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ORIGINAL RESEARCH

Circular RNA Circ_0025033 Promotes the Evolvement of Ovarian Cancer Through the Regulation of miR-330-5p/KLK4 Axis

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Hailing Cheng[1,*]
Ning Wang[1,*]
Jun Tian[1]
Yanyun Li[1]
Lu Ren[1]
Zhenyu Shi[2]

[1]Department of Obstetrics and Gynecology, Huaihe Hospital of Henan University, Kaifeng, Henan, People's Republic of China; [2]Henan Medical School, Henan University, Kaifeng, Henan, People's Republic of China

*These authors contributed equally to this work

Background: Circular RNAs (circRNAs) are significant molecular markers in various types of human cancers. The functional mechanism of circRNA_0025033 (circ_0025033) in ovarian cancer (OC) was discussed in the current study.

Methods: The quantitative real-time polymerase chain reaction (qRT-PCR) was used for determining the circ_0025033 and microRNA-330-5p (miR-330-5p) levels. Cell Counting Kit-8 (CCK-8) and transwell assays were separately exploited to analyze cell viability and migration/invasion. Cell apoptosis was assessed using flow cytometry. The protein levels of epithelial–mesenchymal transition (EMT) related makers and kallikrein-related peptidase 4 (KLK4) were measured by Western blotting. The target combination was confirmed by dual-luciferase reporter assay, RNA immunoprecipitation (RIP) and RNA pull-down assays. And the effect of circ_0025033 on OC in vivo was explored via xenograft tumor assay.

Results: Circ_0025033 was overexpressed in OC tissues and cells. Circ_0025033 knockdown inhibited OC cell viability, migration, invasion and EMT while expedited apoptosis. MiR-330-5p was a target of circ_0025033 and circ_0025033 regulated OC cellular behaviors by sequestering miR-330-5p. Moreover, miR-330-5p targeted KLK4 and circ_0025033 affected the KLK4 expression by sponging miR-330-5p. And miR-330-5p functioned as a tumor inhibitor in OC via targeting KLK4. In vivo, circ_0025033 promoted OC growth by the miR-330-5p/KLK4 axis.

Conclusion: This study demonstrated that circ_0025033 contributed to the progression of OC via the miR-330-5p/KLK4 axis and might be a candidate target in the identification and treatment of OC.

Keywords: circ_0025033, ovarian cancer, miR-330-5p, KLK4

Introduction

Ovarian cancer (OC) is a familiar gynecological malignancy ranked as the seventh lethal cancer among females worldwide.[1] Despite having received the standard therapy, more than 70% of OC patients still underwent the tumor recurrence and eventually got the immedicable outcome in all probability.[2,3] Confronting to this conundrum, seeking more molecular biomarkers seems a sally port to advance the early diagnosis and treatment of OC patients. Accumulating researches have reported the significant roles of non-coding RNAs (ncRNAs) in OC.[4–6]

Circular RNAs (circRNAs), a subgroup of ncRNAs with covalently closed-loop structures, are ubiquitous in eukaryotic cells and can be used as transcriptional regulators by sponging microRNAs (miRNAs) in human cancers.[7,8] The dysregulated

Correspondence: Zhenyu Shi
Henan Medical School, Henan University, Section 229, North of Jinming Avenue, Kaifeng City, Henan Province, People's Republic of China
Tel +86-371-22922953
Email xzmkra@163.com

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circRNAs have testified to be closely associated with the formation and development of OC. For instance, circPLEKHM3 was down-regulated in OC and identified as a tumor inhibitor by the miR-9/BRCA1/DNAJB6/KLF4/AKT1 axis;[9] Guan et al illuminated that circPUM1 was up-regulated and improved the tumorigenesis of OC via targeting miR-615-5p or miR-6753-5p.[10] We noticed the up-regulation of circ_0025033 in OC tissues in a recent study,[11] while the report about its biological function in OC has not been published.

MiRNAs, a special type of short ncRNAs, were shown to participate in the regulation of OC repression or promotion by targeting the 3′-untranslated region (3′UTRs) of messenger RNA (mRNAs).[12,13] MiR-200a-3p was identified as a tumor promotor in OC via the modulation of PCDH9,[14] while miR-331-3p could suppress the proliferation and metastasis of OC cells via negatively targeting RCC2.[15] Some issued reports have proved that the low expression and inhibitory role of miR-330-5p in OC progression, and it could be used as the target of long ncRNA MIAT[16] and EWSAT1[17] in OC cells. However, it is unknown whether miR-330-5p is related to circ_0025033 in OC.

Kallikrein-related peptidase 4 (KLK4) is a subunit of KLK family and acts as an oncogene in many cancers such as triple-negative breast cancer,[18] oral squamous cell carcinoma,[19] and prostate cancer.[20] Gong et al reported that the up-regulation of KLK4 might be regarded as diagnostic index for OC patients.[21] Herein, we attended to investigate the relation between KLK4 and miR-330-5p in OC, as well as circ_0025033 and miR-330-5p or KLK4, which might promote the new understanding for the molecularly pathological mechanism of OC evolvement.

Materials and Methods

Tissue Acquisition and Cell Culture

Pairs of OC tissues and normal para-carcinoma tissues (n=51) were collected from OC patients who have received the ovariectomy at the Huaihe Hospital of Henan University in accordance with the Declaration of Helsinki. All patients provided the written informed consent. All samples were frozen in liquid nitrogen thoroughly. The current study was implemented with the approval of the Institute Review Ethics Committee of Huaihe Hospital of Henan University.

The source of all cells used in our research was TongPai Biotechnology Co., Ltd. (Shanghai, China). Roswell Park Memorial Institute-1640 (RPMI-1640; Hyclone, Logan, UT, USA) and Dulbecco's Modified Eagle's medium (DMEM; Hyclone) were used as the basic medium of OC cells (A2780 and SKOV3) and HOSE (human normal ovarian epithelial cells), respectively. Cell culture was performed using the mixed solution of basic medium, 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and 1% penicillin-streptomycin (Gibco) in a 37°C and 5% CO_2 incubator.

Cell Transfection

Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was exploited for transfecting oligonucleotides or vectors into OC cells, following the manufacturer's protocol. The oligonucleotides small interfering RNA (siRNA) targeting circ_0025033 (si-circ_0025033), miR-330-5p mimic and inhibitor (miR-330-5p and in-miR-330-5p), short hairpin RNA (shRNA) against circ_0025033 (sh-circ_0025033) and matched negative controls (si-NC, miR-NC, in-miR-NC and sh-NC) were purchased from GenePharma (Shanghai, China). The overexpression vectors pcDNA3.1-circ_0025033 and pcDNA3.1-KLK4 (circ_0025033 and KLK4) were acquired after inserting their sequences into the pcDNA3.1 vector (Invitrogen). Particularly, shRNA was transfected through the pSINsi vector (Takara, Beijing, China) to establish the OC cells stably expressed sh-circ_0025033 or sh-NC.

