European Review for Medical and Pharmacological Sciences 2020; 24: 10375-10382

Circ_0005276 aggravates the development of epithelial ovarian cancer by targeting ADAM9

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Abstract. – OBJECTIVE: Our purpose was to assess the relationship between circ_0005276 and clinical features of epithelial ovarian cancer (EOC), and to illustrate the regulatory effect of circ_0005276 on migratory potential in EOC cells.

PATIENTS AND METHODS: EOC tissues and adjacent normal ones were collected from 49 EOC patients. Relative levels of circ_0005276 and ADAM9 in EOC tissues were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The relationship between circ_0005276 and clinical features of EOC patients was analyzed. Moreover, migratory potentials of CAOV3 and SKOV3 cells affected by circ_0005276 were examined by tra... and wound healing assay. Regulatory ef... circ_0005276/ADAM9 feedback loop on t... velopment of EOC were finally detected b... ciferase assay and rescue experiments.

RESULTS: It was found that circ_0005276 w... upregulated in EOC tissues ... level wa... positively linked to rates of ... metasta... sis and distant metastasis ... OC pa... ts. Survival analysis showed wo... OS and ... EOC patients expressing a hig... l of ... than those with a low... el. ... knockdown of circ_0005276 at... ated mig... potentials in EOC cells. AD... was verifie... the target gene bindi... 05276, and ... evel was positively re... ted ... c_0005276. Notably, circ_0005... aggravate... development of EOC by t... eting ADAM9.

CON... SIONS: Circ_000... 6 is highly expres... n EOC ... ues, and its level is positively ... to m... stasis. Serving as an unfavorable ... progn... of EOC, circ_0005276 aggrava... e deve... ment of EOC by upregu... ADA...

Key... rds:
...276, ADAM9, EOC.

Introduction

Epithelial ovarian cancer (EOC) is the number one killer among the top three tumors in the female reproductive ... ste... cent y... ther-apeutic strategi... EOC ... on c... entional treatment, ad... ed surgery, ... notherapy have been ... mproved. N... theless, the overall su... val o... is still unsatisfactory[2,3]. It is reported that in t... ited States, the 5-year sur... EOC was 4... n 2015, which was 3... and 18% in advanced EOC patients with ...O III and FI... IV, respectively[4,5]. The mor... of EOC in ... ina shows an upward trend[6]. T... thogenfes... nd etiology of EOC require to be ... ...lored[7].

The ... epigenetics in tumorigenesis has ...een well concerned[8,9]. Without changing gene ...es, epigenetics can control gene expres... ons ... mediating transcription, histone modification, DNA methylation or chromatin remodeling at various levels[9-11]. Non-coding RNAs have been highlighted during the malignant development of tumors, which mediate oncogenes and tumor suppressors by degrading target mRNAs[12,13].

Unlike traditional linear RNAs, circRNAs display a closed loop structure. Due to the specific structure, circRNAs are stably expressed and hardly degraded by RNA exonucleases[14,15]. The ceRNA hypothesis proposes that circRNAs sponge corresponding miRNAs that share common sequences in the promoter region, and thereafter regulate downstream genes of miRNAs[16-18]. Differentially expressed circRNAs are involved in the development of many types of tumors[18-20]. This paper mainly explores the relationship between circ_0005276 and clinical features of EOC, as well as the regulatory effect of circ_0005276 on migratory potential in EOC cells.

Patients and Methods

EOC Patients and Samples

Baseline characteristics of enrolled 49 EOC patients were listed in Table I. Patients were patho-

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Table I. Association of LINC00675 with clinicopathological characteristics of ESCC patients.

Parameters	No. of cases	circ_0005276 expression		
		Low (%)	High (%)	
Age (years)				0.698
< 60	23	12	11	
≥ 60	26	15	11	
Gender				
Male	28	16	12	
Female	21	11		
T stage				0.656
T1-T2	24	14	10	
T3-T4	25	13		
Tumor size (cm)				0.397
< 4	25	14	11	
≥ 4	24	13	11	
Lymph node metastasis				0.035
No	26	18	8	
Yes	23	9	14	
Distance metastasis				0.019
No	29	20		
Yes	20		13	

logically diagnosed with EOC and older than 18 years old. EOC tissues and adjacent normal tissues were collected. Exclusion criteria: (1) secondary ovarian cancer patients, (2) patients with history of other malignancies or anti-tumor treatment. (3) patients with inadequate clinical data. Tumor staging was conducted based on the guidelines proposed by the Union for International Cancer Control (UICC). This investigation was approved by the Ethics Committee of Binzhou Medical University Hospital and conducted after informed consent was obtained from each subject.

