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Exosomes transferring long non-coding RNA FAL1 to regulate ovarian cancer metastasis through the PTEN/AKT signaling pathway

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Abstract. – OBJECTIVE: Tumor-derived exosomes have been repeatedly studied as tumor antigens, suppressing T-cell signaling molecules and promoting apoptosis in ovarian cancer (OC). Long non-coding RNAs (lncRNAs) have been recognized as major regulators in tumorigenesis, including OC. For this study, we try to find out the mechanism of exosomes and lncRNA FAL1 in OC.

MATERIALS AND METHODS: After the extraction and identification of exosomes, the internalization of exosomes was observed. Invasion and migration experiments were conducted to investigate the effect of SKOV3 cell-secreted exosomes on OC tumorigenesis and metastasis. Furthermore, the *in vivo* findings were verified via xenograft tumors in nude mice. FAL1 was knocked out on exosome-treated cells with exosomes were co-cultured with lncRNA FAL1 or/and PTEN to measure cell invasion and migration.

RESULTS: SKOV3-secreted exosomes were absorbed and internalized by OC cells. After exosome treatment, the migration and invasion of OC cells were enhanced. Tumors in nude mice were larger and heavier, metastasis was increased, and lncRNA FAL1 expression was increased. When lncRNA FAL1 was knocked out, the promoting effects of SKOV3 cells-secreted exosomes on OC cell metastasis were weakened, along with increased PTEN level and decreased AKT phosphorylation level. In HO8910PM cells treated with siRNA-FAL1 exosomes and siRNA-PTEN, cell invasion and migration, and AKT phosphorylation were restored.

CONCLUSIONS: SKOV3-secreted exosomes inhibited the PTEN/AKT signaling pathway by transferring lncRNA FAL1, thus inhibiting OC cell metastasis in vitro and *in vivo*.

Key words:
Ovarian cancer, Exosomes, LncRNA FAL1, PTEN/AKT signaling pathway.

Introduction

Ovarian cancer (OC) is a gynecologic malignant features of rapid growth, frequent metastasis, and rapid drug resistance, which accounts for the No. 1 mortality among all gynecological cancers and the No. 5 major cause of cancers in females. Almost 200,000 women are suffering from this disease worldwide, and it is so lethal that over 100,000 deaths per year are reported[2]. A former study has stated that OC originates from ovarian surface epithelium, which first proliferates from the ovary to pelvic cavity, abdomen and then remote sites, and suggested endometriosis as a forewarning of OC[3]. Family history is the most important causative factor for OC, while pregnancy and breastfeeding have close associations with reduced risk of OC[4]. Researchers and scholars have made great efforts for early detection and novel therapeutic approaches to reduce mortality but failed to reach the expected goals due to incomprehensive understanding on the pathogenesis of OC[3]. To improve the poor clinical prognosis of this disease, understanding the molecular and functional mechanisms involved in OC is of great physiological and clinical importance.

Exosomes, released by cells' endosomal compartment in tumor microenvironment, containing proteins, lipids, mRNAs, long non-coding RNAs (lncRNAs) and microRNAs (miRs)[5], can deliver tumor cells to neighboring cells, leading to drug resistance, increased tumor angiogenesis and metastasis[6,7]. Exosomes have gained more and more attention due to their involvement in promoting OC progression[8]. LncRNAs secreted from exosomes are regarded as potential biomarkers in several cancers[9]. The focally amplified lncRNA on chromosome 1 (FAL1) has a strong link to out-

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comes of thyroid cancer and non-small cell lung cancer, and its abnormal expression works as an oncogenic element in OC[10]. The mutation or loss of phosphatase and tensin homologue (PTEN), a tumor suppressor gene, has been massively reported in various malignancies, including melanomas, breast cancer, and endometrial carcinomas[11]. PTEN loss results in the activation of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway and regulates genome stability and elevates levels of abnormal chromosome numbers in tumors[12]. Aberrant expression of the PTEN/PI3K/AKT signaling pathway has been confirmed to associate with undesirable prognosis of OC[13]. In addition, the regulation of exosomes on PTEN/PI3K/AKT pathway may depend on cancer variety, for instance, colon cancer cells-derived exosomes downregulate PTEN level and increased AKT phosphorylation level[14]. From all above, we may see some underlying connection of exosomes, lncRNA FAL1 and PTEN/AKT signaling pathway in OC. Therefore, we carry out experiments to figure out the effects of exosomes transferring lncRNA FAL1 on OC cell progression *via* the PTEN/AKT signaling pathway.

Materials and Methods

Ethics Statement

This study was approved a[illegible] by the Ethics Committee of the S[illegible]d Affi[illegible]ed Hospital of Kunming Medic[illegible]versity[illegible]nt efforts were made to m[illegible] the [illegible]ber of animals and their suffe[illegible]g.

Extraction a[illegible]tification o[illegible] SKOV3 Cel[illegible]ecre[illegible]Exosomes

Epitheli[illegible] OC cell li[illegible]KOV3 (purchased from Ce[illegible]ank of Chinese [illegible]emy of Sciences, Sh[illegible]hai, C[illegible]na) was cultured in Roswell Park[illegible]emori[illegible]stitute (RPMI)-1640 medium with[illegible]fe[illegible]ovine [illegible]m (FBS) without exosomes [illegible] in a 5[illegible]O_2 incubator for 3 days. [illegible]super[illegible] collected, and cell debris wa[illegible]moved[illegible]entrifugation. The supernatan[illegible]d exosom[illegible] separation reagent (Invitrogen I[illegible]CA, USA) were added into cells [illegible].1 and centrifuged for 1 h at 10000 g and 4ºC [illegible]rnight. The exosome samples were stained [illegible]hosphotungstic acid, observed, and photograp[illegible]d under the transmission electron microscope H-7650 (Hitachi High-Technologies Corporation, Tokyo, Japan). Western blot analysis was applied to detect the surface protein markers of exosome. The antibodies included CD63 (Abcam Inc., Cambridge, MA, USA, ab134045) and secondary antibody goat anti-rabbit (Bios[illegible]nology, Co., Ltd, Beijing, China, bs-[illegible]G). T[illegible] size of exosomes was analyzed by [illegible]osight, and exosomes were diluted with pr[illegible]mount of pure water after centrifugation and p[illegible]itation. Exosomes were injected i[illegible]he detec[illegible]nk with a 1 mL syringe with[illegible]leaving bubbl[illegible] cording to the numbe[illegible] partic[illegible]recorded [illegible] Nanosight-NS300 (M[illegible]n Pa[illegible]tical C[illegible] Ltd, Malvern, Worcest[illegible]hi[illegible]e con[illegible]ation of exosomes wa[illegible]justed t[illegible]10 pa[illegible]es with-in the visual [illegible] which was [illegible]omatically by the mac[illegible]ord and gen[illegible]e reports.