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

Following the RNA extraction by UNlQ-10 Column Trizol Total RNA Isolation Kit (Sangon, Shanghai, China), 1 μg RNA was employed for the complementary DNA (cDNA) synthesis using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Subsequently, the qRT-PCR was administered through QuantiFast SYBR® Green PCR Kit (Qiagen) by the ABI StepOne system (Life Technologies, Carlsbad, CA, USA). Ultimately, the $2^{-\Delta\Delta Ct}$ approach was applied to analyze the relative expression levels.[22] The endogenous controls in our study were glyceraldehyde-3-phosphate dehydrogenase (GAPDH, for circ_0025033) and U6 (for miR-330-5p). The sequences of all primers were shown as below: circ_0025033: 5′-GTGAGCCAGCTTGAGAACAC-3′ (forward) and 5′-TTGAGAATCAGTGGCCGACG-3′ (reverse); miR-330-5p: 5′-TCTCTGGGCCTGTGTCTTAGGC-3′ (forward) and 5′-TTAATGGGGTGATTGGTGGT-3′ (reverse); GAPDH: 5′-CCACATCGCTCAGACAC

CAT-3′ (forward) and 5′-CCAGGCGCCCAATACG-3′ (reverse); U6: 5′-CTCGCTTCGGCAGCACA-3′ (forward) and 5′-ACGCTTCACGAATTTGCGT-3′ (reverse).

Cell Viability Detection

Cell Counting Kit-8 (CCK-8; Sangon) was used for the examination of cell viability of OC cells. At 0 h, 24 h, 48 h and 72 h after transfection, CCK-8 solution was pipetted to OC cells in the 96-well plates with 10 μL/well. Incubating for 2 h, the optical density (OD) value at 450 nm was detected with a microplate reader (Beyotime, Shanghai, China).

Cell Migration and Invasion Detection

The migration and invasion abilities of OC cells were evaluated using transwell 24-well chamber (Corning Inc., Corning, NY, USA). Firstly, 4 × 10^5 cells were resuspended in serum-free medium to produce cell suspension, which was seeded into the upper chamber. And the lower chamber was added with 600 μL corresponding medium with 10% FBS. Whereafter, unmigrated cells were erased by wet cotton swabs post-incubation for 24 h, while cells on the reverse side of the membrane were fastened using 4% paraformaldehyde (Sangon). After cell dyeing by 0.1% crystal violet (Sangon), the images were collected and the number of migrated cells was counted. For the detection of cell invasion, the membrane of the upper chamber was covered with material (Corning Inc.) prior to cell inoculation, then the remaining procedures were performed as depicted above. Eventually, the migration or invasion ability (migrated or invaded cells/total cells × 100%) was calculated.

Cell Apoptosis Assessment

The determination of cell apoptosis was executed by flow cytometry. Concisely, transfected OC cells were digested, centrifuged, and resuspended in 1 × binding buffer (Invitrogen). Then, Annexin V-fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (PI; Invitrogen) were applied for the double staining according to the guidance of the manufacturer. Through the flow cytometer (BD Biosciences, San Diego, CA, USA), the labeled cells of Annexin V+/PI- and Annexin V+/PI+ were discerned as the apoptotic cells. The apoptosis rate was assessed following the formula: apoptotic cells/total cells × 100%.

Western Blotting

After the protein extraction and quantification by the Radio Immunoprecipitation Assay (RIPA) lysis buffer (KeyGen, Nanjing, China) and BCA Protein Assay Kit (Takara) respectively, the mixture of proteins (40 μg per sample) and loading buffer (Takara) was loaded on 10% sodium dodecyl sulfate polyacrylamide gel to conduct the electrophoresis for 2 h. Then, the separated proteins on the gel were transferred onto the polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) and blocked using 5% nonfat milk (Beyotime). Afterwards, the membranes were incubated with primary antibodies at 4°C overnight and secondary antibody for 1 h at indoor temperature, followed by the detection of the SignalFire™ Plus ECL Reagent (Cell Signaling Technology (CST), Boston, MA, USA). The protein bands were observed by ImageLab software version 4.1 (Bio-Rad Laboratories, Hercules, CA, USA) and the signal levels were analyzed as described earlier.[23] The antibodies used in this report were listed as follows: anti-E-cadherin (CST, #3195, 1:1000), anti-N-cadherin (CST, #4061, 1:1000), anti-Vimentin (CST, #5741, 1:1000), anti-KLK4 (Abcam, Cambridge, UK, ab181402, 1:1000), internal control anti-β-actin (CST, #4970, 1:1000), and goat anti-rabbit IgG/HRP-linked secondary antibody (CST, #7074, 1:3000).

Dual-Luciferase Reporter Assay

The recombinant luciferase reporter plasmids needed to be constructed firstly. For circ_0025033, the wild-type (WT) fragment with putative miR-330-5p binding sites and mutant-type (MUT) fragment with mutant sites were severally cloned into the psiCHECK-2 vector (Promega, Madison, WI, USA). The positive recombinant reporters were named as circ_0025033 WT and circ_0025033 MUT. For KLK4, the KLK4 3′-UTR WT and KLK4 3′-UTR MUT were obtained in a similar way as above. In the dual-luciferase reporter assay, OC cells were plated into the 96-well plates with 2 × 10^3 cells per well, and co-transfected with circ_0025033 WT/circ_0025033 MUT/KLK4 3′-UTR WT/KLK4 3′-UTR MUT and miR-330-5p/miR-NC, then the luciferase activities from cell lysates were detected using dual-luciferase assay system (Promega). Renilla luciferase activity was used to standardize the firefly luciferase activity of OC cells.

RNA Immunoprecipitation (RIP) Assay

RIP assay was implemented using the Magna RIP kit (Millipore). Shortly, OC cells transfected with miR-330-5p or miR-NC were lysed with RIP lysis buffer, following the incubation of magnetic beads conjugated with anti-Argonaute2 (anti-Ago2; Abcam, ab32381) and negative control anti-Immunoglobulin G (anti-IgG; Abcam, ab205718). After the digestion of proteinase K (Beyotime), RNA was obtained and qRT-PCR was used for measuring the level of circ_0025033.

RNA Pull-Down Assay

RNA pull-down assay was carried out to analyze the connection between circ_0025033 and miR-330-5p. Briefly, OC cells were transfected with biotinylated miR-330-5p (Bio-miR-330-5p) or mutant type (Bio-miR-330-5p mut) with Bio-NC (Sangon) as the internal control. At 48 h post-transfection, cell lysates were incubated with magnetic beads (Invitrogen) to recognize biotin at 4°C for 4 h, then the relative expression of circ_0025033 was examined by qRT-PCR after RNA isolation from magnetic beads.