Cell Culture

Human ovarian cancer cell lines (SKOV3, OVCAR3, PEO1, A2780, 3AO, HAOV3) and a normal human ovarian surface epithelial cell line (HOSEpiCs) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 mL streptomycin in a 5% CO2 incubator at 37°C.

Transfection

Cells inoculated in 6-well plates were cultured to 30-40% confluence. They were transfected with plasmids constructed by GenePharma (Shanghai, China), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 48 h later, cells were collected for the following use.

Transwell Migration Assay

A total of 200 µL of suspension (5.0×10^5/mL) was inoculated in the upper transwell chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate with 500 µL of medium containing 10% FBS in the bottom. After 48-h incubation, bottom cells were reacted with 15-min methanol, 20-min crystal violet and captured using a microscope. Finally, migratory cells were counted in 5 random fields per sample (magnification 40×).

Wound Healing Assay

A total of 5.0×10^4 cells suspended in culture medium containing 1% FBS were inoculated per well of 6-well plates, and an artificial wound was created. 24 h later, the percentage of wound closure was calculated.

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

RNAs extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNAs underwent qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the internal reference. Each sample was performed

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in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. Circ_0005276: forward: 5'-GCTAAATGGTATCCAGGGTGC-3' and reverse: 5'-CCCTCCTCCACAGTGAAAGC-3', ADAM9: forward: 5'-GCTAGTTGGACTGGAGATTTGG-3' and reverse: 5'-TTATTACCACAGGAGGGAGCAC-3', GAPDH: forward: 5'-GCTTTCTTTCCTTTCGCGCT-3' and reverse: 5'-TTTGCGGTGGAAATGTCCTT-3'.

Western Blotting

Cells were lysed for isolating cellular protein and electrophoresed. Protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h. Membranes were reacted with primary and secondary antibodies for indicated time. Band exposure and analyses were finally conducted.

Luciferase Assay

EOC cells were inoculated in 24-well plates, and co-transfected with circ_0005276 WT/ circ_0005276-MUT and NC/pcDNA-A respectively, using Lipofectamine 2000. ls were lysed for determining relative Lucif activity 48 h later.

Statistical Analysis

Statistical Product Servic (SPSS) 22.0 (IBM, used for data ana . Da expressed as mean ± stand eviation. ences between two gro analyzed t-test. Kaplan-Meie rves depicted for survival analysi followed b -rank test. Pearson cor ion test was ap for evaluating the r onship between exp ession levels of circ 05276 ADAM9 in EOC tissues. $p<0.$ ed tha difference was statistica ificant

Results

c_0005276 Was Highly pressed In EOC

0005276 was highly expressed in EOC tiss than that in normal ones (Figure 1A). Identically, *in vitro* level of circ_0005276 was upregulated in EOC cell lines (Figure 1B).

Circ_0005276 Expression Was Correlated With EOC Metastases

Included EOC patients were assigned into two groups based on the median level of circ By analyzing their clinical data, it w and th circ_0005276 level was positively elated with rates of lymphatic metastasis a tant metastasis in EOC patients (Table I). rmore, Kaplan-Meier curves illu ed wor rall survival (Figure 1C) a isease-free s (Figure 1D) in EOC nts ex sing a l level of circ_000527 abo esults i dicate that circ_0005276 ay ictive l arker for the prognos EOC.

Knockdo c_000527 uppressed Migrato Pote of EOC

Transfection effica sh-circ_0005276 was firs in CAOV3 a KOV3 cells (Figure l Transwell assay uncovered that migratory number wa ecreased after knockdown of 0005276 (F e 2A). Similarly, wound closu ercentage markedly decreased in EOC cell with sh-circ_0005276 (Figure 2B). It luded that circ_0005276 promotes migratory potential of EOC.

05276 Bound to ADAM9

Through online prediction, binding sites in the promoter regions of circ_0005276 and ADAM9 were discovered (Figure 3A). Overexpression of ADAM9 markedly decreased Luciferase activity in wild-type circ_0005276 vector, verifying the binding relationship between circ_0005276 and ADAM9 (Figure 3B). Western blotting and qRT-PCR showed that the protein and mRNA levels of ADAM9 were downregulated in EOC cells transfected with sh-circ_0005276, compared with those in EOC cells transfected with sh-NC (Figure 3C, 3D). Similar to circ_0005276, ADAM9 was upregulated in EOC as well (Figure 3E, 3F). Moreover, a positive relationship was identified between expression levels of circ_0005276 and ADAM9 in EOC tissues (Figure 3G).

