Silencing of Long Noncoding RNA LINC01132 Alleviates the Oncogenicity of Epithelial Ovarian Cancer by Regulating the microRNA-431-5p/SOX9 Axis

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Abstract

Background: To date, long intergenic nonprotein coding RNA 1132 (LINC01132) expression in epithelial ovarian cancer (EOC) and the underlying mechanisms have not been explored. In this study, we measured LINC01132 expression in EOC and assessed the effects of LINC01132 on the malignant behaviours of EOC cells in vitro and in vivo. Additionally, mechanistic studies were performed to elucidate the molecular events that occurred downstream of LINC01132 in EOC cells.

Methods: Reverse-transcription quantitative PCR (RT-qPCR) was utilized to verify LINC01132 expression in EOC. The effects of LINC01132 on the malignant behaviours of EOC cells were determined using a Cell Counting Kit-8 assay, flow cytometry analysis, cell migration and invasion assays and a tumour xenograft model. The targeting interaction among LINC01132, microRNA-431-5p (miR-431-5p) and SRY-Box 9 (SOX9) was verified by RNA immunoprecipitation and luciferase reporter assays.

Results: LINC01132 was overexpressed in EOC and was obviously associated with poor patient prognosis. Functionally, cell experiments revealed that LINC01132 depletion could inhibit EOC cell proliferation, migration and invasion and promote cell apoptosis *in vitro*. Additionally, loss of LINC01132 attenuated tumour growth *in vivo*. Mechanistically, LINC01132 acted as a competing endogenous RNA by sequestering miR-431-5p and thereby increasing SOX9 expression in EOC cells, forming a LINC01132/miR-431-5p/SOX9 axis. In rescue experiments, miR-431-5p inhibition or SOX9 re-expression eliminated the inhibitory effects of LINC01132 silencing on the pathological behaviours of EOC cells.

Conclusions: Generally, LINC01132 exhibited oncogenic activities in EOC cells *in vitro* and *in vivo* by regulating the outcome of the miR-431-5p/SOX9 axis, providing an effective target for EOC diagnosis, therapy and prognosis evaluation.

Background

Ovarian cancer, a lethal gynaecological tumour, is the fifth most common cause of cancer-associated deaths among females [1]. Ovarian cancer ranks first among gynaecological malignancies in terms of mortality, with a 5-year survival rate of less than 35% [2]. Every year, approximately 150,000 patients die from ovarian cancer, and this number is increasing annually [3]. Epithelial ovarian cancer (EOC) is the major type of ovarian cancer, accounting for approximately 85%-90% of all ovarian cancer cases, and is associated with poor clinical outcomes [4]. Currently, EOC is primarily treated with surgical resection along with chemotherapy, radiotherapy and immunological therapy; however, although many patients with EOC experience good therapeutic effects, EOC patients diagnosed at an advanced stage have poor prognoses after treatment with first-line therapies [5, 6]. Furthermore, subtle symptoms and signs of EOC usually result in diagnosis at a late stage, with pelvic dissemination or distant metastasis; thus, patients miss the best opportunities for surgery and have worse survival [7]. Therefore, understanding the mechanisms responsible for EOC pathogenesis is urgently needed to identify promising targets for tumour diagnosis and treatment.

Long noncoding RNAs (lncRNAs) are defined as a heterogeneous class of RNA transcripts consisting of over 200 nucleotides [8]. Although lncRNAs lack the ability to code proteins, they participate in the regulation of gene expression at different levels of epigenetic regulation [9]. Functionally, lncRNAs are confirmed to be crucial modulators of diverse physiological and pathological processes, especially during tumorigenesis and cancer progression[10]. An increasing amount of evidence has shown that an imbalance in lncRNA expression is closely correlated with the progression of EOC [11–13]. The dysregulation of lncRNAs has been clearly shown to exert cancer-inhibiting or cancer-facilitating effects and to contribute to the control of multiple aggressive properties of EOC [14–16].

MicroRNAs (miRNAs) belong to a group of small and nonprotein-coding RNA molecules that are known to negatively regulate gene expression by base pairing with target genes and consequently causing mRNA degradation or translation suppression [17]. Convincing studies have revealed the important regulatory roles of miRNAs in EOC oncogenesis and progression through their tumorigenic or antitumorigenic effects [18–20]. Recently, the competing endogenous RNA (ceRNA) theory was proposed to explain mechanisms of lncRNA action; according to this theory, lncRNAs operate as molecular sponges to sequester miRNAs and thereby modulate gene expression by weakening the miRNA-mediated suppression of gene expression[21, 22]. Thus, exploring cancer-related lncRNAs and miRNAs in EOC as well as the ceRNA theory can be a feasible approach for understanding the mechanisms underlying EOC progression, and this approach may be helpful for the identification of potential diagnostic biomarkers and effective therapeutic targets.

Long intergenic non-protein coding RNA 1132 (LINC01132) expression in EOC and the underlying mechanisms have not been explored until now. In the present work, we measured LINC01132 expression in EOC and examined its correlation with patient prognosis. Next, functional experiments were performed to assess the effects of LINC01132 on the malignant behaviours of EOC cells in vitro and in vivo. Furthermore, we elucidated the molecular events that occurred downstream of LINC01132 in EOC cells.

Methods

Patients and tissue specimens

Fifty-one pairs of EOC tissues and adjacent normal tissues were obtained from Weifang People's Hospital. All the tissues were quickly frozen in liquid nitrogen and then stored in liquid nitrogen until use. Patients who had other types of human cancer or had received chemotherapy or radiotherapy were excluded from this research. The collection and use of human tissues was approved by the Ethics Committee of Weifang People's Hospital, and the protocols were implemented in compliance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all the patients enrolled in this research.

Cell lines

ES-2, an EOC cell line, was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in McCoy's 5a Medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% v/v heat-inactivated foetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). Three EOC cell lines, namely, OVCAR3, CAOV-3, and SK-OV-3, were purchased from the National Collection of Authenticated Cell Cultures (Shanghai, China). The culture conditions for the SK-OV-3 cell line were the same as those for the ES-2 cell lines. Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS was utilized to culture the CAOV-3 cells. The OVCAR3 cells were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 20% FBS and 0.01 mg/ml bovine insulin (Gibco; Thermo Fisher Scientific, Inc.). The human ovarian surface epithelial cell line (OSE) was purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) and grown in ovarian epithelial cell medium (ScienCell Research Laboratories). All the cell lines mentioned above were cultured at 37°C in an incubator containing a humidified atmosphere with 5% CO_2.

Cell transfection

To overexpress miR-431-5p and SOX9, transfection of the miR-431-5p mimic (GenePharma; Shanghai, China) and pcDNA3.1-SOX9 (Shanghai Sangon Co., Ltd.; Shanghai, China) was performed with Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), and the miRNA mimic control (miR-NC; GenePharma) and empty pcDNA3.1 plasmid were used as controls. Small interfering RNAs (siRNAs) targeting LINC01132 (si-LINC01132) and the miR-431-5p inhibitor were synthesized by GenePharma and used to knock down LINC01132 and miR-431-5p expression in EOC cells. The negative control (NC) siRNA (si-NC; GenePharma) and NC inhibitor served as the control for si-LINC01132 and the miR-431-5p inhibitor, respectively.

