-8 assay demonstrated that miRNA-96 knockdown reduced the viability in OC cells relative to controls, suggesting the attenuated proliferative ability (Figure 2B). Wound closure percentage was greatly reduced after 24-hours of incubation in OC cells transfected with miRNA-96 inhibitor (Figure 2C). Similarly, the transwell assay revealed fewer migratory cell numbers in OC cells with miRNA-96 knockdown (Figure 2D). To sum up, the knockdown of miRNA-96 attenuated the proliferative and migratory abilities of OC cells.

FOXO3a Was Lowly Expressed in OC

Based on the online prediction, FOXO3a was screened out as the potential downstream of miRNA-96. Both mRNA and protein levels of FOXO3a were upregulated in OC cells transfected with miRNA-96 inhibitor (Figures 3A, 3B). Conversely to miRNA-96, FOXO3a was lowly expressed in OC tissues and cell lines relative to controls (Figures 3C, 3D). To verify the binding condition between FOXO3a and miRNA-96, pmirGLO-miRNA-96-wt, pmirGLO-miRNA-96-mut, and the negative control pmirGLO were constructed for Dual-Luciferase Reporter Gene Assay. The relative Luciferase activity remarkably decreased in cells co-transfected with pmirGLO-miRNA-96-wt and FOXO3a overexpression vector. However, we did not find evident changes in the Luciferase activity in other groups (Figure 3E). Therefore, we indicated the binding between FOXO3a and miRNA-96.

MiRNA-96 Negatively Regulated FOXO3a

We thereafter speculated that FOXO3a may participate in the miRNA-96-mediated malignant progression of OC. Here, sh-FOXO3a was constructed, and its transfection efficacy was verified by qRT-PCR and Western blot (Figure 4A). The rescue experiments illustrated that the decreased viability due to miRNA-96 knockdown was partially reversed by FOXO3a overexpression (Figure 4B). The inhibited migratory abilities in the OC cells with miRNA-96 knockdown were reversed by the overexpression of FOXO3a as well (Figures 4C, 4D).

Discussion

OC is a common tumor in the female reproductive system with a relatively high incidence. Effective screening and diagnostic strategies of OC are still lacked. Due to the high recurrent rate

Table I. Association of miR-96 expression with clinicopathologic characteristics of ovarian cancer.

Parameters	Number of cases	miR-96 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.298
< 60	13	8	5	
≥ 60	23	10	13	
T stage				0.298
T1-T2	23	13	10	
T3-T4	13	5	8	
Lymph node metastasis				0.070
No	25	15	10	
Yes	11	3	8	
Distance metastasis				0.025
No	18	11	7	
Yes	14	3	11	