Overexpression of ADAM9 Reversed Regulatory Effect of Circ_0005276 on Migratory Potential of EOC

To further uncover the involvement of ADAM9 in EOC development, pcDNA3.1-ADAM9 was constructed. It was found that co-transfection of pcDNA3.1-ADAM9 upregulated the decreased level of ADAM9 in EOC cells with circ_0005276 knockdown (Figure 4A). Notably,

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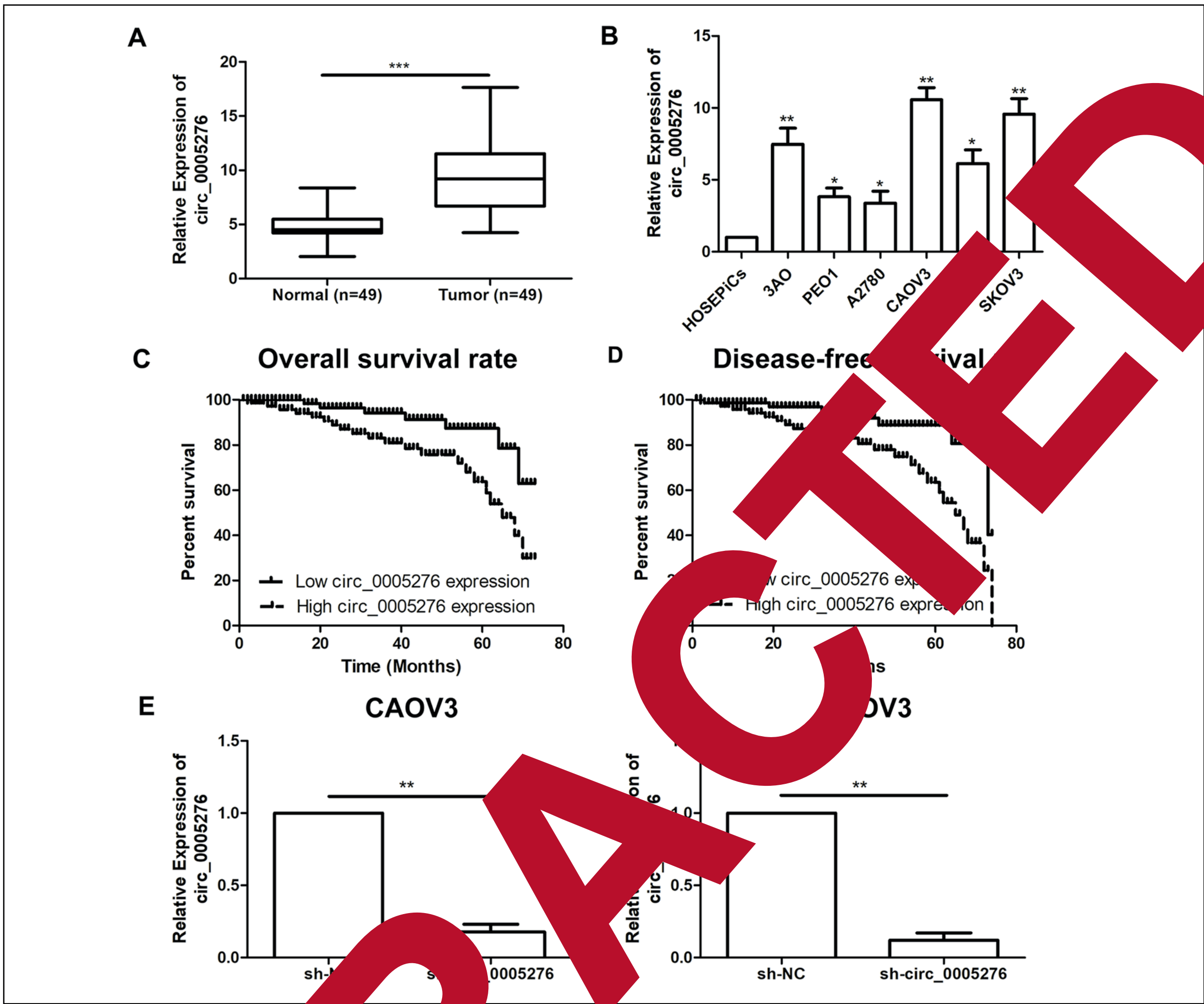