RNA extraction and reverse-transcription quantitative PCR (RT-qPCR)

Total RNA extraction was performed with TRIzol® reagent, and the RNA was quantified with a NanoDrop™ 2000 spectrophotometer (both from Invitrogen; Thermo Fisher Scientific, Inc.). To detect miRNA expression, first-strand cDNA was synthesized from the total RNA by performing reverse transcription using a Mir-X miRNA First-Strand Synthesis Kit (TaKaRa, Dalian, China). Next, PCR quantification was conducted on an Applied Biosystems 7500 Real-time system with a Mir-X miRNA qRT-PCR TB Green® Kit (TaKaRa). For the determination of LINC01132 and SOX9 expression, reverse transcription was carried out utilizing a PrimeScript™ RT reagent kit with gDNA Eraser (Takara), after which the cDNA was used for PCR amplification with TB Green® Premix Ex Taq™ II (Takara). U6 small nuclear RNA was used as the internal control for miRNA, and GAPDH was used as the internal control for mRNA in this assay. The $2^{-\Delta\Delta Cq}$ method was used to process all the data.

Cell Counting Kit-8 (CCK-8) assay

The proliferation of EOC cells was estimated by CCK-8 assay (Dojindo Chemical Laboratory, Kumamoto, Japan). In detail, transfected cells were counted and seeded into 96-well plates. Every well was seeded

with 2000 transfected cells that were suspended in 100 µl complete culture medium. At 0, 1, 2, and 3 days after adherence to the wells, the cells were further incubated with 10 µl CCK-8 solution, and the cells were cultured for an additional 2 h under the conditions described above. Finally, the absorbance at a wavelength of 450 nm was monitored via a microplate reader.

Flow cytometry analysis of cell apoptosis

Transfected cells were digested with trypsin (Gibco; Thermo Fisher Scientific, Inc.), washed with ice-cooled phosphate-buffered saline and centrifuged at 1000 xg for 5 min. The apoptosis of these cells was analysed with an Annexin V–fluorescein isothiocyanate (FITC) apoptosis detection kit (Beyotime Institute of Biotechnology; Shanghai, China). Briefly, the cells were resuspended in 195 µL of annexin V–FITC binding buffer and cultivated with 5 µl Annexin V-FITC and 10 µl PI at 25°C. The incubation was performed in the dark and continued for 20 mins. All the samples were analysed with a flow cytometer (FACScan, BD Biosciences, Franklin Lakes, NJ, USA).

Cell migration and invasion assays

The EOC cell migration and invasion abilities were evaluated with the Transwell method. For the migration assay, the transfected cells were washed with phosphate-buffered saline and centrifuged. The upper compartments of the Transwell chambers (8-µm pores; BD Biosciences) were loaded with 5×10^4 cells resuspended in 200 µl serum-free medium. A volume of 600 µl culture medium supplemented with 20% FBS was added to the lower compartments and served as a chemoattractant. After 1 day of incubation at 37°C, the nonmigrated cells remaining on the top surface of the membranes were removed with a cotton swab, and the migrated cells that crossed the pores were fixed with methanol and dyed with 0.1% crystal violet. The stained cells were photographed using a light microscope (Olympus, Tokyo, Japan), and the number of migrated cells in five random fields of view was counted. For the invasion assay, the experimental steps were the same, except that the Transwell chambers were precoated with Matrigel (BD Biosciences).

Tumour xenograft model

Short hairpin RNA (shRNA) targeting LINC01132 and NC shRNA (sh-NC) were prepared by GenePharma. After these molecules were inserted into a lentiviral vector, the vectors were transfected into 293T cells (National Collection of Authenticated Cell Cultures) with psPAX2 and pMD2.G. Approximately 48 h after transfection, the lentiviruses were collected and used to inject CAOV-3 cells. Three days later, fresh complete culture medium containing puromycin was used to further incubate the CAOV-3 cells, yielding cells stably expressing sh-LINC01132 or sh-NC.

All the experimental steps involving animals were conducted with approval from the Institutional Animal Care and Use Committee of Weifang People's Hospital. Four-week-old female BALB/c nude mice were acquired from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). The stably transfected cells were harvested and subcutaneously injected into the nude mice. One week after cell injection, the sizes of

the subcutaneous tumours were recorded every four days, and their volumes were calculated with the formula: Volume = 1/2 × length × width^2. The mice were euthanized by cervical vertebra dislocation on day 31, and the subcutaneous tumours were harvested for subsequent use.

Bioinformatics prediction

The online software miRDB (http://mirdb.org/) was used to identify the downstream target of LINC01132. The direct target of miR-431-5p was predicted utilizing TargetScan (http://www.targetscan.org/vert_60/) and miRDB.

Subcellular fractionation assay

The lncRNA subcellular localization predictor lncLocator (http://www.csbio.sjtu.edu.cn/bioinf/lncLocator/) was utilized to predict the subcellular distribution of LINC01132 in human cells. The prediction was further verified by a subcellular fractionation assay with a Cytoplasmic & Nuclear RNA Purification kit (Norgen Biotek Corp.). EOC cells were processed with cell fractionation buffer to separate the cytoplasm and nucleus. The relative expression of LINC01132 in both fractions was determined by RT-qPCR. GAPDH and U6 acted as the cytosolic and nuclear controls, respectively.

Luciferase reporter assay

Fragments of LINC01132 and the SOX9 3'-UTR containing wild-type (wt) miR-431-5p-binding sequences were amplified and inserted into the downstream region of the psiCHECK™-2 luciferase reporter vector (Promega Corporation, Madison, WI, USA). The luciferase reporter vectors were labelled psiCHECK™-2-LINC01132-wt and psiCHECK™-2-SOX9-wt. The corresponding mutant (mut) luciferase reporter vectors, namely, psiCHECK™-2-LINC01132-mut and psiCHECK™-2-SOX9-mut, were generated in the same manner. For the reporter assay, EOC cells were seeded into 24-well plates prior to being cotransfected with the miR-431-5p mimic or miR-NC and the wt or mut reporter vectors. Forty-eight hours later, the transfected cells were lysed with a Dual-Luciferase Reporter Assay System (Promega) for luciferase activity determination.

RNA immunoprecipitation (RIP)

RIP was conducted with the EZ-Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). After lysing the cells in complete RIP lysis buffer, the whole-cell lysates were collected and incubated with magnetic beads coupled to an anti-Argonaute2 (Ago2) antibody or normal mouse IgG (Millipore) at 4°C overnight. In addition, 10 μL of whole-cell lysates were aliquoted for use as the input and served as the positive control. IgG acted as the negative control. Proteinase K treatment was used to detach the proteins, and the immunoprecipitated RNAs were then extracted. Eventually, the relative enrichment of LINC01132, miR-431-5p and SOX9 in the immunoprecipitated RNAs was examined via RT-qPCR.

Protein extraction and western blotting

Total protein was extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology). After total protein quantification via a bicinchoninic acid kit (Beyotime Institute of Biotechnology), equal amounts of proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes. The membranes were blocked at room temperature in 5% nonfat milk for 2 h and then incubated overnight at 4°C with primary antibodies against SOX9 (cat. no. ab185966; Abcam, Cambridge, UK) or GAPDH (cat. no. ab181602; Abcam), followed by incubation with an HRP-conjugated goat anti-rabbit secondary antibody (cat. no. ab205718; Abcam) at room temperature for 1 h. Finally, a BeyoECL plus detection kit (Beyotime Institute of Biotechnology) was used to detect the protein signals.