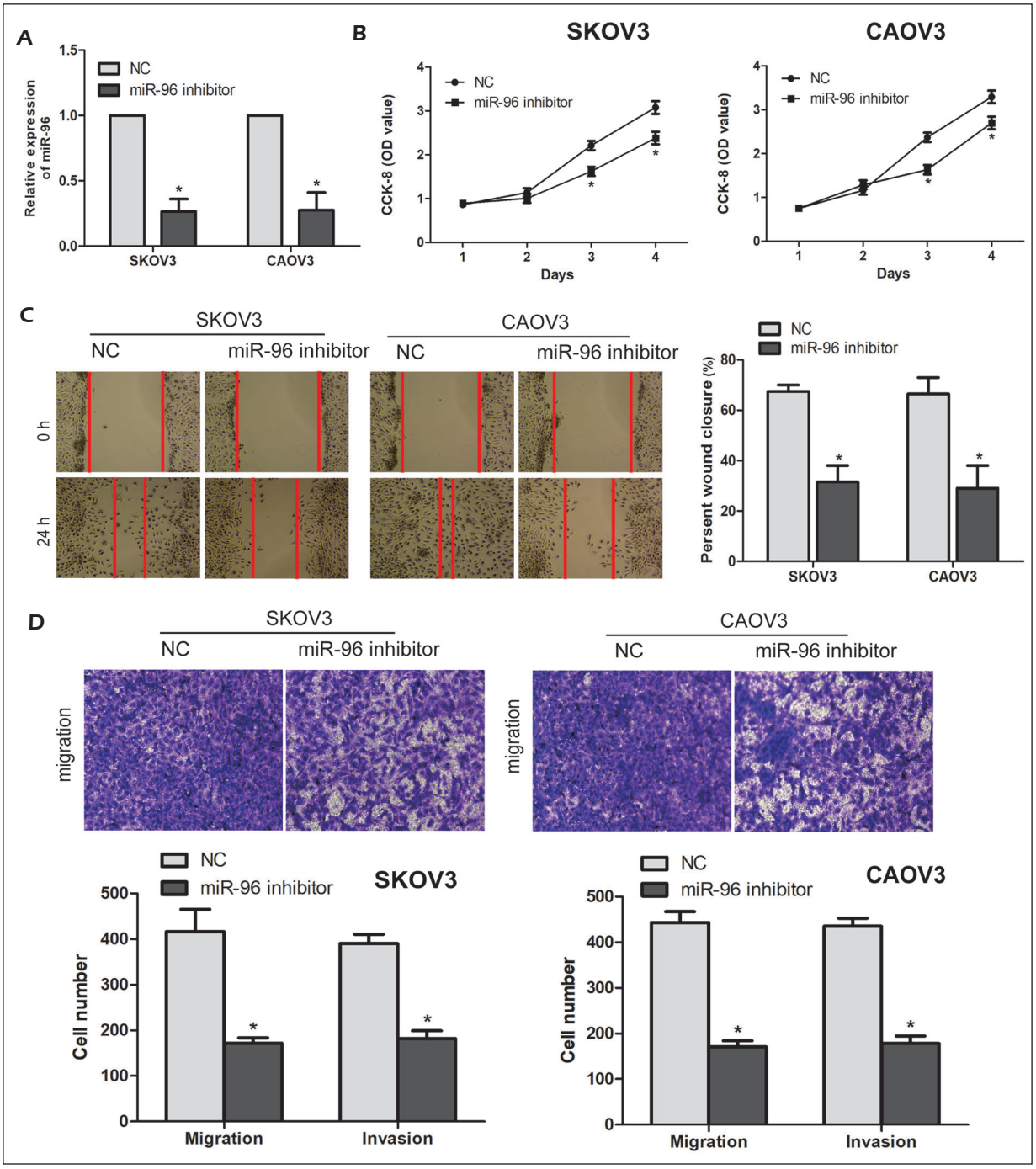

Figure 2. Knockdown of miR-96 inhibited OC cells to migrate and invade. **A,** Transfection efficacy of miR-96 inhibitor in SKOV3 and CAOV3 cells. **B,** CCK-8 assay examined viability in SKOV3 and CAOV3 cells transfected with miR-96 inhibitor or NC. **C,** Wound healing assay examined percentage of wound closure in SKOV3 and CAOV3 cells transfected with miR-96 inhibitor or NC (magnification 20×). **D,** Transwell assay examined migration of SKOV3 and CAOV3 cells transfected with miR-96 inhibitor or NC (magnification: 40×). *$p<0.05$.

and drug-resistance, the mortality of OC ranks first in gynecological malignancies[1-3]. The complex and diverse etiology of OC encourages us to fully elucidate its specific mechanism[4]. A great number of studies have proved that miRNAs are closely related to the occurrence and progression of OC[4,5]. MiRNAs may serve as oncogenes or tumor-suppressor genes during the progression