Figure 1. Circ_0005276 is hig... ...ression levels of circ_0005276 in EOC tissues and normal ovarian tissues. **B,** Expression lev... f c... in EOC ... lines. **C, D,** Kaplan-Meier curves show overall survival (**C**) and disease-free survival (**D**... OC pati... h high or low level of circ_0005276. **E,** Transfection efficacy of sh-circ_0005276 in CAOV3 and SKOV... s. Data are ex... as mean ± SD. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

overexpr... on of ADAM9 ... ased migratory cell n... er (Fig... e 4B) and w... und closure per-cent... (Figu... C) in EOC cells transfected with ... ir... 05276 ... is demonstrated that circ_00... aggra... s the development of ... by up... lati... ADAM9.

Discussion

...Malignant biological behaviors, pathogenesis, ... ogression mechanisms of EOC are research foc... s[1-3]. Atypical symptoms in the early stage of EOC result in the poor prognosis since most of EOC patients are initially diagnosed in middle or advanced stage[3-5]. Seeking abnormally expressed genes in EOC and analyzing their potential functions contribute to improve survival of affected patients[5,6]. Epigenetics is a hot topic in cancer researches, i.e., gene expressions are heritably changed while nuclear DNA sequences remain unchangeable[6-8]. Genetics provides information on various proteins that synthesize epigenetic modified proteins, and epigenetic information regulates a set of expressed genes and the degree of expressions[8,9]. Precise expressions of genes are not only controlled by DNA sequences, but also subject to epigenetics. Posttranscriptional and transcriptional regulations are two major components of epigenetics[10]. By regulating protein