Xenograft Tumor Assay

Female BALB/c nude mice (n=10, seven-week-old) from LAC Laboratory Animal Co. Ltd (Shanghai, China) were subcutaneously injected with A2780 cells (1×10^6) stably expressed sh-circ_0025033 or sh-NC. After one week, tumors were observable and tumor volume was estimated using the formula: length × width^2 ×0.5 weekly. 4 weeks later, mice were performed euthanasia and tumors were dissected. Following the weighing of tumors on an electronic scale, RNA and protein were isolated and the expression levels of circ_0025033, miR-330-5p and KLK4 were promptly determined using qRT-PCR or Western blotting. The animal assay was ratified by the Animal Ethics Committee of Huaihe Hospital of Henan University and strictly carried out in accordance with the NIH guidelines for Care and Use of Laboratory Animals.

Statistical Analysis

The statistical analysis was executed through SPSS 20.0 and GraphPad Prism 7. Data in our study were exhibited as mean ± standard deviation (SD) with three independent repetitions of all assays. Spearman correlation coefficient was performed to analyze the associated linear relationship. The difference comparison was conducted by Student's *t*-test and one-way analysis of variance (ANOVA) followed by Tukey test. Statistically, $P < 0.05$ indicated a significant difference.

Results

The Up-Regulation of circ_0025033 Was Exhibited in OC Tissues and Cells

To observe the involvement of circ_0025033 in OC, the expression of circ_0025033 was examined. The results of qRT-PCR demonstrated that the relative expression level of circ_0025033 was evidently increased in OC tissues compared with the normal tissues (Figure 1A). And this overexpression phenomenon of circ_0025033 was also verified in two OC cell lines (A2780 and SKOV3) relative to normal HOSE cells (Figure 1B). It was obvious that circ_0025033 was up-regulated in OC tissues and cells.

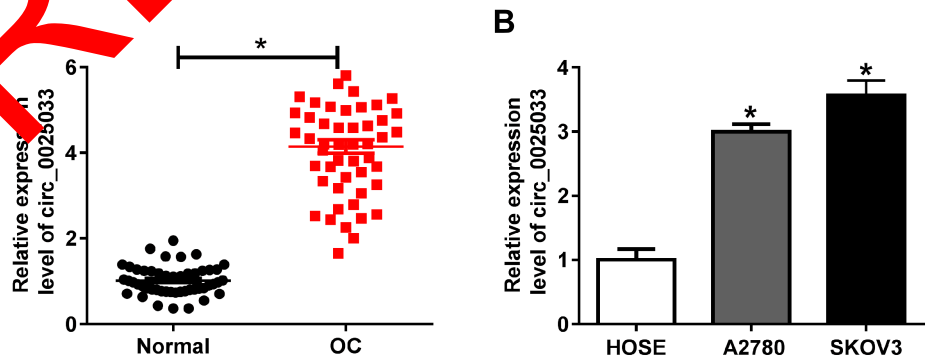

Figure 1 The up-regulation of circ_0025033 was exhibited in OC tissues and cells. (**A, B**) The qRT-PCR was conducted for the detection of circ_0025033 expression in OC tissues (**A**), A2780 and SKOV3 cells (**B**) and their controls. *$P < 0.05$.

Circ_0025033 Knockdown Reduced Cell Viability, Migration, Invasion and Epithelial–Mesenchymal Transition (EMT) While Promoted Apoptosis of OC Cells

For investigating the role of circ_0025033 in the biological processes of OC, si-circ_0025033 transfection was executed and its knockdown effect on circ_0025033 expression was successful in A2780 and SKOV3 cells (Figure 2A). The decreased expression of circ_0025033 refrained cell viability by contrast with si-NC group in CCK-8 assay (Figure 2B and C). Also, A2780 and SKOV3 cells transfected with si-circ_0025033 displayed the lower cell migration (Figure 2D) and invasion (Figure 2E) abilities in comparison to these cells transfected with si-NC. Additionally, flow cytometry indicated that the apoptosis rate of si-circ_0025033 group was considerably higher than that of si-NC group (Figure 2F). With respect to the EMT process, E-cadherin is deemed as an typical anti-EMT marker while N-cadherin and Vimentin are pro-EMT markers in cancer cells.[24,25] Through the analysis of Western blotting, we found the E-cadherin protein level was elevated but N-cadherin and Vimentin exhibited the opposite trend after circ_0025033 was knocked down in A2780 and SKOV3 cells (Figure 2G). Hence, circ_0025033 knockdown impeded the progression of OC cells in vitro.

Circ_0025033 Acted as a miR-330-5p Sponge

Numerous researches about circRNAs have clarified that they can serve as the "sponges" of different miRNAs to regulate cancer development. Herein, CircRNA Interactome