Internalization of [illegible]omes

E[illegible]s were labele[illegible] PKH67 fluorescent c[illegible]nker kits (Sigma-Aldrich Chemical Compa-St Louis, MO[illegible]SA), and a sterile slide coated [illegible] 0.01% poly[illegible]e was carefully placed in a 2[illegible]l plate. A[illegible]l of (0.5-1) × 10^5 OC cell lines SK[illegible]d U[illegible]910PM were inoculated into the culture [illegible]or incubation in a 37ºC incubator with 5% CO_2. The exosomes labeled with PKH67 [illegible] cultured with OC cell lines in the slide. [illegible]n, the culture medium was removed, and exosomes were washed with PBS 3 times (5 min/time) to discard the exosomes labeled with fluorescence but did not internalized by OC cells. The exosomes were then fixed with 4% paraformaldehyde for 20-30 min. After 3 PBS washes (5 min/time), exosomes were stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min. The distribution of fluorescence was observed under the confocal laser microscope (Nikon Instruments (Shanghai) Co., Ltd, Shanghai, China) followed by 3 PBS washes (5 min/time) of exosomes.

Co-Culture of SKOV3 Cells-Secreted Exosomes with OC Cells

SKOV3 cells and HO-8910PM cells (Cell Bank of Chinese Academy of Sciences, Shanghai, China) were cultured in RPMI-1640 medium containing 5% FBS without exosomes at 5 × 10^5 cells/well in a 6-well plate. SKOV3-secreted exosomes at 100 μg/mL were respectively co-cultured with SKOV3 cells and HO-8910PM cells for 3 days.

Transwell Assay

The transwell chamber was put into a 24-well plate (Millipore Corp., Billerica, MA, USA). A total of 2 × 10^4 cells were cultivated in 200 μL of

serum-free medium in the apical chamber. While 500 μL of medium with 10% FBS but no exosome was paved in the basolateral chamber. After 48-h of cultivation, cells that did not migrate through the filter were gently removed with a cotton swab. The migrated cells were fixed with 100% pre-cooled methanol and stained with 0.05% crystal violet for 30 min. Six visual fields were randomly selected from each well and the cell number in each visual field was recorded. The experiment was repeated 3 times.

A total of 100 mL of Matrigel matrix (Becton, Dickinson Company, NJ, USA) was added to the polycarbonate membrane within the transwell chamber and placed overnight in an incubator to solidify the Matrigel matrix. Other steps were similar to those in the migration experiments.

Xenograft Tumor in Nude Mice

Twenty female thymus-free nude mice (4-5 weeks, about 20 g) (from Walvax Biotechnology Co., Kunming, Yunnan, China) were reared in specific pathogen-free (SPF) grade animal rooms. Animal feed and water sources were disinfected. The constructed luc-SKVO3 were treated with trypsin, counted and re-suspended in serum medium at 5 × 10^8 cells/well. After construction of nude mice model of *in situ* tumors, the nude mice were randomly assigned into 4 groups with 5 mice in each group. On the first day after inoculation of *in situ* tumors, the mice in the exosome group were intraperitoneally injected with 50 μg of exosomes every three days, while mice in the control group were intraperitoneally injected with PBS every three days. The first bioluminescent *in vivo* imaging was performed 7 days after *in situ* modeling. After anesthesia, the nude mice were intraperitoneally injected with 150 mg/kg D-luciferin. Ten minutes after injection, the nude mice were placed in the imaging room and the luminescence intensity and location of nude mice were measured by a small animal bioluminescence imaging system. *In vivo* imaging was performed once a week for 8 weeks totally, and nude mice were euthanized by intraperitoneal injection with pentobarbital sodium (100 mg/kg body weight, Sigma-Aldrich Chemical Company, St Louis, MO, USA) to weigh the tumors.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

RNA extraction was performed based on the instructions of corresponding kit (Guangzhou RiboBio Co., Ltd, Guangzhou, Guangdong, China). The reverse transcription of total RNA was performed according to transcription kit (TaKaRa Holding Inc., Otsu, Shiga, Japan) operating instructions. RT-PCR was conducted based on the instructions of SYBR I Premix Ex Taq™ kit (TaKaRa Holding Inc., Otsu, Shiga, Japan), and PCR was conducted according to ABI 7500 Real-Time PCR system (Applied, Inc., Foster City, CA, USA). The primers were synthesized by Shanghai GenePharma Co, Ltd (Shanghai, China) and the sequences are shown in Table I. Reaction conditions were set as pre-denaturation at 95ºC for 3 min, denaturation at 95ºC for 10 s, and 59ºC for 30 s. The fluorescence signals were collected after annealing. With 3 duplicated wells set in each plate, the experiment was repeated 3 times. The relative expression was calculated by $2^{-\Delta\Delta Ct}$. $\Delta\Delta Ct = [Ct\ (target\ gene) - Ct\ (reference\ gene)]_{experimental\ group} - [Ct\ (target\ gene) - Ct\ (reference\ gene)]_{control\ group}$.

Gene Silencing and Cell Transfection

According to the lncRNA FAL1 and PTEN sequences provided by GenBank, siRNA-FAL1, siRNA- negative control (NC), and siRNA-PTEN were designed and synthesized, as shown in Table II. SKOV3 cells were inoculated into RPMI-1640 medium containing 10% FBS without exosomes. SKVO3 cells in logarithmic growth phase were inoculated into a 6-well plate at about 2 × 10^5 cells/well for 14-18 h. Next, siRNA-FAL1 and siRNA-NC were respectively transfected into

Table I. Primer sequences of RT-qPCR.