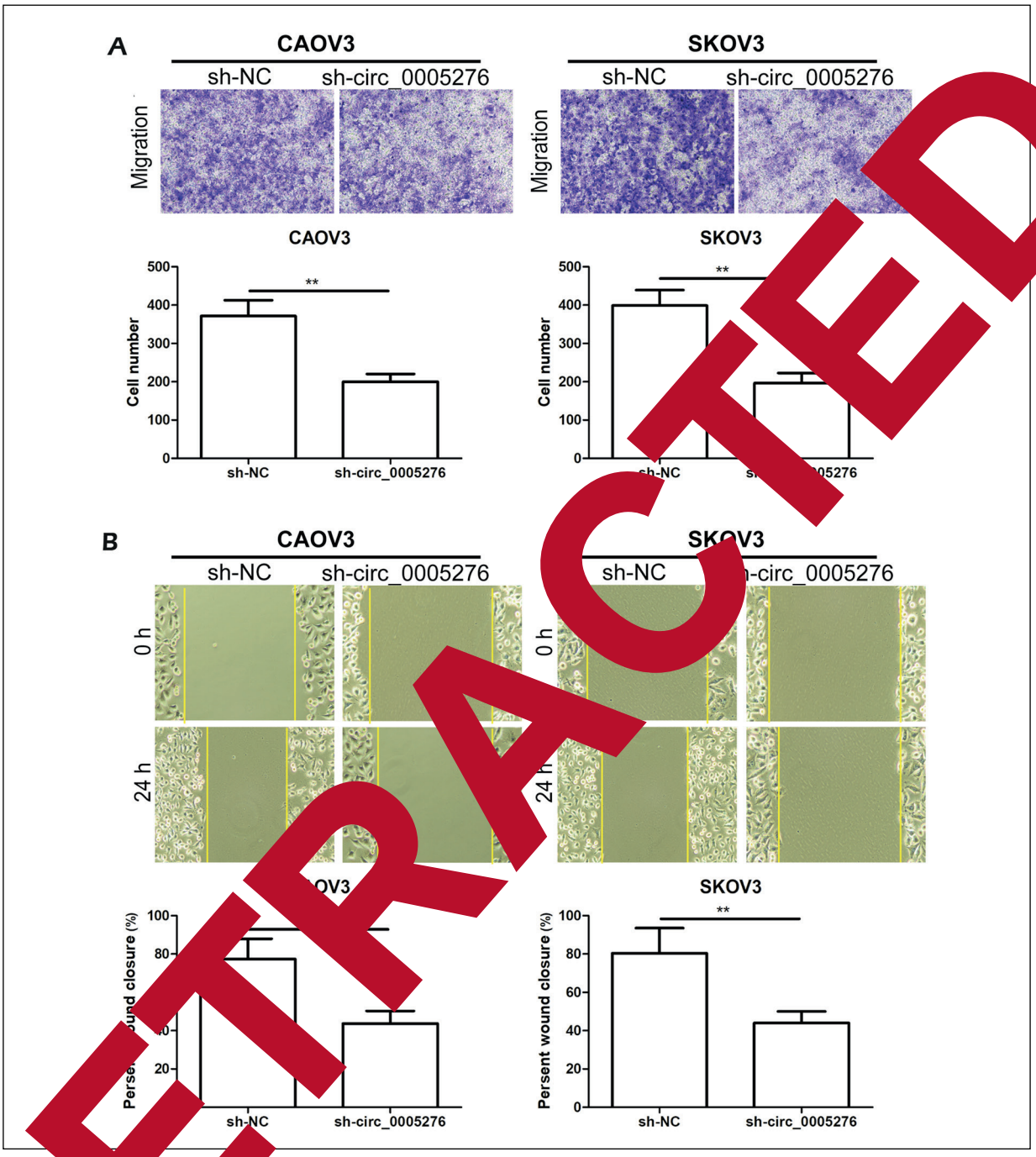

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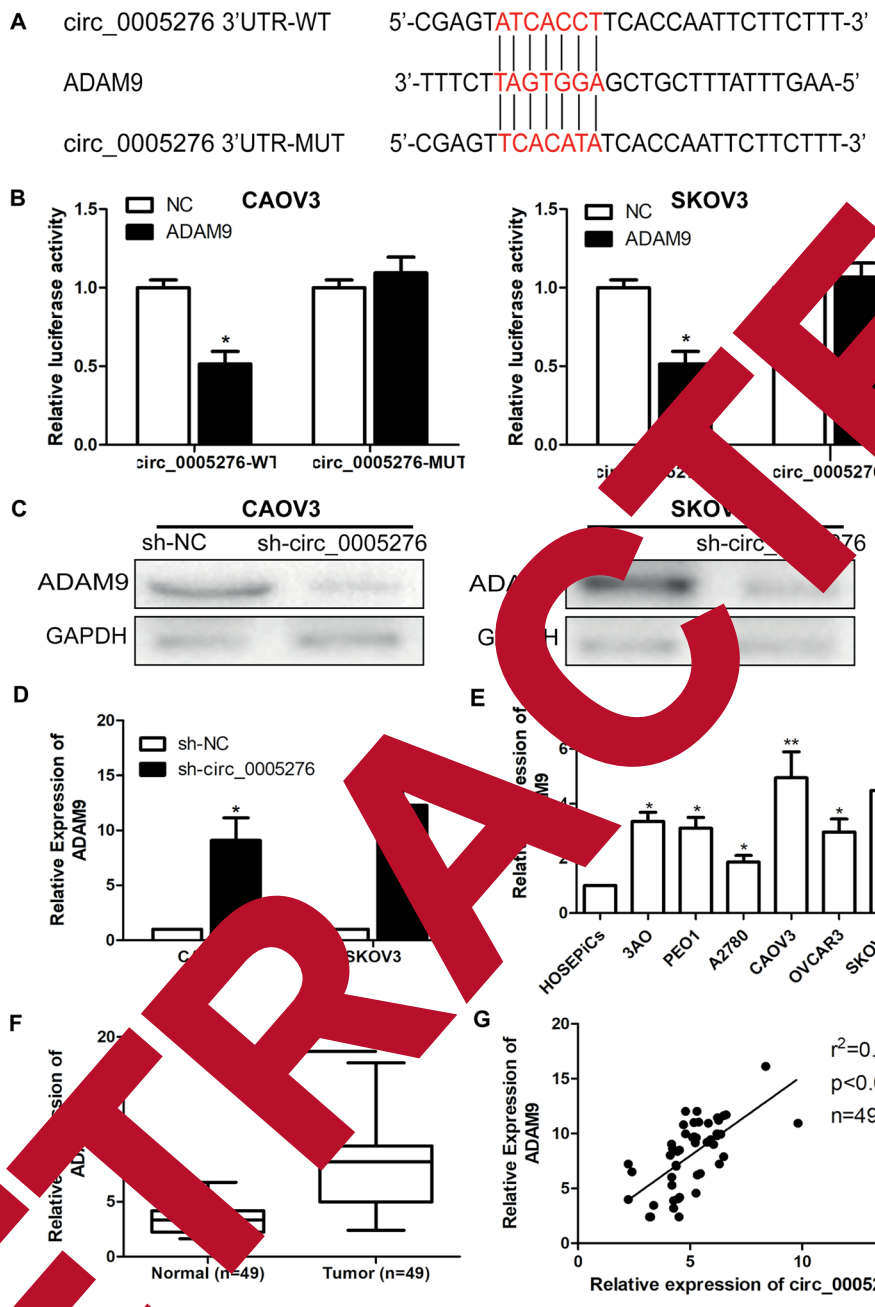
Figure 2. Knockdown of circ_0005276 suppresses migratory potential of EOC. **A,** Migration in CAOV3 and SKOV3 cells transfected with sh-circ_0005276 or sh-NC (magnification: 40×). **B,** Wound closure percentage in CAOV3 and SKOV3 cells transfected with sh-circ_0005276 or sh-NC (magnification: 40×). Data are expressed as mean ± SD. $^{**}p<0.01$.