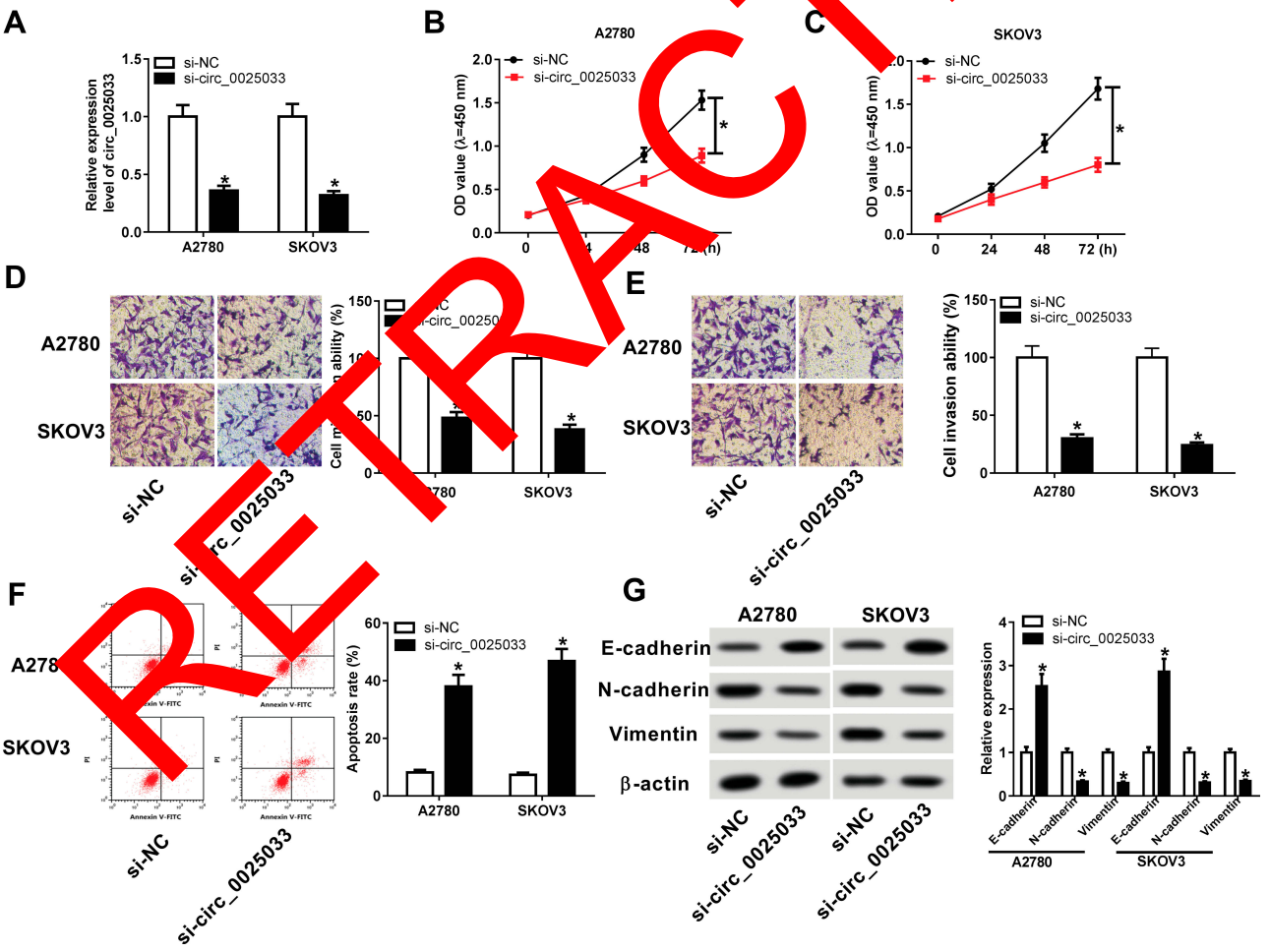

Figure 2 Circ_0025033 knockdown reduced cell viability, migration, invasion and epithelial–mesenchymal transition (EMT) while promoted apoptosis of OC cells. Si-circ_0025033 and si-NC were severally transfected into A2780 and SKOV3 cells. (**A**) The transfection efficiency of si-circ_0025033 was evaluated via qRT-PCR. (**B** and **C**) CCK-8 was used for determining cell ability of transfected OC cells. (**D** and **E**) Cell migration (**D**) and invasion (**E**) abilities were analyzed using transwell assay. (**F**) Flow cytometry was applied to examine the apoptosis rate. (**G**) The protein expression levels of EMT-related markers were measured by Western blotting. *$P < 0.05$.

analysis demonstrated that circ_0025033 contained a hypothetic combinative region (red sign) of miR-330-5p (Figure 3A). In order to prove the actual interaction between circ_0025033 and miR-330-5p, the red binding sites of circ_0025033 were mutated into CGUCUG (the green underline) and the luciferase reporter plasmids (circ_0025033 WT and circ_0025033 MUT) were constructed to perform the dual-luciferase reporter assay. As the illustration of Figure 3B and C, the overexpression of miR-330-5p lessened the luciferase activity of luciferase plasmid circ_0025033 WT but not circ_0025033 MUT in A2780 and SKOV3 cells, compared with miR-NC group. RIP assay also showed that miR-330-5p transfection elevated the precipitation of circ_0025033 in Ago2 relative to miR-NC, while circ_0025033 was undetectable in IgG all along (Figure 3D). Moreover, the expression of circ_0025033 was distinctly higher in Bio-miR-330-5p group

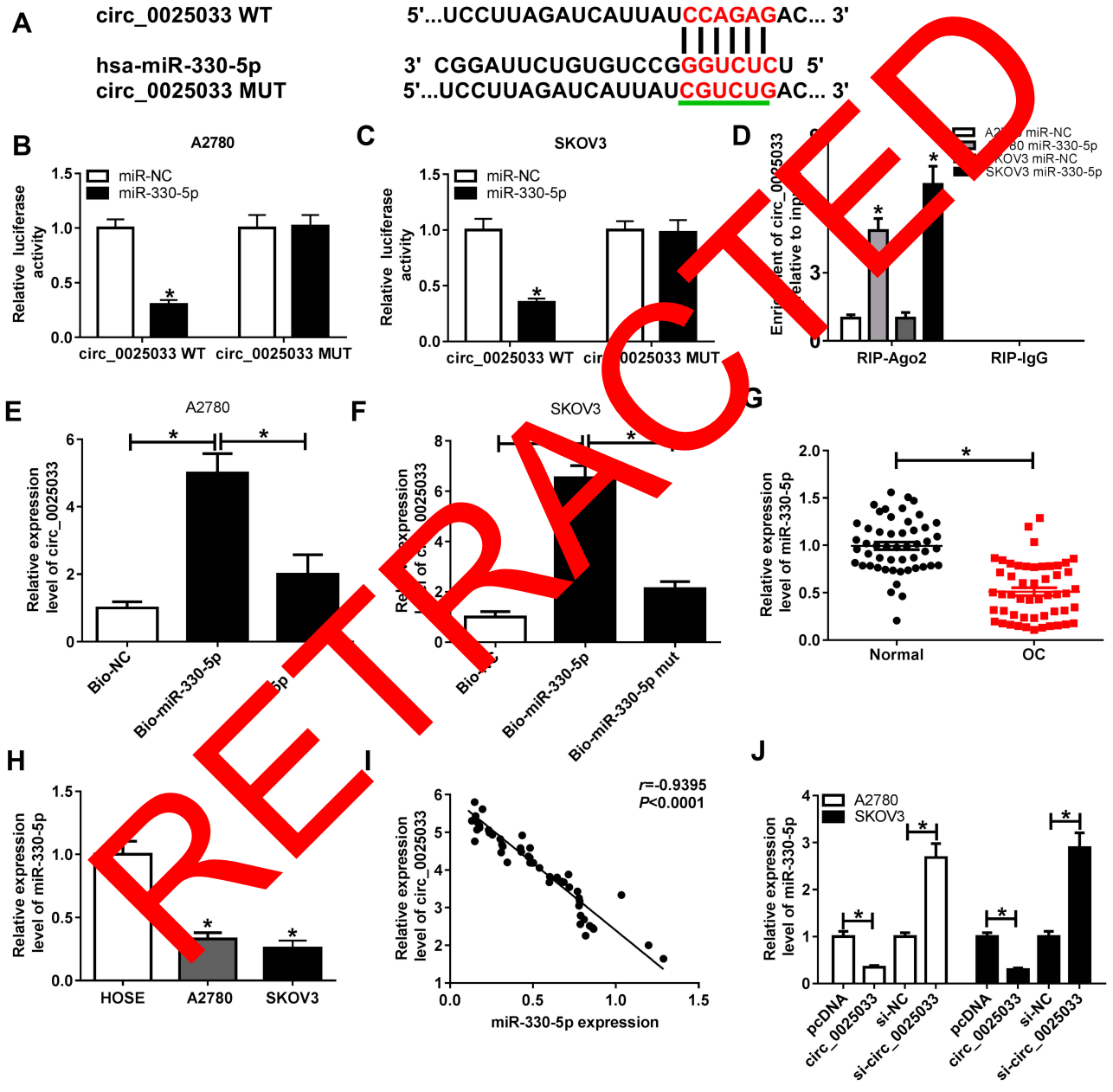

Figure 3 Circ_0025033 acted as a miR-330-5p sponge. (**A**) CircRNA Interactome was implemented to analyze the binding region between circ_0025033 and miR-330-5p. (**B–F**) The interaction between circ_0025033 and miR-330-5p was affirmed through the dual-luciferase reporter assay (**B** and **C**), RIP assay (**D**) and RNA pull-down assay (**E** and **F**). (**G** and **H**) The miR-330-5p expression in OC tissues and cells (A2780 and SKOV3) was determined using qRT-PCR. (**I**) The linear relation between circ_0025033 and miR-330-5p was analyzed via the Spearman correlation coefficient. (**J**) The effects of circ_0025033 overexpression or inhibition on the miR-330-5p level were assayed by qRT-PCR. *$P < 0.05$.