Statistical analysis

All the experiments were conducted in triplicate, and the experimental data are expressed as the mean ± standard deviation. One-way analysis of variance with Tukey's post hoc test was used to compare multiple groups to determine statistical significance. Comparison of the significance between two groups was conducted with Student's t test. The overall survival curves were calculated with the Kaplan-Meier method and compared with the log-rank test. Pearson's correlation analysis was used to examine the expression correlations. All the tests were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Deletion of LINC01132 represses the progression of EOC

This research first analysed LINC01132 expression in ovarian cancer tissues through The Cancer Genome Atlas (TCGA) and GTEx databases. The expression level of LINC01132 was higher in ovarian cancer tissues than in normal tissues (Fig. 1A). The expression status of LINC01132 was also determined in 51 pairs of EOC tissues and adjacent normal tissues that were obtained in our hospital. Higher LINC01132 expression in EOC tissues than in adjacent normal tissues was confirmed (Fig. 1B). Similarly, LINC01132 was upregulated in the EOC cell lines compared with the OSE human ovarian surface epithelial cell line (Fig. 1C). Next, we classified all patients in these datasets into either the LINC01132-high group or the LINC01132-low group according to the median value of LINC01132 in the EOC tissues. Patients with EOC characterized by high LINC01132 levels presented reduced overall survival compared with patients characterized by low LINC01132 levels (Fig. 1D).

Given the upregulation of LINC01132 in EOC, we wondered whether dysregulation of LINC01132 is related to the aggressive process of EOC cells. To this end, the OVCAR3 and CAOV-3 cell lines, which have higher LINC01132 expression than the other EOC cell lines, were selected for loss-of-function experiments. To avoid off-target effects, two siRNAs, si-LINC01132#1 and si-LINC01132#2, were transfected into the OVCAR3 and CAOV-3 cells to effectively induce LINC01132 silencing. RT-qPCR revealed that the two siRNAs both exerted satisfactory silencing effects (Fig. 1E). The CCK-8 assay showed that the inhibition of LINC01132 clearly inhibited the proliferative ability of EOC cells (Fig. 1F). Additionally, the transfection of si-LINC01132 led to a clear increase in the apoptosis of EOC cells (Fig. 1G). Furthermore, cell migration

(Fig. 1H) and invasion (Fig. 1I) were strikingly suppressed due to LINC01132 downregulation. Hence, LINC01132 plays a tumorigenic role in EOC cells.

LINC01132 acts as a ceRNA by sequestering miR-431-5p in EOC

To reveal the mechanism underlying the oncogenic actions of LINC01132 in EOC, the subcellular localization of LINC01132 was predicted by applying lncLocator. The outcomes showed that most LINC01132 was located in the cell cytoplasm (Fig. 2A), which was subsequently proven by an subcellular fractionation assay (Fig. 2B). This observation suggested that the functions of LINC01132 were performed via a ceRNA mechanism. The miRDB tool was used to search for the putative target miRNA of LINC01132. Totally 75 miRNAs contained binding sites for LINC01132. Five miRNAs, including miR-125b-5p, miR-134-3p (Fig. 2C), miR-199a-3p, miR-199b-3p, and miR-431-5p (Fig. 2D), were selected for experimental verification as their downregulation in EOC. Furthermore, the expression of these candidates in EOC cells upon LINC01132 silencing was assessed by RT-qPCR. miR-431-5p was overexpressed by si-LINC01132 transfection, whereas the levels of the other miRNAs were unaffected (Fig. 2E). Furthermore, miR-431-5p was dramatically weakly expressed in EOC tissues (Fig. 2F), and its expression was negatively correlated with LINC01132 (Fig. 2G).

The two binding sites between miR-431-5p and LINC01132 are shown in Fig. 2H. Subsequently, a luciferase reporter assay allowed us to determine whether LINC01132 can directly bind to miR-431-5p in EOC cells. As shown in Fig. 2I, miR-431-5p overexpression clearly lowered the luciferase activity of psiCHECK™-2-LINC01132-wt (1 and 2), but these regulatory effects on the luciferase activity were offset when the binding sites were mutated (1 and 2). Furthermore, as shown by the RIP assay, LINC01132 and miR-431-5p were dramatically enriched in the Ago2 group compared with the IgG group (Fig. 2J), indicating the coexistence of two RNAs in the RNA-induced silencing complexes. In summary, LINC01132 acts as a ceRNA in EOC cells by sponging miR-431-5p.

LINC01132 operates as a ceRNA to positively regulate SOX9 expression by sequestering miR-431-5p

The detailed roles of miR-431-5p in EOC cells were further examined. The overexpression of miR-431-5p in EOC cells by transfecting with miR-431-5p mimic was verified by qRT-PCR (Fig. 3A). The proliferation of OVCAR3 and CAOV-3 cells was reduced following the overexpression of miR-431-5p (Fig. 3B). Flow cytometry analysis confirmed that compared with that in the cells transfected with miR-NC, apoptosis was clearly promoted in the EOC cells transfected with the miR-431-5p mimic (Fig. 3C). Additionally, exogenous miR-431-5p expression resulted in a notable reduction in EOC cell migration and invasion (Fig. 3D).

Bioinformatics analysis was implemented to identify the potential downstream target of miR-431-5p. The 3’-UTR of SOX9 contained two binding sites for miR-431-5p (Fig. 3E) and was further studied due to its critical tumour-promoting activities during EOC oncogenesis. A luciferase reporter assay revealed that the miR-431-5p mimic clearly decreased the luciferase activity of psiCHECK™-2-SOX9-wt (1 and 2) but exerted no effect on the activity of psiCHECK™-2-SOX9-mut (1 and 2; Fig. 3F). Subsequently, our study determined

the regulatory actions of miR-431-5p on SOX9 expression in EOC cells, and we found that the SOX9 mRNA (Fig. 3G) and protein (Fig. 3H) levels were downregulated after miR-431-5p mimic transfection. Furthermore, high SOX9 expression in EOC tissues (Fig. 3I) exhibited an inverse relationship with miR-431-5p levels (Fig. 3J). All these results collectively validated SOX9 as a direct target of miR-431-5p in EOC cells.

Then, we attempted to test whether LINC01132 acts as a miR-431-5p molecular sponge to regulate SOX9 expression. The absence of LINC01132 significantly reduced the SOX9 mRNA (Fig. 4A) and protein (Fig. 4B) levels, whereas this regulation was largely abrogated by cotransfection with the miR-431-5p inhibitor (Figs. 4C and D). More importantly, the RIP assay affirmed that LINC01132, miR-431-5p and SOX9 coexisted in RNA-induced silencing complexes (Fig. 4E). In addition, a positive correlation between LINC01132 and SOX9 was verified in EOC tissues (Fig. 4F). In short, LINC01132 acts as a ceRNA by competitively binding to miR-431-5p and consequently elevating SOX9 expression.

The oncogenic activities of LINC01132 in EOC cells are dependent on the miR-431-5p/SOX9 axis

A series of rescue experiments was designed to elucidate whether the modulatory effects of LINC01132 on EOC cells are attributed to the miR-431-5p/SOX9 axis. Transfection of the miR-431-5p inhibitor significantly decreased miR-431-5p expression, suggesting that miR-431-5p was successfully silenced in EOC cells (Fig. 5A). The loss of LINC01132 reduced cell proliferation, which was largely restored by miR-431-5p inhibition (Fig. 5B). In addition, cotransfection with the miR-431-5p inhibitor abolished the increase in apoptosis in LINC01132-silenced EOC cells (Fig. 5C). Furthermore, treatment with the miR-431-5p inhibitor recovered the migratory and invasive (Fig. 5D) abilities of EOC cells that were impaired by LINC01132 knockdown.

In addition, the SOX9 overexpression plasmid pcDNA3.1-SOX9 (Fig. 6A) was introduced into LINC01132-deficient EOC cells, and functional experiments were then performed. Re-expression of SOX9 antagonized the suppression of cell proliferation (Fig. 6B) and promotion of cell apoptosis (Fig. 6C) induced by si-LINC01132. Moreover, the reduced cell migration and invasion (Fig. 6D) caused by si-LINC01132 was neutralized by overexpressing SOX9. Taken together, LINC01132 plays tumour-promoting roles in EOC cells by modulating the miR-431-5p/SOX9 axis.