modification, non-coding RNAs, and DNA methylation, epigenetics is responsible for cell phenotypic changes[11,12], and it is also closely involved in tumor development[12,13].

CircRNAs contain exon sequences and are spliced at classical splice sites[14,15]. Circ_0005276 has been identified to participate in cell apoptosis, cell cycle, and other phenotypes. It is upregulated in many types of tumor cells and related to tumor development[19]. However, the mechanism of circ_0005276 in EOC is not clear. Therefore, the objective of this study was firstly to elucidate the



re 3. 0005276 binds to ADAM9. **A,** Binding sites in the promoter regions of circ_0005276 and ADAM9. **B,** se activities in CAOV3 and SKOV3 cells co-transfected with circ_0005276-WT/circ_0005276-MUT and NC/pcDNA-AD **C, D,** C) and mRNA levels (**D**) of ADAM9 in CAOV3 and SKOV3 cells transfected with sh-circ_0005276 or sh-N Expressio levels of ADAM9 in EOC cell lines. **F,** Expression levels of ADAM9 in EOC tissues and normal ovarian tis ve correlation between expression levels of circ_0005276 and ADAM9 in EOC tissuees. Data are expressed an <0.05, **p<0.01, ***p<0.001.

enic role of circ_0005276 in the progression of C, as well as the specific mechanism of circ_0005276 regulating ADAM9. Circ_0005276 was upregulated in EOC tissues, and its level was positively linked to rates of lymphatic metastasis and distant metastasis. Survival analysis showed worse OS and DFS in EOC patients expressing a high level of circ_0005276. Besides, the