than that in Bio-miR-330-5p mut and Bio-NC groups, implying that circ_0025033 was pulled down by miR-330-5p (Figure 3E–F). And it was conspicuous that miR-330-5p expression was declined in OC tissues (Figure 3G) as well as A2780 and SKOV3 cells (Figure 3H). Besides, there was a negative relationship (r=−0.9395, P<0.0001) between circ_0025033 and miR-330-5p in OC tissues (Figure 3I). Furthermore, circ_0025033 transfection inhibited the miR-330-5p expression but si-circ_0025033 introduction caused the rising of miR-330-5p level (Figure 3J). All data have validated that circ_0025033 could sponge miR-330-5p.

Down-Regulation of miR-330-5p Abrogated the si-circ_0025033-Induced Effects on OC Cells

The regulatory relation of circ-0025033 and miR-330-5p in OC was researched by further rescue experiments. As shown in Figure 4A, the up-regulation of miR-330-5p expression induced by si-circ_0025033 was abolished by miR-330-5p inhibitor, implicating the excellent inhibition of in-miR-330-5p. CCK-8 and transwell assays testified that miR-330-5p down-regulation largely ameliorated the suppression of cell viability (Figure [illegible]–C), migration

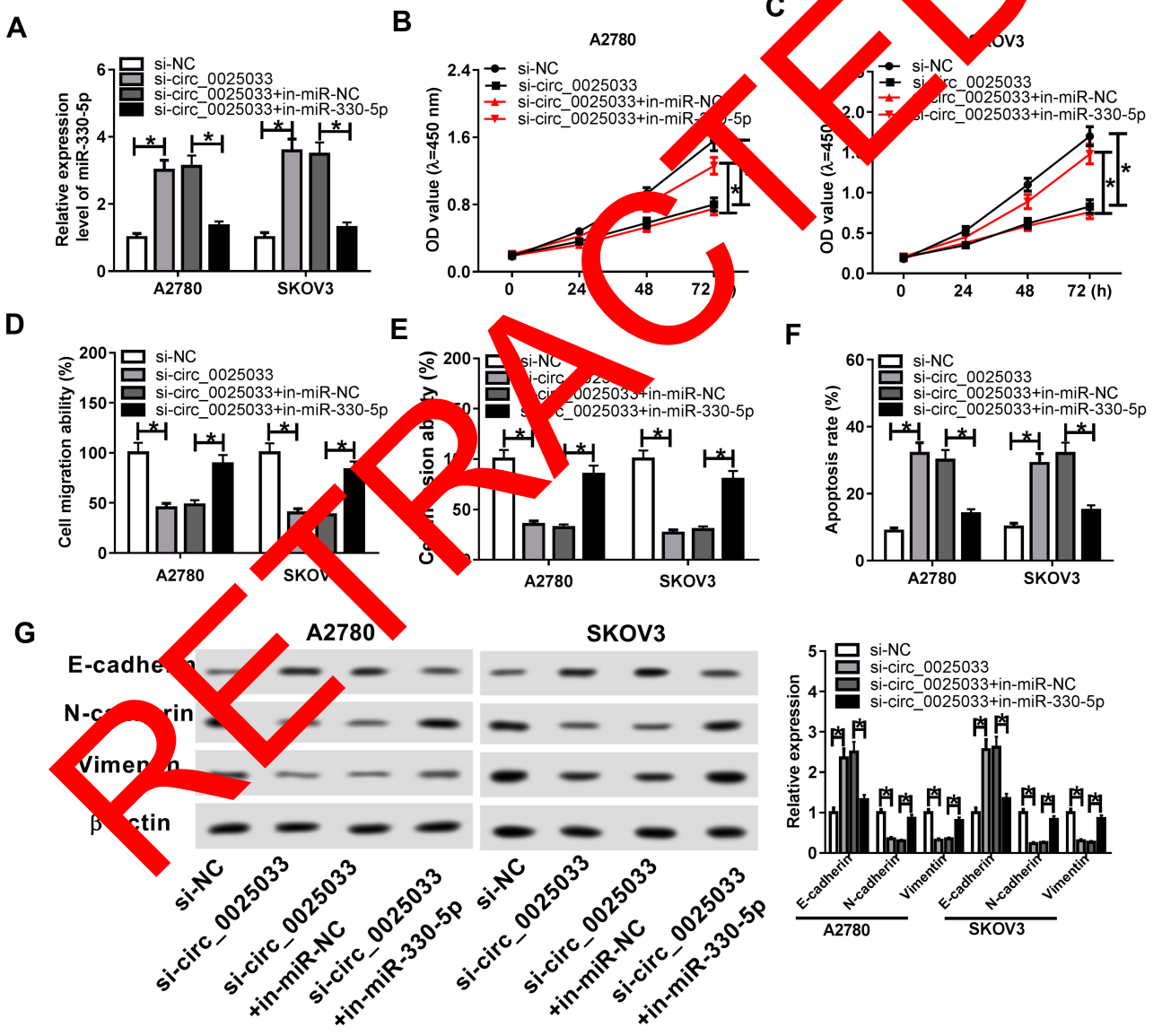

Figure 4 MiR-330-5p inhibition abrogated the si-circ_0025033-induced effects on OC cells. A2780 and SKOV3 cells were transfected with si-circ_0025033, si-circ_0025033 +in-miR-330-5p or corresponding controls. (**A**) The qRT-PCR was administrated to examine the miR-330-5p expression in transfected OC cells. (**B–E**) CCK-8 and transwell assays were respectively executed for the assessment of cell viability (**B** and **C**), and migration (**D**) invasion (**E**). (**F**) Cell apoptosis was evaluated using flow cytometry. (**G**) Western blotting was applied for detecting the expression of EMT-associated markers. *P < 0.05.

(Figure 4D) and invasion Figure 4E) in A2780 and SKOV3 cells transfected with si-circ_0025033. As a result of in-miR-330-5p transfection, the increased apoptosis rate caused by circ_0025033 knockdown was mostly recovered (Figure 4F). Likewise, the alterations in the levels of E-cadherin, N-cadherin and Vimentin evoked by the down-regulation of circ_0025033 were neutralized by repressing the expression of miR-330-5p (Figure 4G). Thus, miR-330-5p down-regulation reverted the effects on OC cells induced by circ_0025033 knockdown.