	Forward primers	Reverse primers
FAL1	5'-GCAAGCGGAGACTTGTCTTT-3'	5'-TTGAACTCCTGACCTCGTGA-3'
PTEN	5'-TTTGAAGACCATAACCCACCAC-3'	5'-ATTACACCAGTTCGTCCCTTTC-3'
GAPDH	5'-GCCGTCTAGAAAAACCTGCC-3'	5'-ACCACCTGGTGCTCAGTGTA-3'

Note: FAL1, focally amplified lncRNA on chromosome 1; PTEN, phosphatase and tensin homologue; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

Table II. Primer sequences of siRNAs.

	Forward primers	Reverse primers
siRNA-FAL1	5'-GCGGAGACUUGUCUUUAAATT-3'	5'-UUUAAAGACAAGUCCGC
siRNA-NC	5'-UUCUCCGAACGUGUCACGUTT-3'	5'-ACGUGACACGUUCGG ATT-3'
siRNA-PTEN	5'-CCGAUACUUCUCUCCAAAUTT-3'	5'-AUUUGGAGAGAAG GGTT-3'

Note: FAL1, focally amplified lncRNA on chromosome 1; NC, negative control; PTEN, phosphatase and tensi gue.

SKOV3 cells using Lipofectamine 2000 (Invitrogen Inc., Carlsbad, CA, USA). Following a 3 d-cultivation in a 37°C incubator with 5% CO_2, cell supernatant was collected, and cell debris was removed by centrifugation. The extraction and identification of exosomes was identical to the above methods.

The expression of lncRNA FAL1 in siRNA-FAL1 cells and siRNA-NC cells, siRNA-FAL1 exosomes and siRNA-NC exosomes was separately detected by RT-qPCR to verify the knockout effect of FAL1 and its expression in exosomes. The siRNA-FAL1 exosomes and siRNA-NC exosomes were co-cultured with SKVO3 and HO-8910PM cells respectively to verify ir effects on OC metastasis *in vitro* and *in v* same method as above).

Western Blot Analysis

OC cells co-cultured with ex in diffe ent groups were collected to e proteins. The standard protein dilut rve w ade by bicinchoninic acid (BCA thod, tein concentration of ch A total of 50 g prot was se d by sodium dodecyl sulfate-p rylamide g trophoresis (SDS-PAGE trophoresis uct was transferred t e difluoride (PVDF) membrane d sealed 5% bovine serum albumin BA). The mem was then cultured prima antibodies: monoclonal rabbit anti an PT antibody (1:1000) and monoclo bit -huma KT antibody (1:1000), monoc bbit a uman p-AKT antibody 00), a al mouse anti-human β-actin body ( ) overnight. Then, the membra was washe in Tris-Buffered Saline Tween ((10 min/time) and incubated with ondary antibody goat anti-rabbit (or mouse) 2000) labeled by horseradish peroxidase (HRP) h at room temperature. Molecular imager Ch Doc XRS system (Bio-Rad Laboratories, CA, USA) and enhanced chemiluminescence (ECL) reagent (Millipore Corp, Billerica, MA, USA) were used for ex re. All bodies w brought from Cell ing nology (CST, Beverly, MA, US

Statistica

Statisti Pro and Service Solution (SPSS) 17.0 (SPSS In icago, IL, USA) and Gr Prism 5.0 w employed for data a sis. Comparisons between two groups were yzed using st and among multi-groups analyzed u g one-way analysis of variance (ANOVA) wo-way ANOVA. The pairwise after one-way ANOVA were conduct ukey's multiple comparisons test, nd pairwise comparisons after two-way ANOVA conducted by Sidak's multiple comparisons t. $p < 0.05$ meant statistical difference, and $p < 0.01$ meant significant statistical difference.

Results

Successful Extraction of Exosomes Derived from SKOV3 Cells

Typical round or oval cup-shaped exosomes with diameters ranging from 40 nm to 100 nm can be seen under the transmission electron microscope (Figure 1A). Western blot analysis detected the marker of OC SKOV3 cells-secreted exosomes – CD63 (Figure 1B). Nanosight, used for the detection of the size, distribution and concentration of exosomes, found that most particles were in the range of exosome diameter (30-100 nm). The diameter of the most abundant particles was about 88 nm (Figure 1C).

SKOV3 and HO-8910PM Cells Can Absorb and Internalize Extracted Exosomes

To verify whether extracted exosomes can be re-absorbed and internalized by OC cell lines SKOV3 and HO-8910PM, purified exosomes la-