LINC01132 downregulation inhibits xenograft tumour growth in vivo

To confirm the function of LINC01132 in EOC tumour growth in vivo, CAOV-3 cells stably transfected with sh-LINC01132 or sh-NC were subcutaneously injected into nude mice, and tumour growth was analysed. Downregulation of LINC01132 dramatically slowed tumour growth (Fig. 7A) and reduced tumour size (Fig. 7B). The subcutaneous tumours were harvested at day 31 after injection. We found that cells transfected with sh-LINC01132 form tumours with clearly lower weights (Fig. 7C). Further detection of LINC01132 and miR-431-5p showed that the subcutaneous tumours in the sh-LINC01132 group exhibited a low expression level of LINC01132 (Fig. 7D) and a high expression level of miR-431-5p (Fig. 7E). Furthermore, the LINC01132-downregulated cell-derived tumours exhibited decreased SOX9 expression

compared with the sh-NC cell-derived tumours (Fig. 7F). Collectively, these results show that depletion of LINC01132 decreased the tumour growth of EOC cells in vivo.

Discussion

At present, the clinical relationship between lncRNAs and human cancer is a research hotspot in tumour molecular biology given their importance in tumorigenesis and tumour development [23–25]. Therefore, lncRNAs may be attractive therapeutic targets for tumour intervention. As high-throughput sequencing technologies progress, the number of lncRNAs known to be dysregulated in EOC is rapidly increasing[26]; however, the detailed contributions of the majority of these lncRNAs remain poorly understood and require further research. In this study, we attempted to investigate the cellular function and underlying mechanisms of LINC01132 in EOC to offer novel directions and insights for the clinical diagnosis and treatment of EOC.

Multiple studies performed by different scientists have revealed the critical roles of lncRNAs in the oncogenicity of EOC. For instance, NORAD[27], LINC00673 [28] and TC0101441[29] are upregulated in EOC and identified as tumour promoters. In contrast, low expression of WDFY3-AS2[30], MAGI2-AS3 [31] and AOC4P[32] in EOC results in antitumorigenic effects. However, it is unknown whether LINC01132 participates in the malignancy of EOC. In the present work, high levels of LINC01132 in EOC was observed in the TCGA database and our own cohort. The overall survival of EOC patients with high LINC01132 levels was strikingly lower than that of patients with low LINC01132 levels. Functionally, cell experiments revealed that knockdown of LINC01132 was capable of inhibiting the proliferation, migration and invasion of EOC cells and promoting apoptosis. The results of the tumour-forming experiment in nude mice were consistent with the *in vitro* results.

Next, we aimed to understand the detailed mechanism by which LINC01132 participates in EOC, which remains mostly uncharacterized. The important roles of lncRNAs in regulating physiological and pathological behaviours are achieved through various mechanisms. The lncRNA-mediated ceRNA theory is a classic mechanism that explains the mechanism of lncRNA action in EOC[12]. Cytoplasmic lncRNAs can act as endogenous molecular sponges to sequester certain miRNAs and indirectly modulate gene expression by protecting mRNAs from miRNA-induced mRNA deregulation or translation inhibition[33]. Accordingly, we used lncLocator and subcellular fractionation assays to show that LINC01132 is mostly distributed in the cytoplasm of EOC cells. Then, bioinformatics analysis indicated that miR-431-5p might be a target of LINC01132. A luciferase reporter assay revealed that LINC01132 possessed a miR-431-5p-binding site.

To explore the ceRNA network mediated by LINC01132 in EOC, the direct target of miR-431-5p was explored, and our mechanistic analysis identified SOX9 as a downstream target of miR-431-5p in EOC cells. Interestingly, our data further showed that SOX9 was positively regulated by LINC01132 in EOC cells. In rescue experiments, the effects of LINC01132 knockdown on SOX9 expression were abrogated after the inhibition of miR-431-5p. More importantly, the RIP assay verified the coexistence of LINC01132,

miR-431-5p and SOX9 in the RNA-induced silencing complexes. Above all, a new ceRNA network, composed of LINC01132, miR-431-5p and SOX9, was shown to exist in EOC.

Differentially expressed miR-431-5p is observed in many human cancer types, including EOC[34]. Consistent with these studies, our research confirmed the downregulation of miR-431-5p in EOC and the cancer-inhibiting roles of miR-431-5p in EOC progression. SOX9, a member of the sex-determining region Y box family, was identified as the downstream effector of miR-431-5p in EOC cells. Although a number of studies have demonstrated the effects of SOX9 in driving EOC initiation and progression [35–37], the upstream regulatory mechanisms that cause the overexpression of SOX9 in EOC are still largely unknown. Herein, the experimental results revealed a novel LINC01132/miR-431-5p axis that controls SOX9 expression in EOC cells both *in vitro* and *in vivo*. Furthermore, miR-431-5p inhibition or SOX9 re-expression eliminated the tumour-suppressive effects of LINC01132 downregulation on the pathological behaviours of EOC cells, demonstrating that SOX9 plays critical roles in mediating LINC01132/miR-431-5p axis-triggered biological functions in EOC.

Generally, our study first found that LINC01132 was overexpressed in EOC and exhibited a significant association with poor clinical prognosis. LINC01132 exerted oncogenic effects in EOC cells *in vitro* and *in vivo* by controlling the outcome of the miR-431-5p/SOX9 axis. LINC01132/miR-431-5p/SOX9 is expected to be an effective target for the diagnosis, treatment and prognostic evaluation of EOC.

List Of Abbreviations

EOC Epithelial ovarian cancer

FBS Fetal bovine serum

NC Negative control

PBS Phosphate buffer saline

WT Wild-type

MUT mutant

lncRNA long non-coding RNA

miRNA microRNA

SOX9 SRY-Box 9

LINC01132 long intergenic nonprotein coding RNA 1132

Declarations

Ethics approval and consent to participate

The collection and use of human tissues was approved by the Ethics Committee of Weifang People's Hospital, and the protocols were implemented in compliance with the principles of the Declaration of Helsinki. All the experimental steps involving animals were conducted with approval from the Institutional Animal Care and Use Committee of Weifang People's Hospital.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors have made a significant contribution to the findings and methods. They have read and approved the final draft.