MiR-330-5p Negatively Interacted with KLK4 and circ_0025033 Regulated the KLK4 Level via Targeting miR-330-5p

Based on the prediction of DianaTools, we found there were seven binding sites between KLK4 3′UTR and miR-330-5p (CCCAGAG-GGGUCUC) as Figure 5A depicted. After the construction of KLK4 3′UTR WT and KLK4 3′UTR MUT, the determination of dual-luciferase reporter system manifested that the relative luciferase intensity of KLK4 3′UTR WT group was strikingly decreased following the transfection of miR-330-5p, which can hardly affect the luciferase activity of KLK4 3′UTR MUT group (Figure 5B–C). Subsequently, we detected the protein expression of KLK4 in OC and the results indicated that KLK4 was highly expressed in OC tissues (Figure 5D) and cells (A2780 and SKOV3) (Figure 5E) using the normal tissues and cells as controls. Interestingly, the linear relationship between miR-330-5p and KLK4 was negative (r=−0.9086, P<0.0001) (Figure 5F) while circ_0025033 was positively related to KLK4 (r=0.8790, P<0.0001) (Figure 5G) in OC tissues. Therefore, we further explored the impacts of miR-330-5p and circ_0025033 on KLK4 expression. As the data of Western blotting in Figure 5H and I, miR-330-5p up-regulation or circ_0025033 down-regulation induced the inhibition of KLK4 protein expression in A2780 and SKOV3 cells. Judging from these findings, we concluded that KLK4 was a target of miR-330-5p and circ_0025033 could upregulate the KLK4 expression by sponging miR-330-5p.

MiR-330-5p Functioned as a Tumor Repressor of OC Cells by Negatively Targeting KLK4

To ascertain whether miR-330-5p exerted the inhibition in OC progression by regulating KLK4, A2780 and SKOV3 cells were transfected with miR-330-5p, miR-330-5p+KLK4 or respective controls. QRT-PCR showed that KLK4 overexpression rescued the miR-330-5p-induced inhibitory effect on KLK4 protein level (Figure 6A). After performing CCK-8 and transwell assays, we found the intervention of miR-330-5p led to the memorable decrease of cell viability (Figure 6B–C), migration (Figure 6D) and invasion (Figure 6E) of A2780 and SKOV3 cells, whereas ectopic overexpression of KLK4 returned these effects to a great extent. In addition, the apoptosis rate of A2780 and SKOV3 cells was reduced after the transfection of miR-330-5p, which was also attenuated following the promotion of KLK4 expression (Figure 6F). Meanwhile, Western blotting exhibited that E-cadherin protein level was elevated while N-cadherin and Vimentin were lessened in A2780 and SKOV3 cells transfected with miR-330-5p compared with miR-NC control, but these effects were mitigated in miR-330-5p and KLK4 co-transfection group by comparison with miR-330-5p+pcDNA group (Figure 6G). The above data provided proofs that miR-330-5p played as a tumor inhibitor in OC via inhibiting KLK4.

Circ_0025033 Expedited the Tumor Growth of OC in vivo Through Regulating the miR-330-5p/KLK4 Axis

We used the xenograft tumor assay to prove the oncogenic role of circ_0025033 in OC in vivo. As Figure 7A and B described, circ_0025033 knockdown triggered the decrease of tumor volume and weight in vivo contrasted with sh-NC group. Additionally, the expression of circ_0025033 was down-regulated (Figure 7C) while miR-330-5p had a contrary tendency (Figure 7D) after inhibiting circ_0025033 in vivo. And Western blotting analysis demonstrated that the KLK4 protein expression was lower in sh-circ_0025033 group than that in sh-NC group (Figure 7E). As Figure 7F summarized, circ_0025033 targeted miR-330-5p to increase the KLK4 expression, consequently promoting tumor growth of OC.

Discussion

As a genre of novel ncRNA, circRNAs have become the research focus in various biological behaviors, including cell viability, apoptosis and metastasis of OC.[28,29] It is extremely significant to study how circRNAs generate their functions at the molecular level. In the current report, circ_0025033 was identified to regulate OC cellular processes through the miR-330-5p/KLK4 axis. The functional mechanism of circ_0025033 in OC was successfully uncovered.