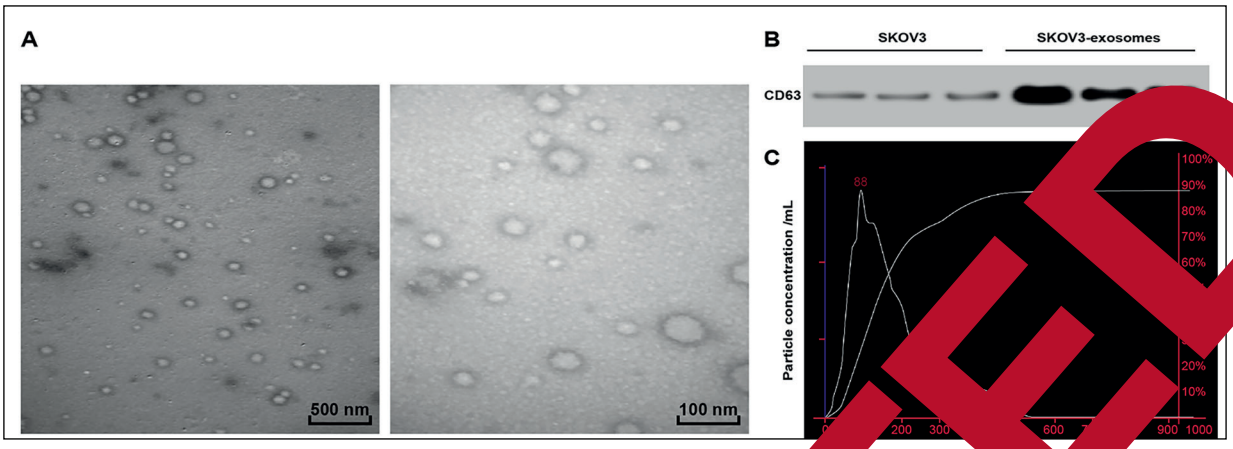
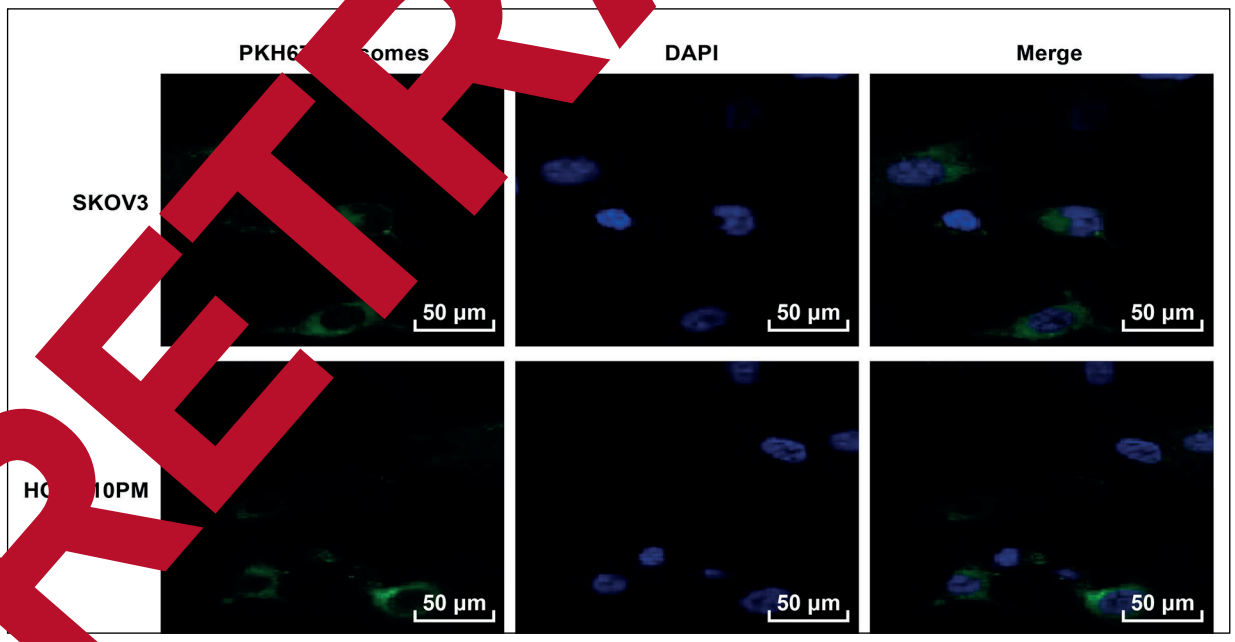


Figure 1. Identification of exosome. **A,** The morphology of exosomes observed [illegible] transmissio[illegible] microscope (Left: 20000 ×; right: 10000 ×). **B,** Level of exosome marker CD63 detecte[illegible] blot analysis. **C,** Diameter and concentration (1: 40) of exosome observed by Nanosight.

beled with PKH67 dyeing were applied to stimulate OC cell lines SKOV3 and HO-8910PM for 12 h. After nucleation with DAPI, the distribution and intensity of fluorescence were observed under a laser confocal microscope. The confocal results indicated that SKOV3 and HO-8910PM ca[illegible] sorb and internalize exosomes, and fluo[illegible] labeled exosomes mainly distributed in t[illegible] toplasm, especially around the perinuclear [illegible] (Figure 2).

S[illegible]V3-Secreted Exosomes Accelerate [illegible]ration and [illegible]vasion of OC Cells

KOV3 cell-[illegible]eted exosomes were separa[illegible] co-cultur[illegible] with SKVO3 cells and HO-891[illegible] [illegible] erify the invasion and migration abi[illegible] OC cells before and after exosome treatment. The results indicated that the migra[illegible] invasion of OC cells were greatly en[illegible] after exosome treatment (all $p < 0.05$) (Figure 3A-B).



Figu[illegible] 2. Representative images of the internalization of OC cells and exosomes (200 ×). PKH67 (green fluorescence) labeled exosomes, DAPI (blue fluorescence) stained nucleus, Merge observed co-localization of fluorescence. OC, ovarian cancer; DAPI, 4',6-diamidino-2-phenylindole.