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Figures

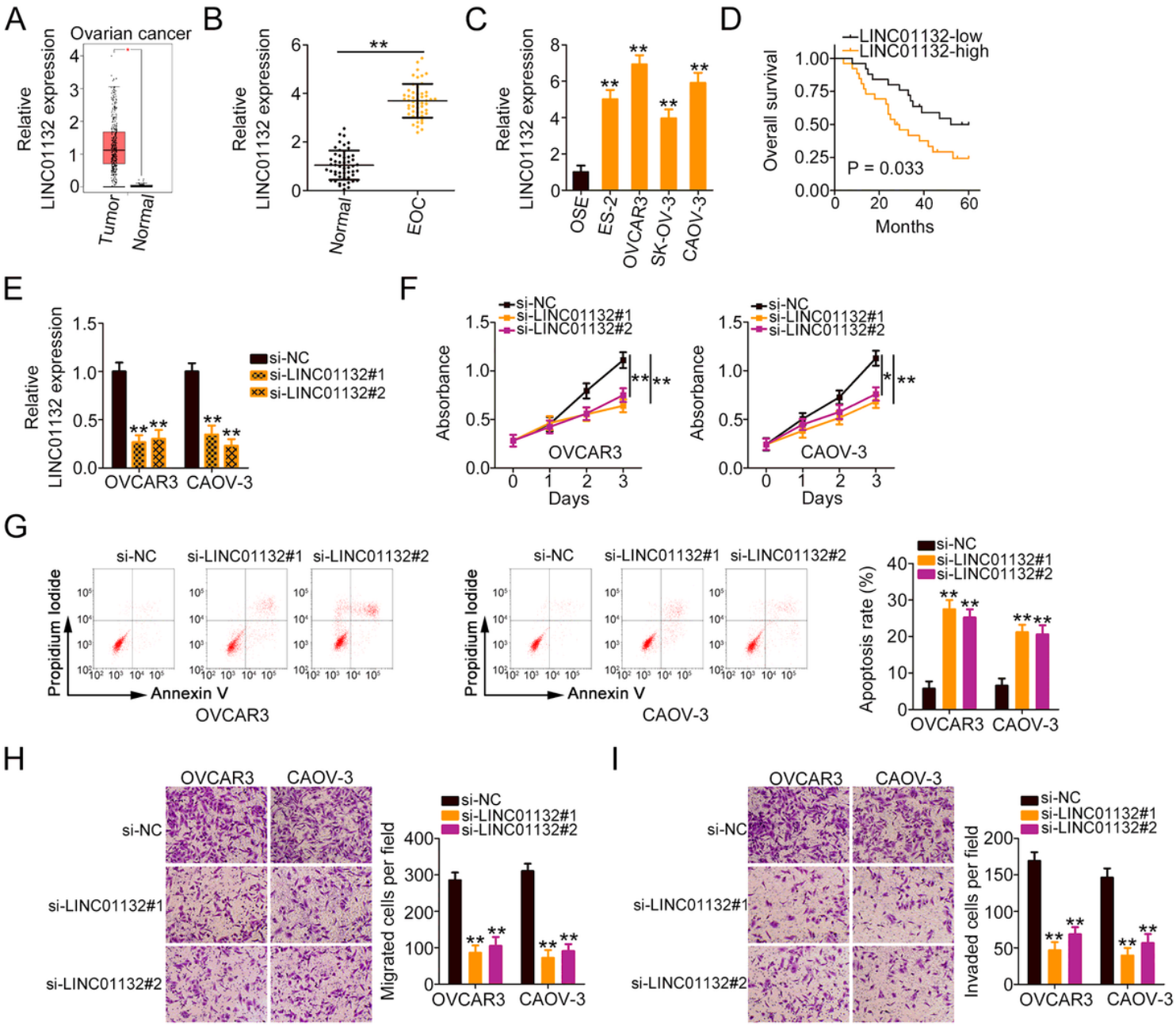

Figure 1

Expression of LINC01132 in EOC tissues and cell lines. (A) The LINC01132 expression pattern in ovarian cancer was analysed via The Cancer Genome Atlas (TCGA) and GTEx databases. (B) RT-qPCR was carried out to detect LINC01132 expression in 51 pairs of EOC tissues and adjacent normal tissues. (C) The expression level of LINC01132 in EOC cell lines (ES-2, OVCAR3, CAOV-3, and SK-OV-3) and a human ovarian surface epithelial cell line (OSE) was determined via RT-qPCR. (D) The overall survival of patients with high LINC01132 expression was poorer than that of patients with low LINC01132 expression. (E) The knockdown efficiency of si-LINC01132 in EOC cells was evaluated by RT-qPCR. (F) The proliferative ability of EOC cells after LINC01132 silencing was analysed by CCK-8 assay. (G) Flow cytometry analysis detected alterations in the apoptosis of EOC cells after si-LINC01132 transfection. (H, I) Cell migration and invasion assays revealed the migration and invasion of EOC cells after LINC01132 silencing. *P<0.05 and **P<0.01

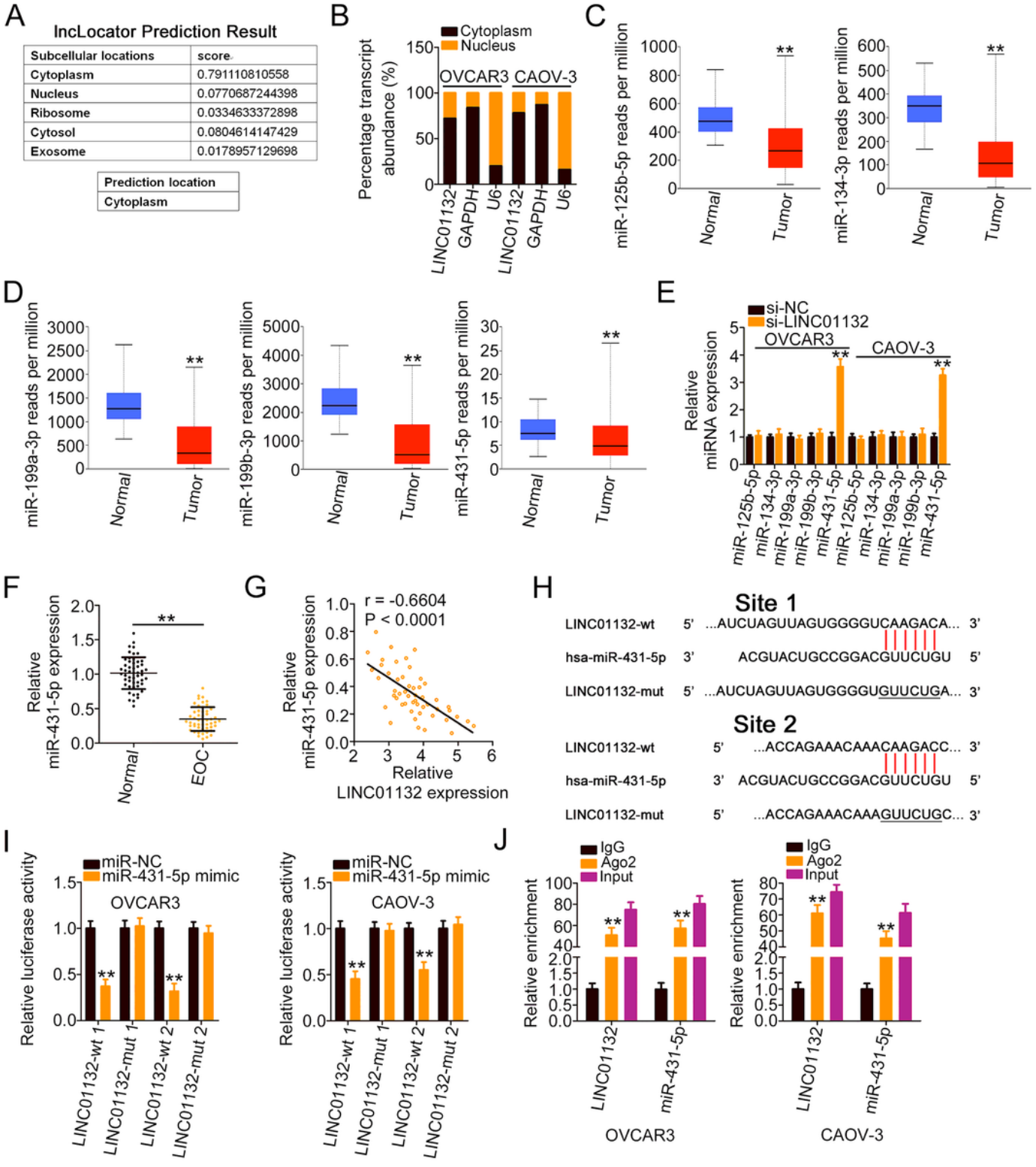

Figure 2

LINC01132 is capable of sponging miR-431-5p in EOC cells. (A) lncLocator was used to predict the subcellular distribution of LINC01132. (B) The localization of LINC01132 in EOC cells was assessed by subcellular fraction assay. (C, D) Expression of miR-125b-5p, miR-134-3p, miR-199a-3p, miR-199b-3p, and miR-431-5p in ovarian cancer was analysed via The Cancer Genome Atlas (TCGA) database. (E) Expression of miR-125b-5p, miR-134-3p, miR-199a-3p, miR-199b-3p, and miR-431-5p in EOC cells

following LINC01132 silencing was measured by RT-qPCR. (F) miR-431-5p expression in 51 pairs of EOC tissues and adjacent normal tissues was analysed by RT-qPCR. (G) An inverse correlation between the LINC01132 and miR-431-5p levels in EOC tissues was observed by Pearson's correlation analysis. (H) The predicted binding sequences between LINC01132 and miR-431-5p are shown. The sequences contained underscores is the site that was mutated in LINC01132. (I) Luciferase activity was quantified in OVCAR3 and CAOV-3 cells that were transfected with miR-431-5p mimic or miR-NC with psiCHECK™-2-LINC01132-wt or psiCHECK™-2-LINC01132-mut reporter plasmids. (J) RIP demonstrated the coexistence of LINC01132 and miR-431-5p in RNA-induced silencing complexes. **P<0.01