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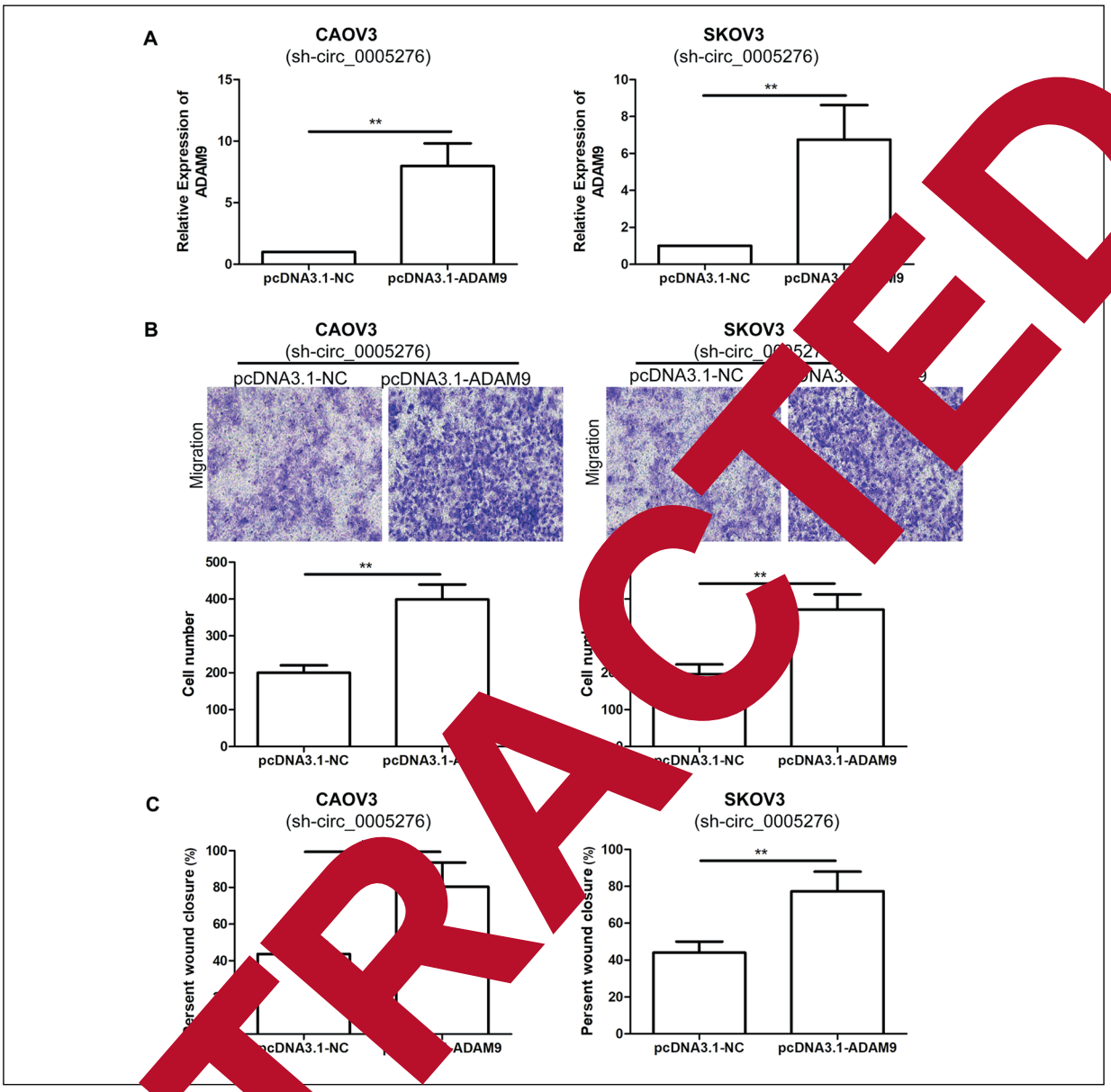

Figure 4. C[illegible]xpression of A[illegible]9 reverses regulatory effect of circ_0005276 on migratory potential of EOC. **A,** ADAM9 levels in C[illegible]3 and SKOV3 cells [illegible]sfected with sh-circ_0005276 and pcDNA3.1-NC/pcDNA3.1-ADAM9. **B,** Migration in CAO[illegible]nd SKOV3 cells co-tran[illegible]ected with sh-circ_0005276 and pcDNA3.1-NC/pcDNA3.1-ADAM9 (magnification: 40×). [illegible]ound c[illegible]e percentage in CAOV3 and SKOV3 cells co-transfected with sh-circ_0005276 and pcDNA3.1-NC/pcB[illegible]ADA[illegible](magnifi[illegible]tion: 40×). Data are expressed as mean ± SD. ***p*<0.01.

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[illegible]dow[illegible]ci[illegible]005276 attenuated migration potential of EOC cells. The above findings demonstrate that circ_0005276 aggravates the d[illegible]EOC.

CircRNA abolishes the inhibitory effect of miRNAs on their downstream genes, thus upregulating their levels, that is, the ceRNA theory[16-18]. CircRNAs are able to directly bind to proteins, thus inhibiting their activities or components of protein complexes. They also guide protein synthesis as translation templates[21,22]. Through bioinformatics prediction and Luciferase assay verification, ADAM9 was confirmed to be the downstream gene binding circ_0005276. ADMA9 is a transcription factor involved in malignant development of tumors[23,24]. ADAM9 participates in angiogenesis, tumor growth, and metastasis. Here, ADAM9 level was positively regulated by circ_0005276. Of note, ADAM9 was found to be responsible for migratory potential of EOC reg-

ulated by circ_0005276. This research provides novel targets for clinical treatment and prognosis of EOC.

Conclusions

These results showed that circ_0005276 is highly expressed in EOC tissues, and its level is positively linked to metastasis. Serving as an unfavorable gene in the prognosis of EOC, circ_0005276 aggravates the development of EOC by upregulating ADAM9.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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