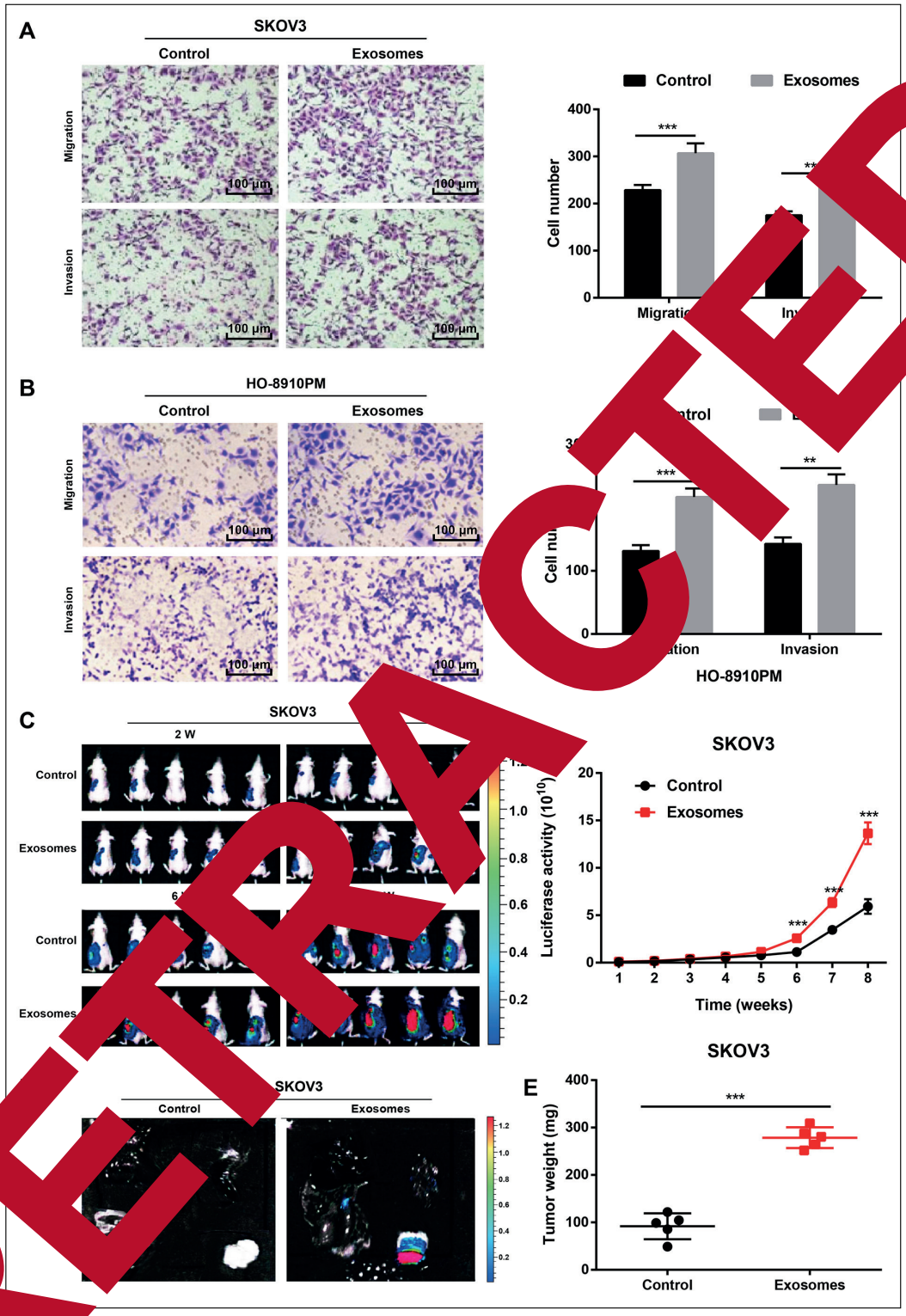


Figure 3. SKOV3-secreted exosomes accelerate migration and invasion of OC cells. **A,** The invasion and migration of SKOV3 cells before and after exosome treatment (100 ×). **B,** The invasion and migration of HO-8910PM cells before and after exosome treatment (100 ×). **C,** The tumorigenicity of SKOV3 cells in nude mice before and after exosome treatment. **D,** The metastasis of SKOV3 cells in nude mice before and after exosome treatment. **E,** Weight of xenograft tumors produced by SKOV3 cells before and after exosome treatment. Data in panel A, B, and C were analyzed by two-way ANOVA, and pairwise comparisons after two-way ANOVA were conducted by Sidak's multiple comparisons test; data in panel E were checked by independent *t*-test. $^{**}p < 0.01$; $^{***}p < 0.001$; OC, ovarian cancer.

In order to elucidate the effect of SKOV3-secreted exosomes on the tumorigenesis and migration of OC cells *in vivo*, we constructed nude mice model of *in situ* tumors of SKOV3 cells, in which SKOV3 cells were injected intraperitoneally, and the growth and organ metastasis of OC *in situ* were continuously observed *in vivo* by small animal imaging technology. The results demonstrated that intraperitoneal injection of SKOV3 cell exosomes boosted the growth and metastasis of nude mice *in situ* tumors. In the control group, 2 nude mice had live metastasis, 2 spleen metastasis, 2 kidney metastasis, and 1 pancreas-omentum metastasis, while in the exosome group, 3 nude mice had live metastasis, 4 spleen metastasis, 2 kidney metastasis, and 4 pancreas-omentum metastases (Figure 3C-E).

LncRNA FAL1 is Up-Regulated in OC Cells Treated with SKOV3-Secreted Exosomes

SKOV3-derived exosomes affect the invasion and migration of OC cells, but its mechanism remained unknown. It has been reported that lncRNA FAL1 affects the invasion, migration and apoptosis of epithelial OC cells. The abnormal increase of lncRNA FAL1 expression may be a critical molecular mechanism for the occurrence and progression of OC. To explore whether lncRNA FAL1 exerted functions in cell growth after exosome treatment, RT-qPCR was applied to detect lncRNA FAL1 expression in OC cells before and after exosome treatment. Results displayed that lncRNA FAL1 expression in cells after exosome treatment was higher than that before treatment ($p < 0.001$) (Figure 4). Thus, we stated that lncRNA FAL1 played an important role in the invasion and migration of OC cells after exosome treatment.

siRNA-FAL1 Inhibits Migration and Invasion of OC Cells

To verify the effect of lncRNA FAL1 on the invasion and migration of OC cells treated by SKOV3-secreted exosomes, lncRNA FAL1 was firstly knocked down (siRNA-FAL1) and siRNA-FAL1 was then transferred into SKOV3 cells. The exosomes from SKOV3 cells were extracted with siRNA-NC and control siRNA-FAL1 expression in siRNA-FAL1 cells and siRNA-NC cells, siRNA-FAL1 exosomes, and siRNA-NC exosomes were detected by RT-qPCR. The results (Figure 5A) revealed that lncRNA FAL1 was knocked out in cells and lncRNA FAL1 expression in exosomes was also decreased, which was significantly different from that in the siRNA-NC group.

SiRNA-FAL1 exosomes and siRNA-NC exosomes were separately co-cultured with SKVO3 cells and HO-8910PM cells to verify the effect of exosomes on invasion and migration of OC cells after lncRNA FAL1 knockout. The results revealed that after lncRNA FAL1 knockout, the migration and invasion abilities of siRNA-FAL1 exosomes-treated OC cells were noticeably lower than those of siRNA-NC exosomes-treated OC cells (Figure 5B-C), suggesting that lncRNA FAL1 showed potent effects on SKOV3-secreted exosomes regulating invasion and migration of OC cells.