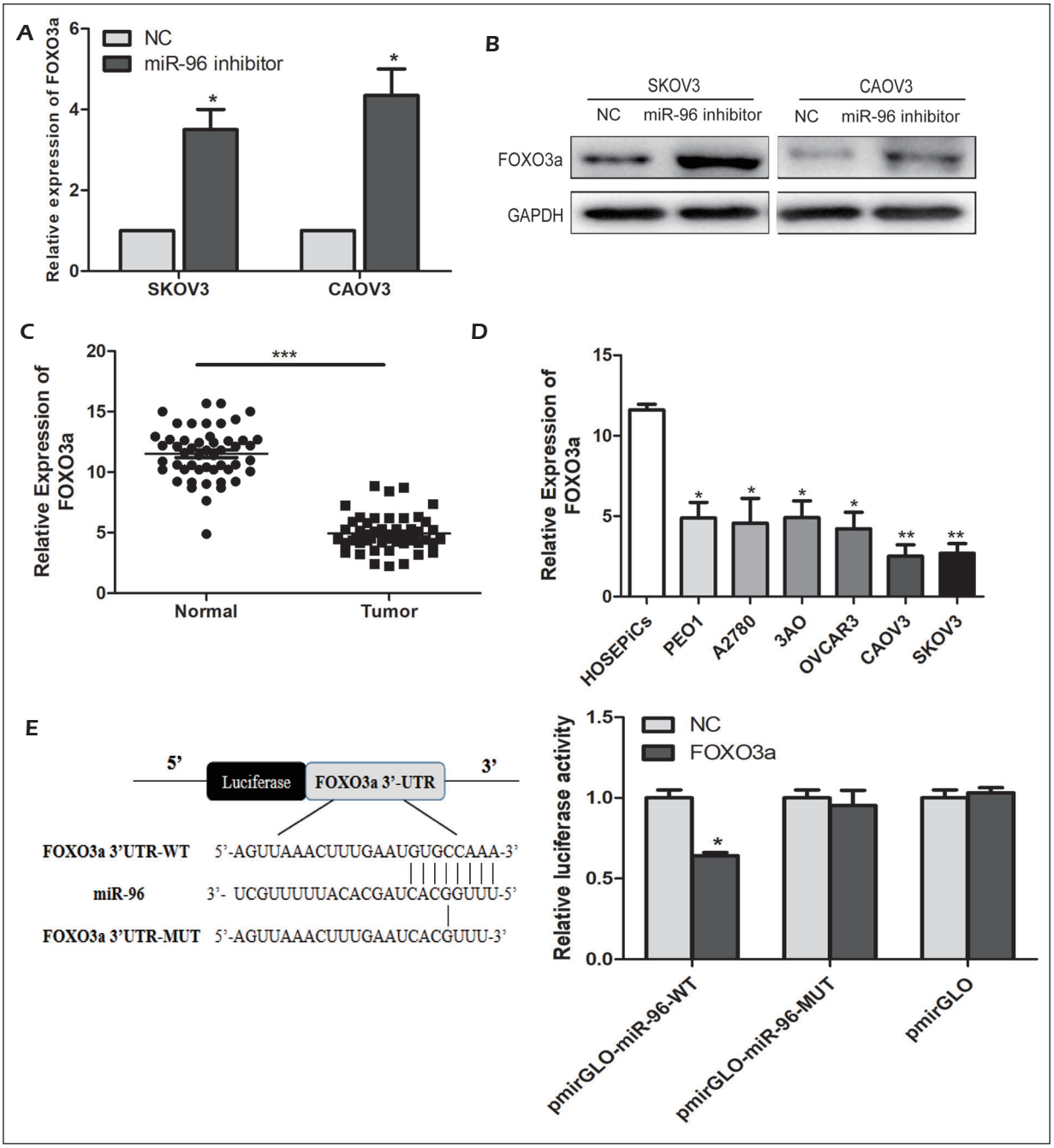

Figure 3. FOXO3a was lowly expressed in OC. **A,** Relative level of FOXO3a in SKOV3 and CAOV3 cells transfected with miR-96 inhibitor or NC determined by qRT-PCR. **B,** Protein level of FOXO3a in SKOV3 and CAOV3 cells transfected with miR-96 inhibitor or NC determined by Western blot. **C,** Relative level of FOXO3a in ovarian cancer tissues and adjacent normal tissues determined by qRT-PCR. **D,** Relative level of FOXO3a in ovarian cancer cell lines and controls determined by qRT-PCR. **E,** pmirGLO-miR-96-wt, pmirGLO-miR-96-mut and negative control pmirGLO were constructed for dual-luciferase reporter gene assay. Luciferase activity examined in SKOV3 and CAOV3 cells co-transfected with pmirGLO-miR-96-wt/pmirGLO-miR-96-mut/pmirGLO and FOXO3a overexpression vector/NC, respectively. *p<0.05, **p<0.01, ***p<0.001.

of OC, which are required to be further investigated[6-8]. MiRNAs are endogenous non-coding RNAs, with 18-25 bp in length. They exert vital functions by binding to target mRNAs, thus mediating the expressions and activities of the downstream gene expressions and activities[7-9]. A variety of biological progressions could be mediated by certain miRNAs, such as organ