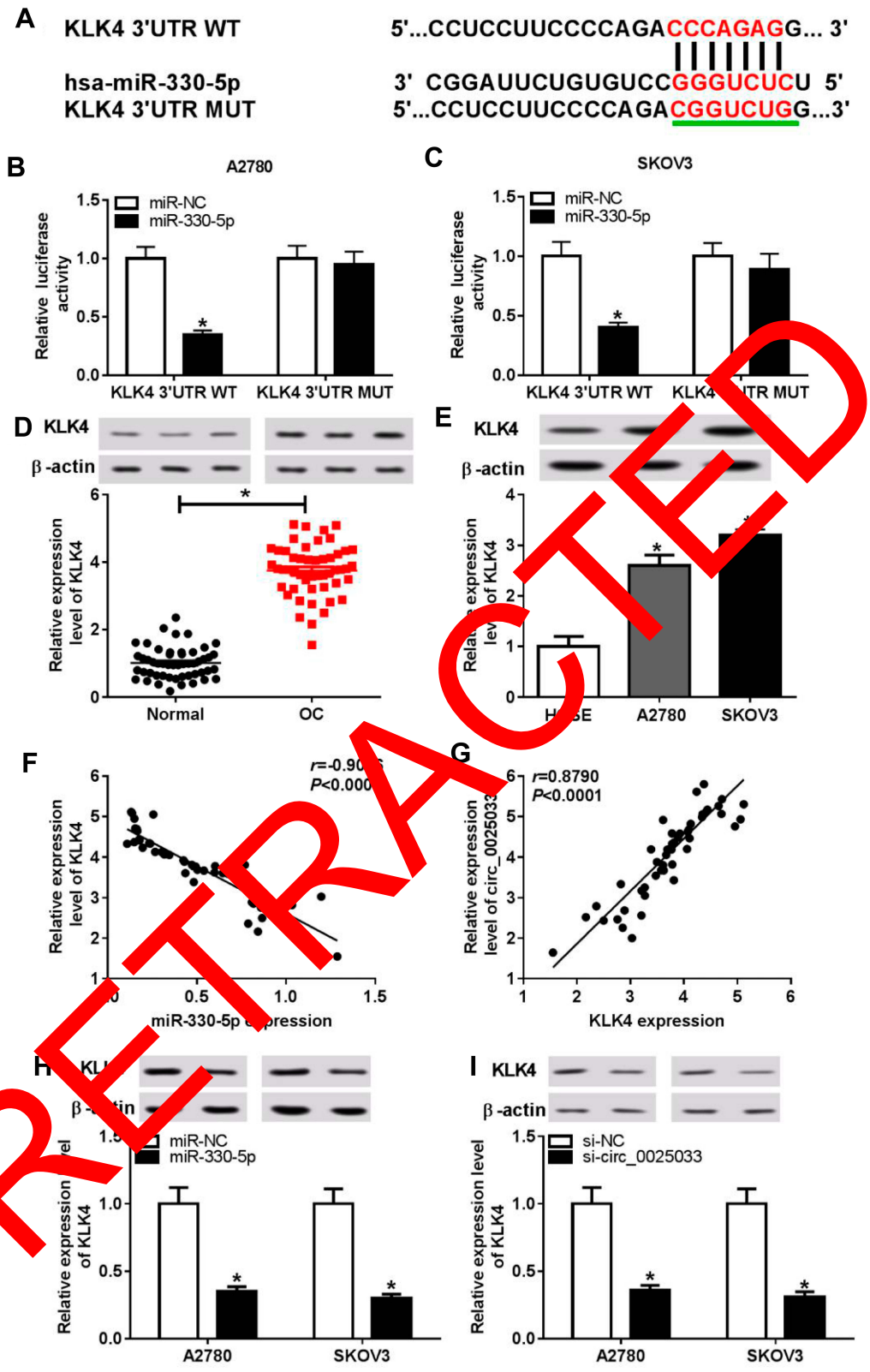

Figure 5 MiR-330-5p negatively interacted with KLK4 and circ_0025033 regulated the KLK4 level via targeting miR-330-5p. (**A**) The analysis of the binding sites between miR-330-5p and KLK4 was executed by DianaTools. (**B** and **C**) Dual-luciferase reporter assay was exploited to verify the combination between miR-330-5p and KLK4. (**D** and **E**) The KLK4 protein expression in OC tissues and cells was measured using Western blotting. (**F, G**) Spearman correlation coefficient was applied to analyze the linear relation between miR-330-5p and KLK4, as well as circ-0025033 and KLK4, in OC tissues. (**H** and **I**) The protein level of KLK4 was examined by Western blotting in A2780 and SKOV3 cells transfected with miR-330-5p (**H**), si-circ_0025033 (**I**) or matched controls. *$P < 0.05$.

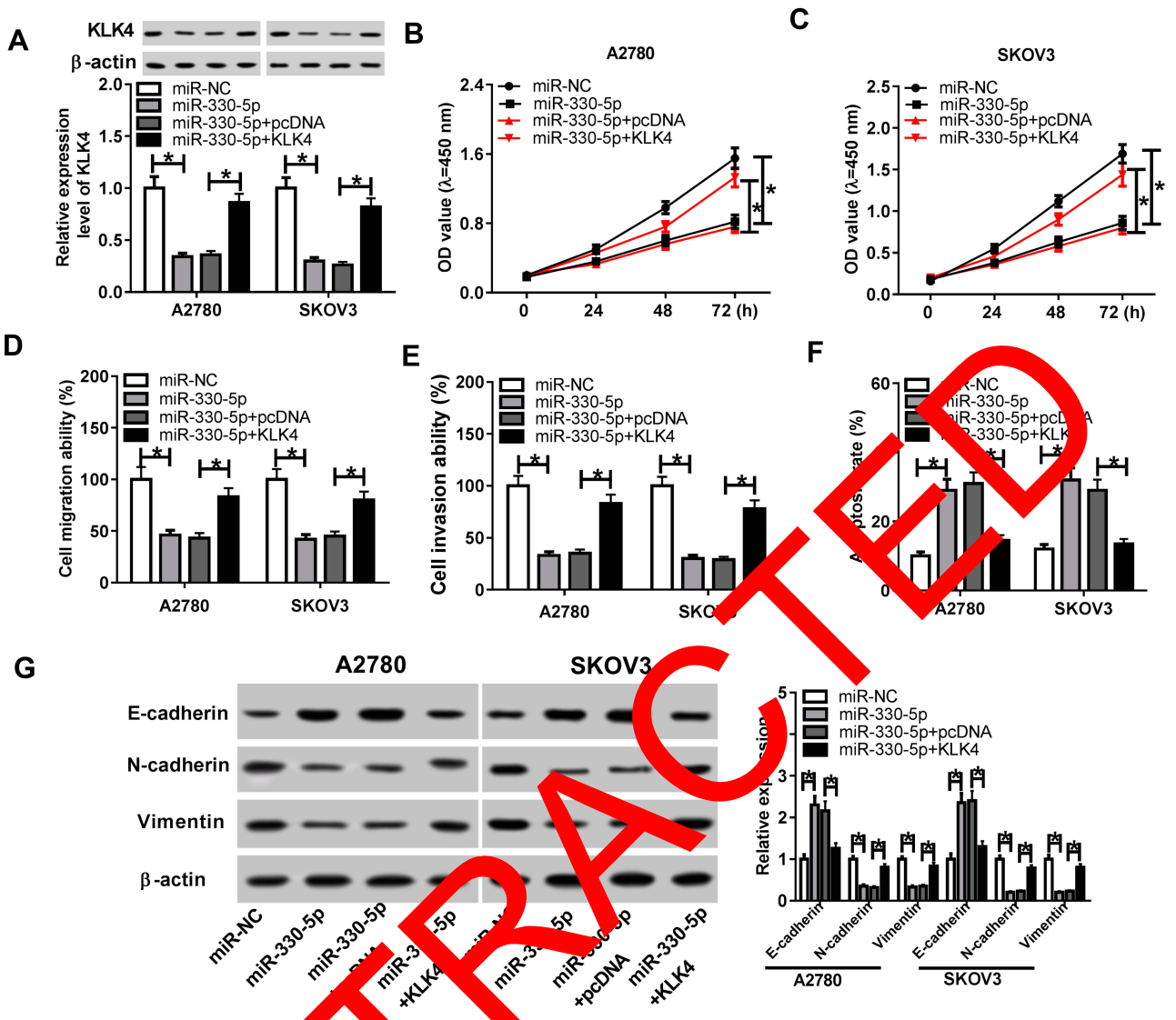

Figure 6 MiR-330-5p functioned as a tumor repressor of OC cells by negatively targeting KLK4. The transfection of miR-NC, miR-330-5p, miR-330-5p+pcDNA or miR-330-5p+KLK4 was executed in A2780 and SKOV3 cells. (**A**) KLK4 protein expression was assayed by Western blotting in transfected OC cells. (**B, C**) Cell viability was determined by CCK-8 assay. (**D, E**) Transwell assay was exploited for estimating the abilities of cell migration (**D**) and invasion (**E**). (**F**) The assessment of cell apoptosis was implemented using flow cytometry. (**G**) EMT process was evaluated by the detection of related proteins via Western blotting. *$P < 0.05$.