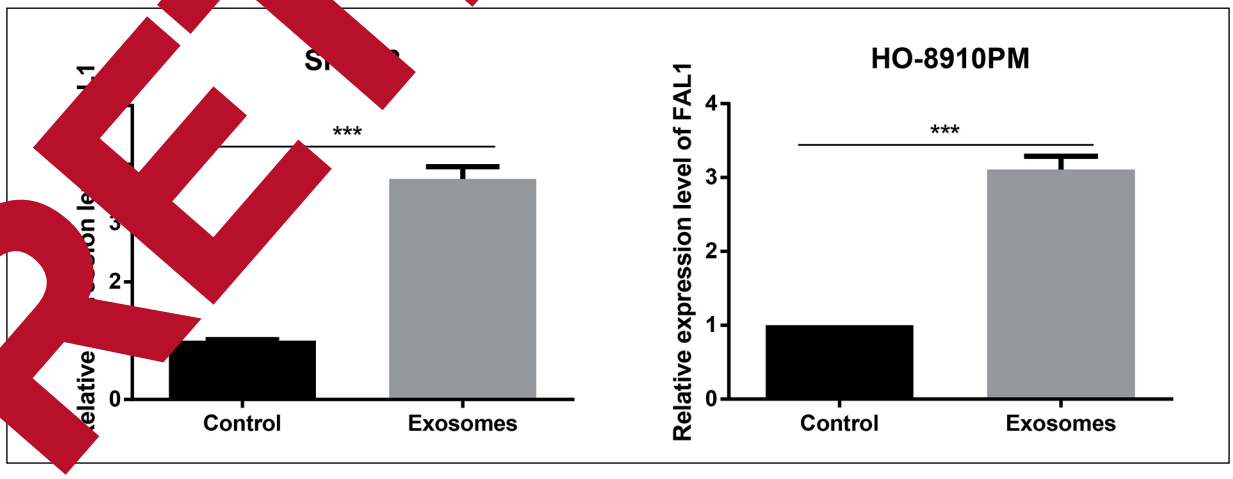


Figure 4. LncRNA FAL1 is upregulated in OC cells after exosome treatment. ***$p < 0.001$, compared with the control group; data were checked by independent *t*-test. OC, ovarian cancer; FAL1, focally amplified lncRNA on chromosome 1.