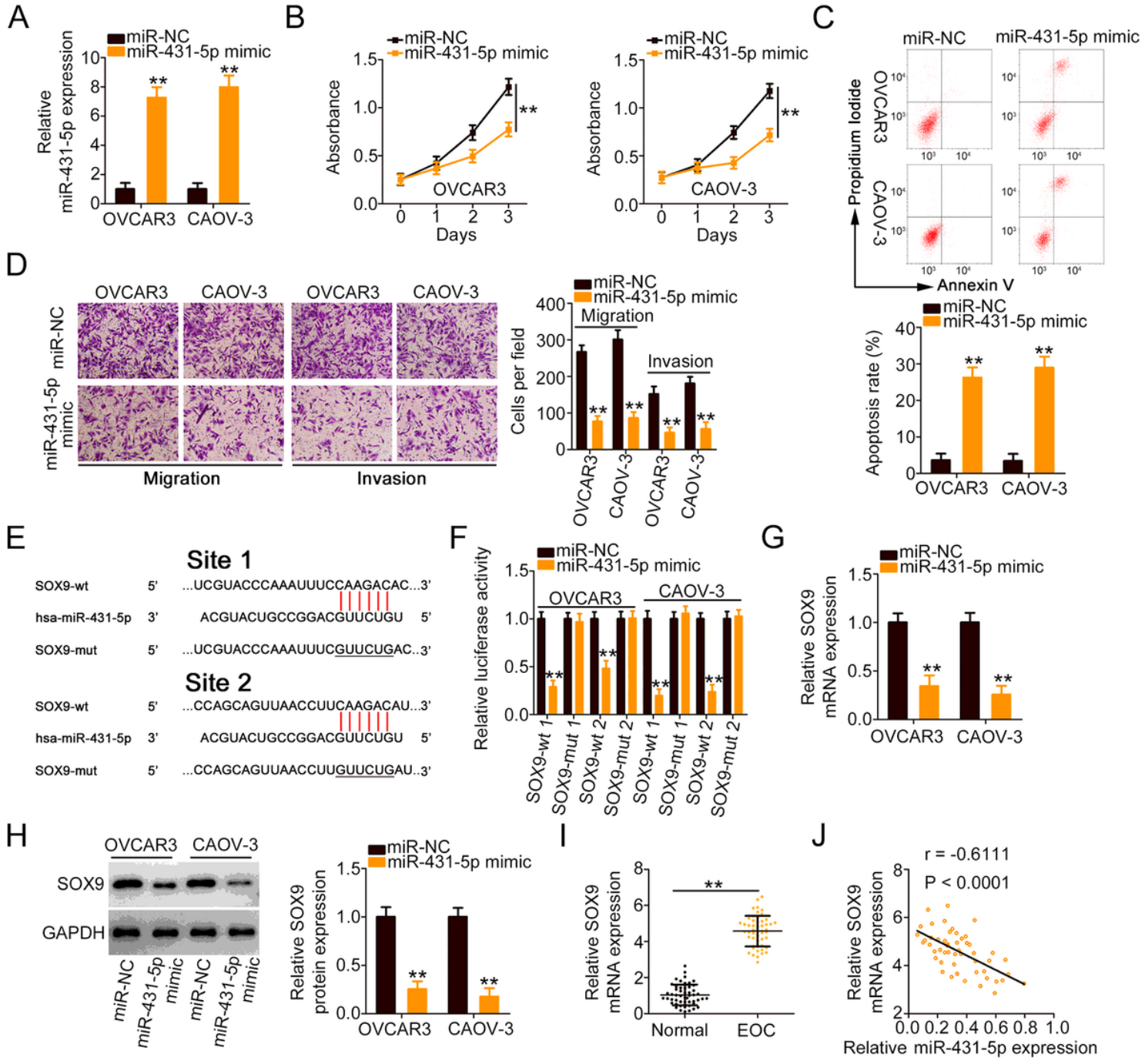

Figure 3

Identification of SOX9 as the downstream target of miR-431-5p in EOC cells. (A) miR-431-5p expression in miR-431-5p mimic-transfected or miR-NC-transfected EOC cells was measured utilizing RT-qPCR. (B, C) CCK-8 assay and flow cytometry analysis revealed the effects of miR-431-5p upregulation on the proliferation and apoptosis of EOC cells, respectively. (D) Cell migration and invasion assays showed alterations in the migration and invasion of EOC cells after transfection with the miR-431-5p mimic or miR-NC. (E) The 3′-UTR of SOX9 contained two miR-431-5p-binding sites. The mutated sites in the 3′-UTR of SOX9 are also indicated. (F) OVCAR3 and CAOV-3 cells were transfected with the miR-431-5p mimic or miR-NC with the psiCHECK™-2-SOX9-wt or psiCHECK™-2-SOX9-mut reporter plasmids, and then, the luciferase activity was detected. (G, H) The SOX9 mRNA and protein levels in EOC cells upon miR-431-5p overexpression was determined by RT-qPCR and western blotting, respectively. (I) The expression status of SOX9 in 51 pairs of EOC tissues and adjacent normal tissues was determined by RT-qPCR. (J) Pearson's correlation analysis was used to determine the correlation between LINC01132 and SOX9 expression in the 51 EOC tissues. **P<0.01