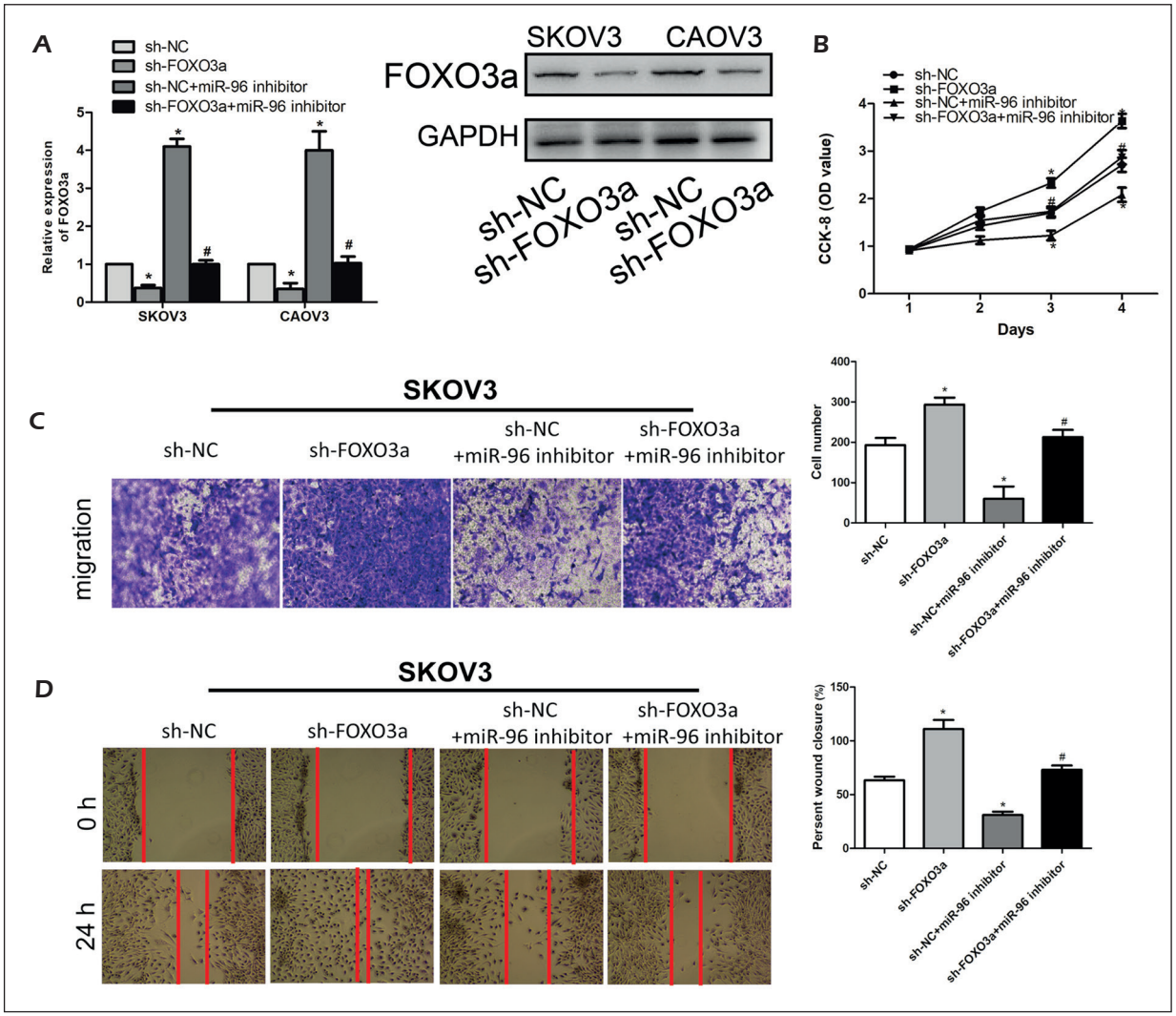

Figure 4. MiR-96 negatively regulated FOXO3a. SKOV3 and CAOV3 cells were transfected with sh-NC, sh-FOXO3a, sh-NC+miR-96 inhibitor, or sh-FOXO3a+miR-96 inhibitor, respectively. **A,** Relative level of FOXO3a in each group determined by qRT-PCR and Western blot. **B,** CCK-8 assay examined viability in each group. **C,** The transwell assay examined migration in each group (magnification: 40×). **D,** Wound healing assay examined percentage of wound closure in each group (magnification: 20×) $*^{\#}p<0.05$.

development, cellular behaviors, aging, etc.[9-12]. In molecular biology researches[13,14], the potential targets of miRNAs are generally predicted by online bioinformatics method. Here, we predicted the downstream of miRNA-96 on TargetScan (http://www.targetscan.org) and FOXO3a was screened out. We first examined the expression level of miRNA-96 in OC tissues and cell lines. MiRNA-96 was highly expressed in OC. The transfection of miRNA-96 inhibitor attenuated the migratory and invasive abilities of SKOV3 and CAOV3 cells. Some researchers[15,16] reported that the gene mutation and promoter methylation are the two major reasons for abnormal upregulation of miRNA in tumors. MiRNA-96 is a recently discovered member of the miR-183 family and locates on chromosome 7q32.2, a high-risk region for gene deletions and mutations[21,22]. The biological functions of miRNA-96 vary a lot in different tumors. Through relative analyses, we found that miRNA-96 gene sequence was not located on the reported region of the OC-relevant gene deletion[23-25]. Hence, the gene mutations are considered to be involved in miRNA-96-mediated progression of OC, and the specific mechanism still needs to be further explored.

According to additional domains and sequence conservation[26-28], FOX family is classified into several subfamilies, namely FOXM, FOXK, FOXA, and FOXO families. FOXO1, FOXO3a,

FOXO4, and FOXO6 exist in mammals[29-31]. Under different pathological conditions, FOXO3a could be regulated by different miRNAs[32-34]. FOXO3a is directly targeted by miR-155 in ischemic renal diseases and some types of cancer[35-36]. Besides, FOXO3a in the malignant progression of OC have been identified in previous studies, ant it is utilized as a therapeutic target for tumor treatment[37,38]. In this analysis, FOXO3a was observed to be the target gene of miRNA-96. Moreover, FOXO3a was negatively regulated by miRNA-96 in OC at both mRNA and protein levels.

Through a series of rescue experiments, we illustrated the role of miRNA-96/FOXO3a axis in accelerating the malignant progression of OC. The knockdown of miRNA-96 attenuated the proliferative and migratory abilities of OC cells, which were partially reversed by FOXO3a knockdown. Our findings provide novel directions in the targeted therapy of OC.

Conclusions

In summary, we found that the knockdown of miRNA-96 attenuated proliferative and migratory abilities of OC cells by targeting FOXO3a. MiRNA-96 accelerated the malignant progression of OC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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