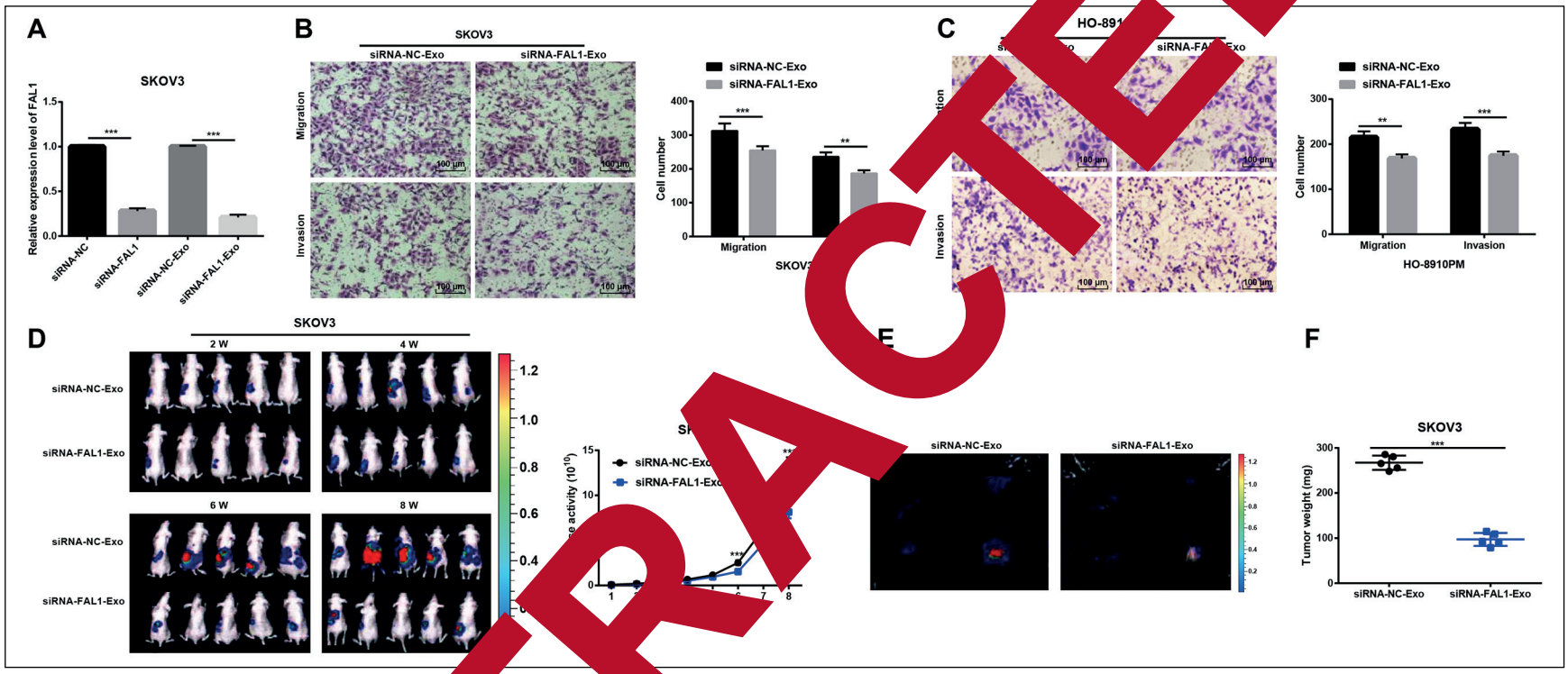


Figure 5. siRNA-FAL1 inhibits the promotion e secreted exo on the metastasis of HO-8910PM cells. **A,** LncRNA FAL1 expression in SKOV3 cells and exosomes after lncRNA FAL1 knockout. **B,** The invasion mig of SKOV3 cells after SKOV3 exosome treatment and lncRNA FAL1 knockout (100 ×). **C,** The invasion and migration of HO-8910PM cells after SKOV3 exosome eatment and IA FAL1 knockout (100 ×). **D,** The tumorigenicity of SKOV3 cells in nude mice after SKOV3 exosome treatment and lncRNA FAL1 knockout. **E,** The met s of SKOV3 c er exosome treatment and lncRNA FAL1 knockout. **F,** Weight of xenograft tumors produced by SKOV3 cells after exosome treatment and lncRNA F knockout. Data in p A were analyzed by one-way ANOVA, and pairwise comparisons after one-way ANOVA were conducted by Tukey's multiple comparisons test. D n panel C, and D were analyzed by two-way ANOVA, and pairwise comparisons after one-way ANOVA were conducted by Sidak's multiple comparisons test. Data i l F w hecked by independent *t*-test. $^{**}p < 0.01$; $^{***}p < 0.001$; OC, ovarian cancer; lncRNA, long non-coding RNA; FAL1, focally amplified lncRNA on chromosome 1

siRNA-FAL1 exosomes and siRNA-NC exosomes were used to treat nude mice model of *in situ* tumors of SKOV3 cells. The results showed that compared to the nude mice treated with siRNA-NC exosomes, siRNA-FAL1 exosomes could significantly inhibit the formation and metastasis of tumors in nude mice, presenting with slowed down growth rate of tumors, decreased weight of tumors, and increased metastasis of liver, spleen, kidney, pancreas, and omentum in nude mice in the siRNA-FAL1 exosome group (Figure 5D-F). These results suggested that siRNA-FAL1 significantly inhibited the promotion of SKOV3 exosomes on SKOV3 cell metastasis *in vivo*.

LncRNA FAL1 Inhibits Activation of PTEN/AKT Signaling Pathway

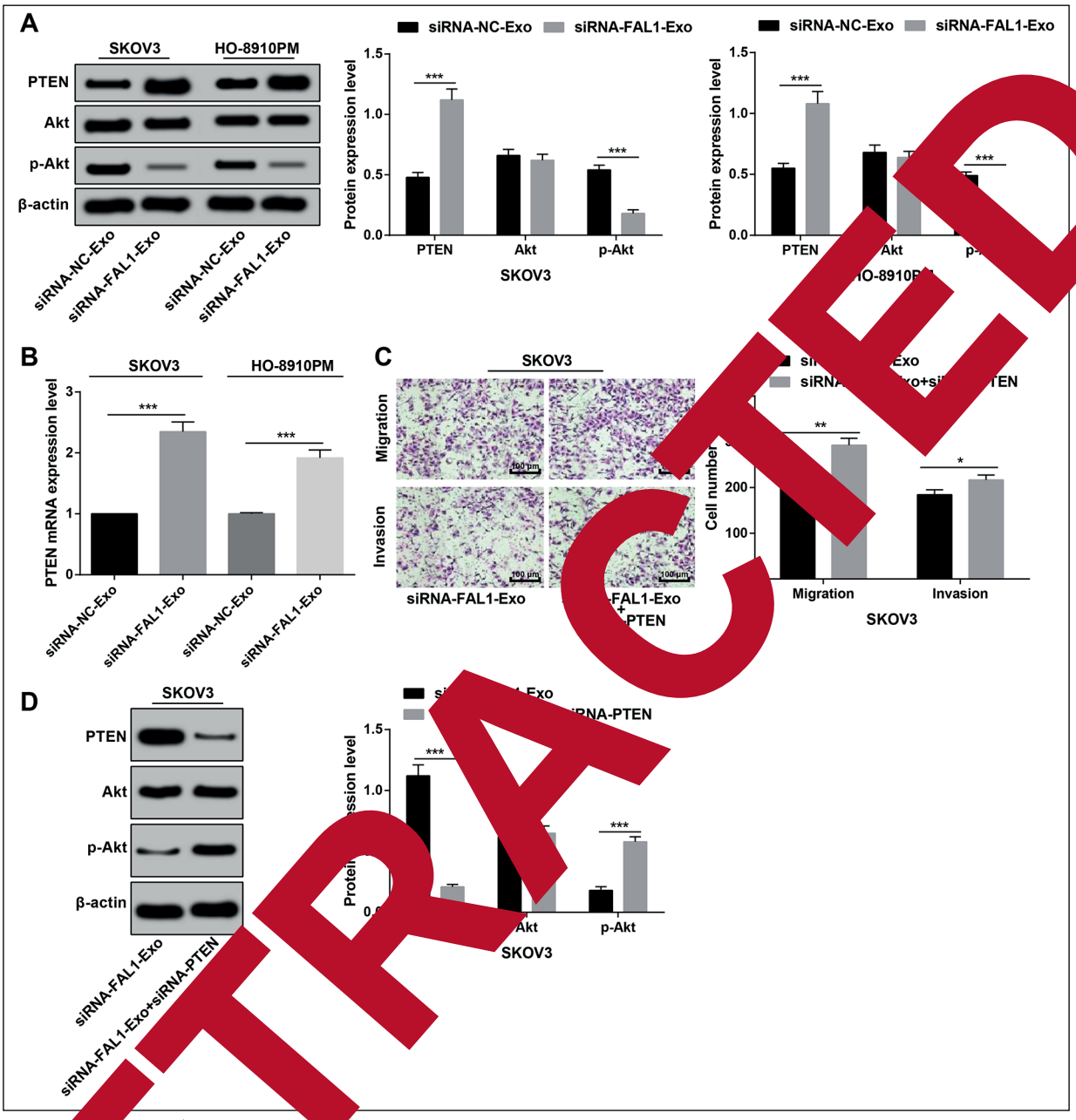
Western blot analysis showed that compared with the siRNA-NC exosomes, PTEN level in OC cells treated with siRNA-FAL1 exosomes was increased, and AKT phosphorylation level was decreased, while the total AKT level remained unchanged (Figure 6A). RT-qPCR detected the mRNA expression of PTEN and found that PTEN was highly expressed in OC cells treated wit[h] siR-NA-FAL1 exosomes (Figure 6B). In SKO[V] treated both with siRNA-FAL1 exosomes a[nd] siR-NA-PTEN, siRNA-PTEN significantly recov[ered] the suppression of cell invasion and migration a[bil]ity by siRNA-FAL1 exosomes to [an] extent (Fig[ure] ure 6C). Western blot analys[is] [re]sented that when compared with th[e] [si]NA-F[AL1] group, AKT phosphorylation lev[el] [was] notab[ly] [in]creased after PTEN knockout (Figu[re]