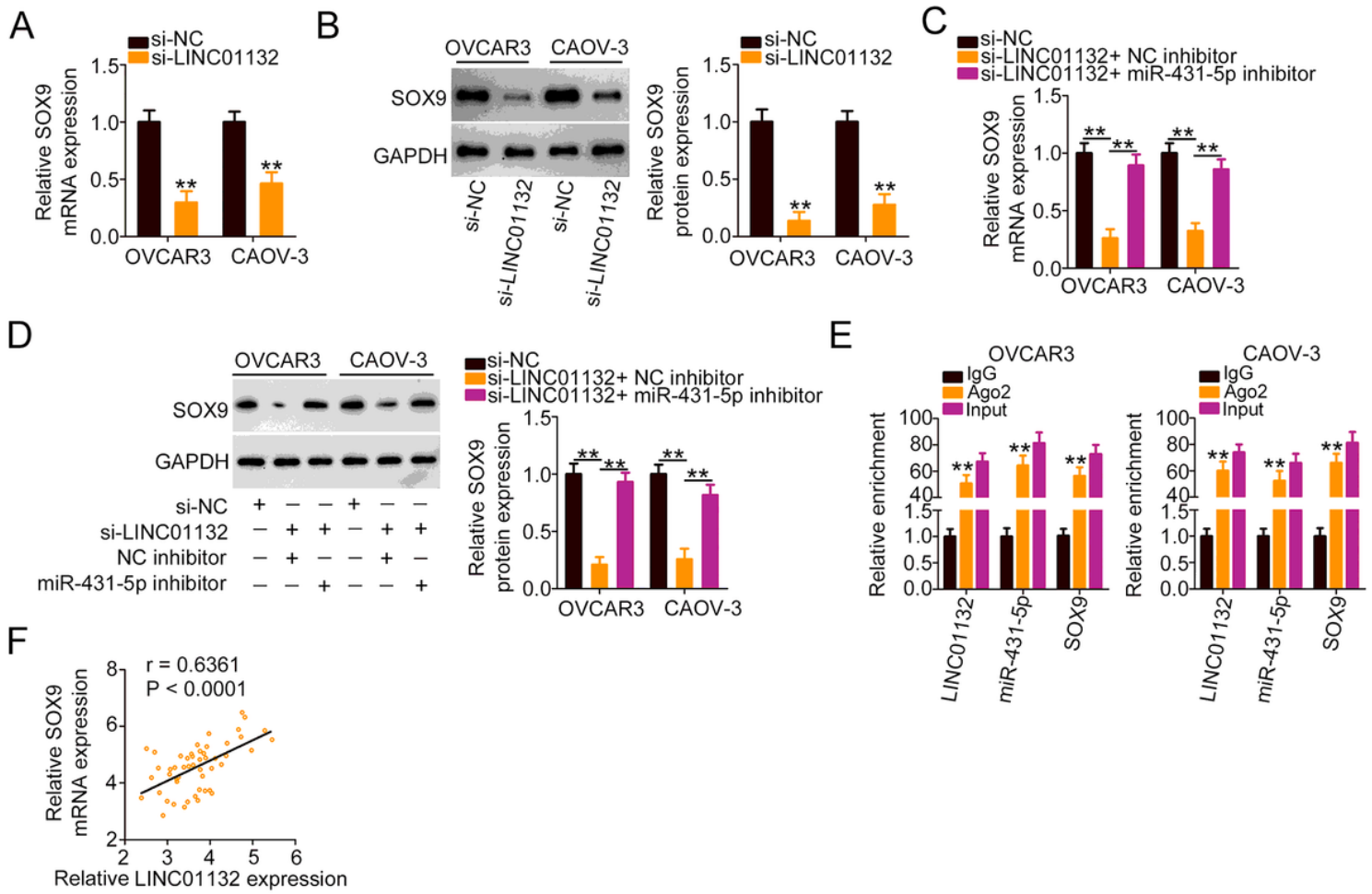

Figure 4

LINC01132 sequesters miR-431-5p and consequently positively regulates SOX9 expression in EOC. (A, B) RT-qPCR and western blotting were used to determine the changes in the SOX9 mRNA and protein levels in EOC cells when LINC01132 was knocked down. (C, D) Expression levels of SOX9 mRNA and protein were measured in EOC cells after cotransfection with si-LINC01132 and the miR-431-5p inhibitor or NC

inhibitor using RT-qPCR and western blotting, respectively. (E) The coexistence of LINC01132, miR-431-5p and SOX9 in RNA-induced silencing complexes was verified by RIP. (F) The positive correlation between LINC01132 and SOX9 mRNA expression in EOC tissues was confirmed by Pearson's correlation analysis. **P<0.01

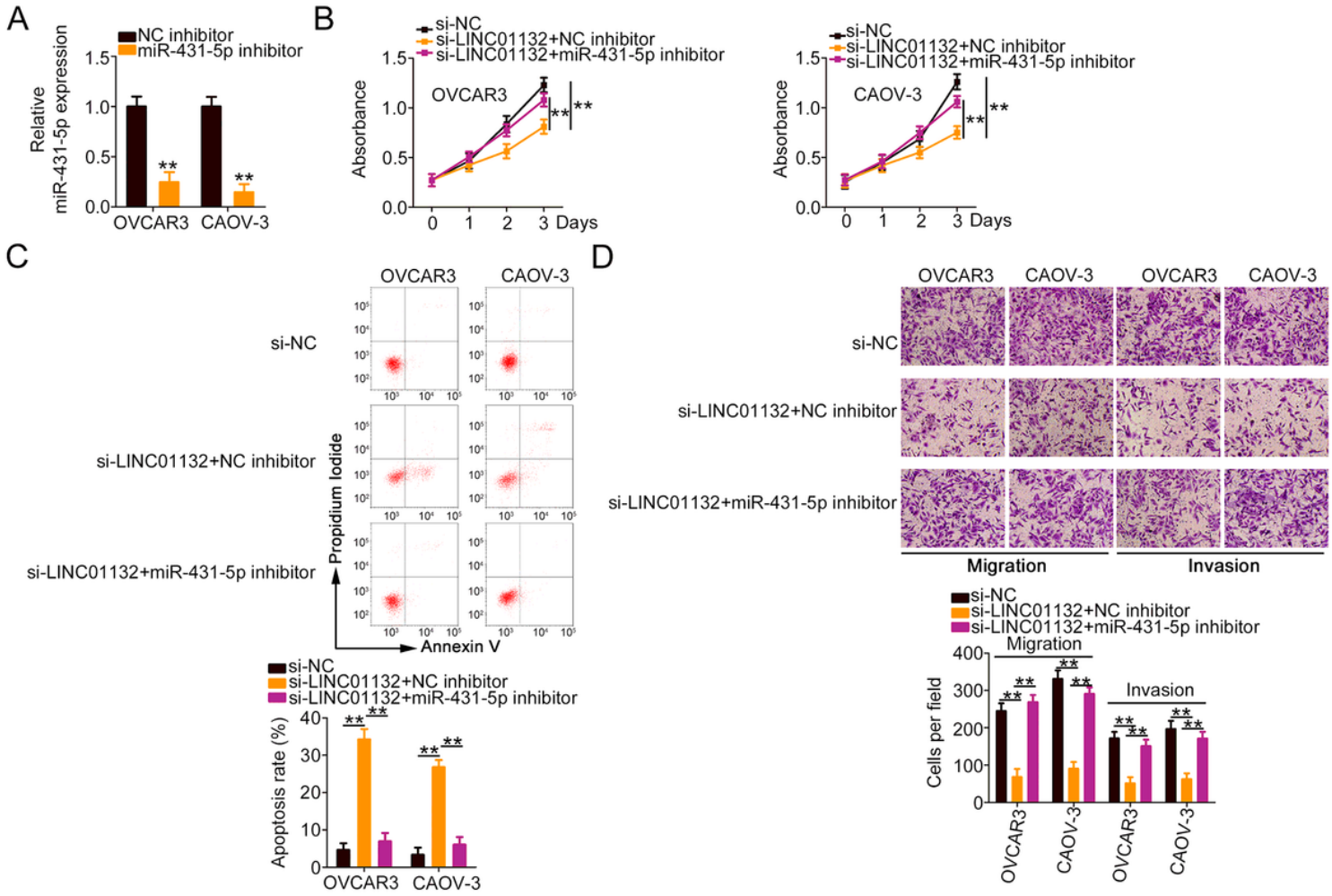

Figure 5

Inhibition of miR-431-5p reverses the cancer-inhibiting activities of si-LINC01132 in EOC cells. (A) The efficiency of the miR-431-5p inhibitor in silencing miR-431-5p expression was determined by RT-qPCR. (B, C) si-LINC01132 with the miR-431-5p inhibitor or NC inhibitor were introduced into EOC cells. Then, cell proliferation and apoptosis were assessed by CCK-8 assay and flow cytometry analysis, respectively. (D) The migratory and invasive capacities of EOC cells treated as described above were determined by cell migration and invasion assays. **P<0.01