It is undisguised that up-regulated circRNAs contribute to tumorigenesis of OC according to the published documents. For example, Cheng et al disclosed that circ_0061140 overexpression enhanced cell growth and metastasis of OC by the miR-370/FOXM1 pathway;[30] Sheng et al declared that circUBAP2 functioned as a tumor promoter in OC occurrence via sponging miR-144.[31] And circWHSC1 was showed to accelerate the progression of OC through the regulation of MUC1 and hTERT by directly targeting miR-145 and miR-1182.[32] Coincident with these findings, our outcomes indicated that circ_0025033 was abnormally overexpressed in OC tissues and cells. Moreover, knockdown of circ_0025033 brought about the repression of cell viability, migration, invasion and EMT process but the motivation of apoptosis in OC cells, giving the potent explanation for the pro-tumor role of circ_0025033 in OC evolution. Pan et al reported that circ_0025033 up-regulation exacerbated cell proliferation, migration and invasion but had a suppressive effect on cell apoptosis of papillary thyroid cancer (PTC).[33] The promoted effect of circ_0025033 on OC progression in our study was in accordance with the report of it in PTC.

It has been clarified that the roles of circRNAs in cancers possibly depended on adsorbing miRNAs.[34] Circ_0059655 modulated tumorigenesis in salivary adenoid cystic carcinoma via sequestering miR-338-3p,[35] and circRNA_100290 played a regulatory role in oral cancer by functioning as the sponge of the miR-

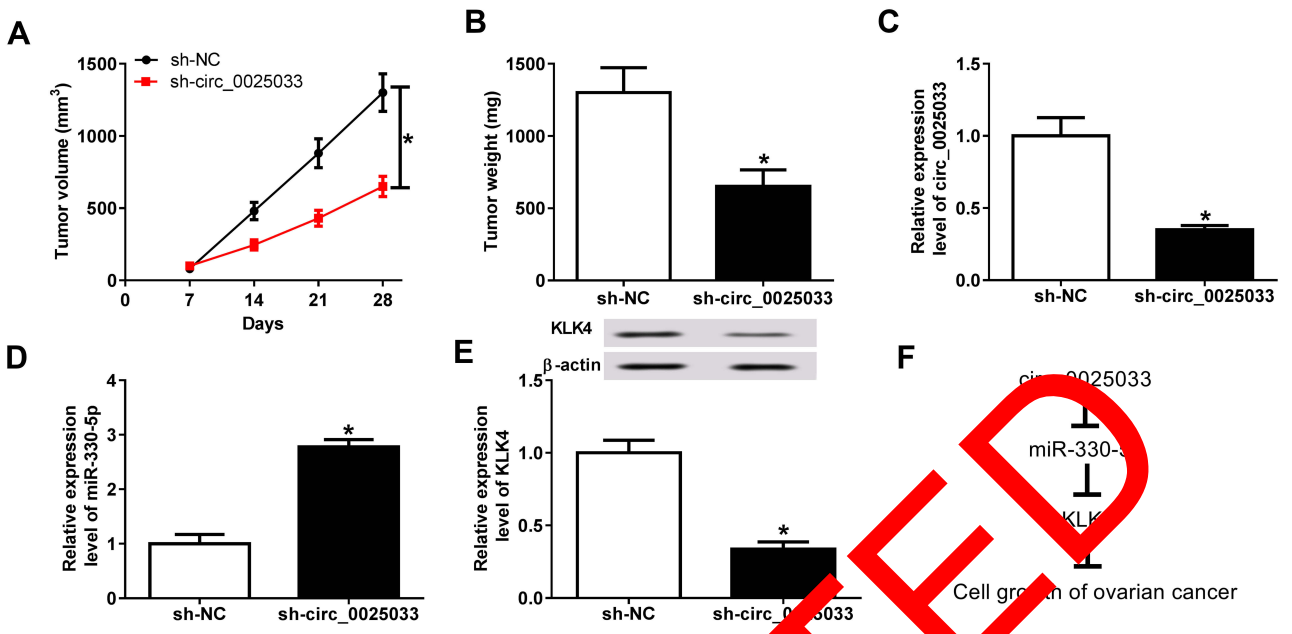

Figure 7 Circ_0025033 expedited the tumor growth of OC in vivo through regulating the miR-33 5p/KLK is. (**A**) After t injection of A2780 cells stably expressed sh-circ_0025033 or sh-NC, tumor volume of mice was assayed weekly. (**B**) Tumors were weighed after disse n. (**C, D**) The qRT-PCR was applied for examining the circ_0025033 and miR-330-5p expression in excised tumors. (**E**) The KLK4 protein level mined through estern blotting. (**F**) Circ_0025033 promoted tumor growth of OC in vivo by sponging miR-330-5p to upregulate KLK4. *$P < 0.05$.

29 family.[36] Also, the advancement of circ_0025033 in PTC was achieved by sponging miR-1231 and miR-1304.[33] Our port validated that miR-330-5p, a common tumor inhibitor, was a rget of circ_0025033. And miR-330-5p down-regulation could we en the anti-cancer influence of circ_0025033 knock down, plicati the oncogenic role of circ_0025033 in OC as accor lished vi acting as the sponge of miR-330-5p.

Significant progresses in und standing th physiological functions and pathological i olve nt of the K family have been developed in ome disease.[37] An earlier article documented that KL 2 and KLK3 coul modulate tumor growth of prostate ncer.[38] Also, KLK4 was considered to serve as an indicator in dicting s ort-term relapse of colorectal canc and oor pr nos of OC patients[40] as well as discri ating th malignant or benign prostate tumors.[20] Herein, w fied that KLK4 was a target of miR-330-5p and miR-330 impeded the evolution of OC by inhibiting KLK4 expression. Besides, the expression of KLK4 was regulated by circ_0025033 through targeting miR-330-5p. Furthermore, circ_0025033 expedited tumorigenesis by the miR-330-5p/KLK4 axis in vivo. These experiments in vitro and in vivo jointly interpreted the regulation of circ_0025033/miR-330-5p/KLK4 signal in OC progression.

In conclusion, our work unveiled that the aberrant up-regulation of circ_0025033 in OC and circ_0025033 enhanced cell viability, migration, invasion and EMT while inhibited optosis by e miR-330-5p/KLK4 axis. The explicit regulatory ch ism of circ_0025033/miR-330-5p/KLK4 may lay e foundation for exploring the pathomechanism of OC and circ_0025033 may be a novel therapeutic target for OC. What is more, hampering the expression of circ_0025033 has the potential to be an alternative treatment strategy for OC patients.

Highlights

1. Circ_0025033 is up-regulated in OC tissues and cells.
2. Knockdown of circ_0025033 refrains cell viability, migration, invasion and epithelial–mesenchymal transition (EMT) but enhances apoptosis of OC cells.
3. Circ_0025033 targets miR-330-5p and KLK4 is a target of miR-330-5p.
4. Circ_0025033 promotes tumor growth of OC in vivo via the miR-330-5p/KLK4 axis.

Disclosure

The authors declare that they have no financial conflicts of interest.

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