[D]iscussion

Macroph[ages]-derived [a] tumor necrosis factor-li[ke] weak inducer [of] apoptosis (TNF-TWE[AK] treated exosomes inhibited epithelial OC[] progressi[on] by upregulating miR-7 and inacti[vat]ing epiderm[al] growth factor receptor (EGFR)/extracellular signal-regulated ki-nase (ERK) signaling pathway[15]. Based on this in[for]mation, we p[ro]posed a possible involvement of lncRNA FAL1 transported by exosomes with the PTEN/AKT signaling pathway in OC development. In summary, we offered evidence to support that SKOV3-secreted exosomes inhibited the PTEN/AKT signaling pathway by transferring lncRNA FAL1, thus inhibiting OC cell metastasis.

The first major result was that SKOV3-secreted exosomes promoted migration and invasion of OC cells. Exosomes could regulate cell proliferation, metastasis, angiogenesis, and immune regulation, thus exerting key functions in OC progression and drug resistance[16]. Consistent [with] results, Dorayappan et al[17] stated th[at exo]somes derived from OC cells and induced by hypoxia were able to promote cell migration, invasion, and tumor metastasis. Gang et al[18] demonstrated that exosomes derived from renal cancer cells increased cell migration and invasion, thus contributing to renal cancer development. Furthermore, we observed a high expression of lncRNA FAL1 in OC cells after exosome treatment. LncRNA FAL1 also presented high expression in osteosarcoma tissues, locally recurrent tissues, and increased lncRNA FAL1 expression was positively associated with tumor metastasis and higher stages, osteosarcoma occurrence and short survival time, serving as a prognostic indicator and diagnostic marker for osteosarcoma. Convincingly, research made in 2014 found that lncRNA FAL1 expression was elevated in OC at advanced stages and can be considered as a biomarker for poor prognosis of OC[19]. Circulating exosomes derived from serum of cancer tissues promoted hepatocellular carcinoma cell proliferation and invasion by transporting lncRNA FAL1[9]. Subsequently, our data discovered that siRNA-FAL1 inhibited the promotion of SKOV3-secreted exosomes-induced metastasis in HO-8910PM cells. Interestingly, siRNA-FAL1 was verified to suppress tumor growth in a mouse model of OC at advanced stages[19]. Similarly, lncRNA FAL1 knockdown was also able to repress proliferation, invasion, and migration activity of H1299 cell and in colorectal cancer[20,21].

Importantly, our evidence supported siRNA-FAL1 upregulated PTEN expression and downregulated AKT. AKT activation and PTEN loss were associated with disease recurrence and reduced survival in several cancers[22]. Acting as a potent tumor suppressor, PTEN could induce tumor cell apoptosis and block angiogenesis, thus suppressing the growth, invasion, and metastasis of tumors[11]. In addition, Martins et al[23] noted that PTEN loss was frequently observed in high-grade serous OC. While high AKT phosphorylation level had a strong link with poor overall survival and progression-free survival in OC. Recently, lncRNA FAL1 was identified to inhibit PTEN expression in gastric cancer and PTEN overexpression partially reversed the promoting effects of lncRNA FAL1 overexpression on gastric cancer development[24]. Besides, our results



Figur siRNA l exosomes inhibits OC cell metastasis by repressing the PTEN/AKT signaling pathway. **A,** Levels of P AKT, T in OC cells treated with siRNA-FAL1 exosomes. **B,** mRNA expression of PTEN in OC cells treated with exosom siRNA-NC exosomes. **C,** Cell invasion and migration ability of SKVO3 cells treated with siRNA-F somes RNA-PTEN (100 ×). **D,** Levels of PTEN, AKT, p-AKT in OC cells treated with siRNA-FAL1 mes a A-E . Data in panel A, C, and D were analyzed by two-way ANOVA, and pairwise comparisons after ANO nducted by Sidak's multiple comparisons test. Data in panel B were analyzed by one-way ANOVA, and wise com ons after one-way ANOVA were conducted by Tukey's multiple comparisons test. *$p < 0.05$; **$p < 0.01$ < 0.001; OC, ovarian cancer; lncRNA, Long non-coding RNA; FAL1, focally amplified lncRNA on chromosome 1; P e and tensin homologue; AKT, protein kinase B.

strated that siRNA-PTEN saved OC cell inv on and migration. PTEN can induce G1 cell arrest and then suppress OC cell growth through negative regulation of the PI3K/AKT signaling pathway[13]. LncRNA FAL1 silencing restrained cell proliferation, invasion, and migration in non-small cell lung cancer *via* the PTEN/AKT signaling pathway[20]. PTEN knockdown combined with

increased AKT phosphorylation level reversed the repressive roles of miR-216a downregulation in OC development[25]. Additionally, exosomes from TWEAK-stimulated macrophages inhibited phosphorylation levels of AKT and ERK1/2 in epithelial OC cells[15]. Taken together, siRNA-FAL1 exosomes inhibits OC cell metastasis by inhibiting the PTEN/AKT signaling pathway.

Conclusions

In summary, we provided compelling evidence to state that tumor-secreted exosomes promoted OC metastasis, while lncRNA FAL1 silencing reversed the promotion effects *via* the PTEN/AKT signaling pathway. This study may offer new perspective for further understanding of the mechanism of OC and finding new targets for molecular targeted therapy. Further researches should be conducted to find out exact application approach for OC based on results obtained from this study.

Conflict of Interest

The Authors declare that they have no conflict of in[illegible]s.

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