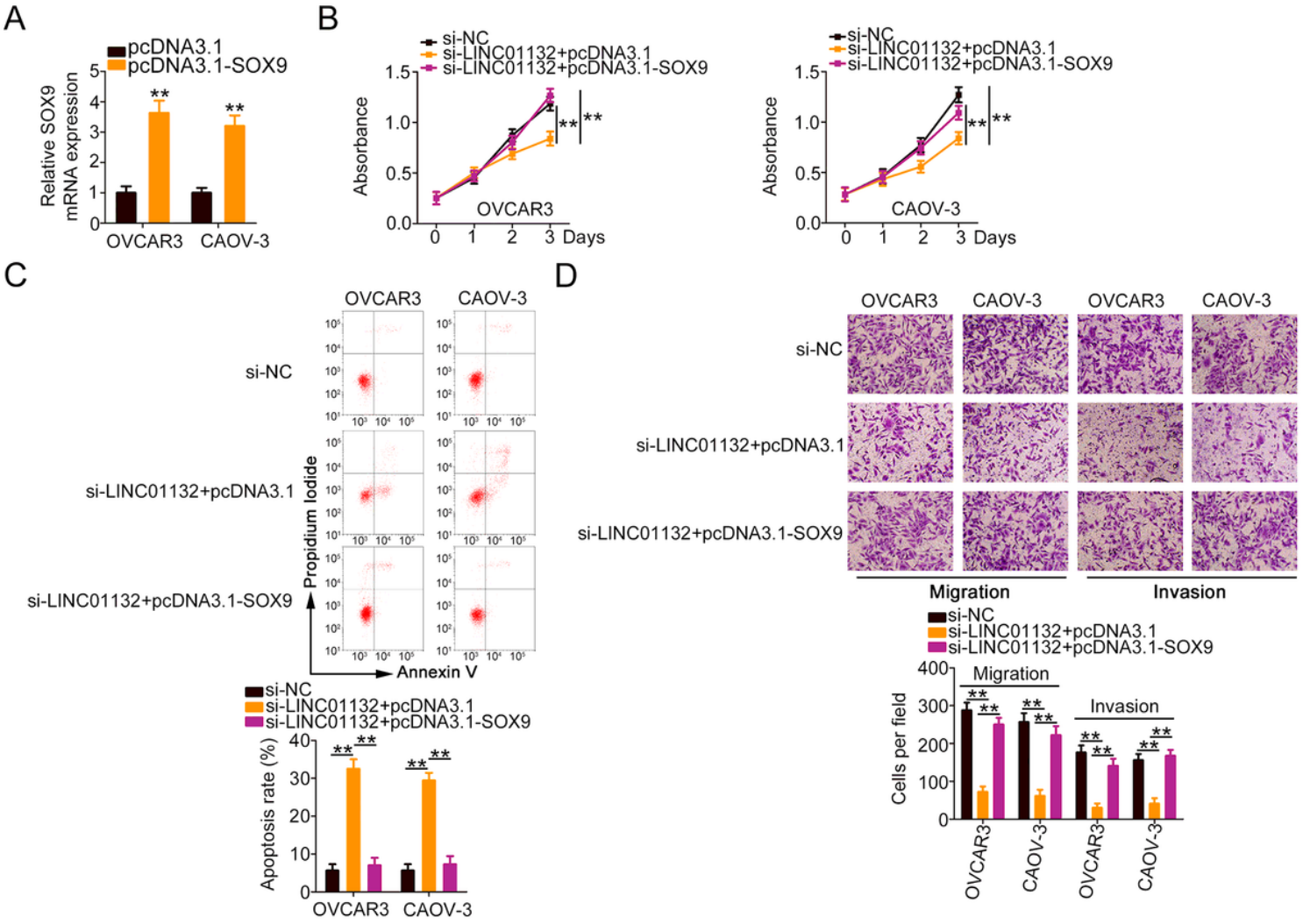

Figure 6

Reintroduction of SOX9 reverses the effects of si-LINC01132 on EOC cells. (A) The mRNA level of SOX9 in EOC cells was tested after pcDNA3.1-SOX9 or pcDNA3.1 transfection using RT-qPCR. (B, C) EOC cells were cotransfected with si-LINC01132 and pcDNA3.1-SOX9 or pcDNA3.1 and then analysed CCK-8 assay and flow cytometry analysis to measure cell proliferation and apoptosis. (D) Cell migration and invasion assays were conducted on the cells described above to measure cell migration and invasion. **P<0.01

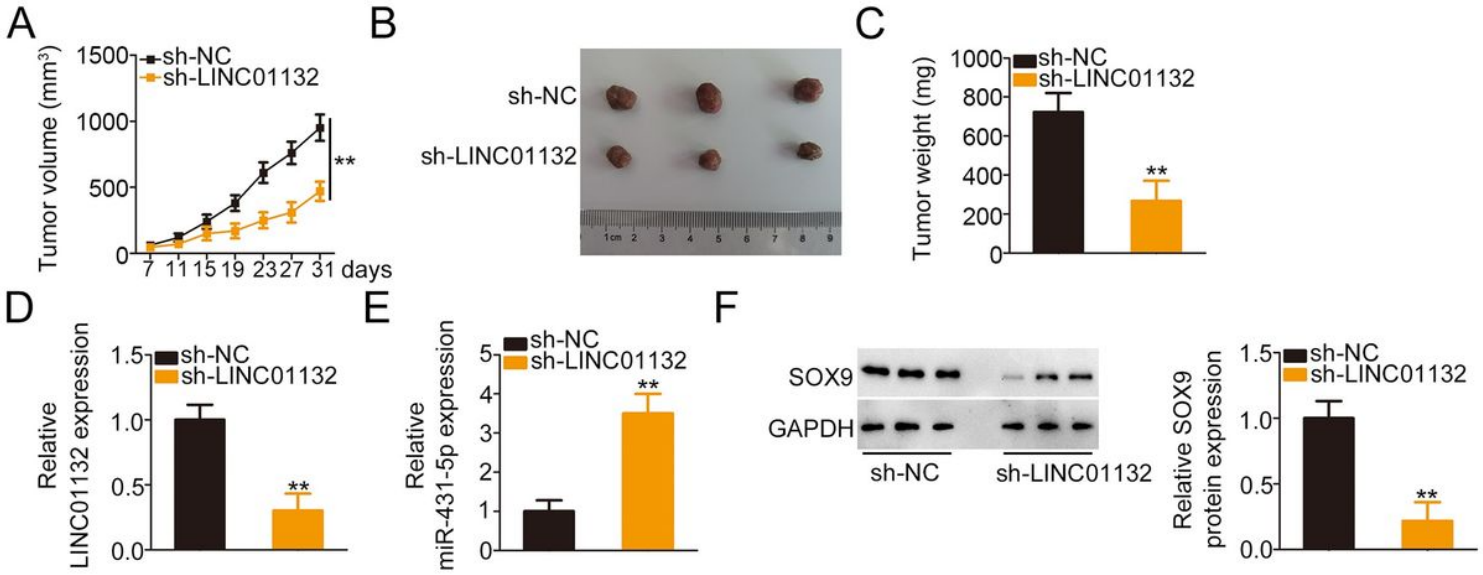

Figure 7

Loss of LINC01132 inhibits tumour growth in vivo. (A) Tumour growth curves were plotted based on the tumour volumes monitored at different times after tumour cell injection. (B) Representative images showing the tumour xenografts collected from the sh-LINC01132 and sh-NC groups. (C) Weights of the tumour xenografts on the 28th day. (D, E) LINC01132 and miR-431-5p levels in the tumour xenografts were analysed by RT-qPCR. (F) Western blotting showed SOX9 protein expression in tumour xenografts. **P<0.01