Long non-coding RNA EPB41L4A-AS2 suppresses progression of ovarian cancer by sequestering microRNA-103a to upregulate transcription factor RUNX1T1

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Abstract
Ovarian cancer (OC) is a malignant tumour with a poor prognosis. Emerging evidence has shown that long non-coding RNAs (lncRNAs) are regulators that can be used for prognosis, diagnosis and targeted therapy of cancers. Therefore, our purpose was to investigate the possible regulatory role of the lncRNA EPB41L4A-AS2 in the progression of OC. Initially, EPB41L4A-AS2 expression was determined in OC tissues and matched paracancerous tissues. Then the RNA crosstalk among EPB41L4A-AS2, miR-103a and RUNX1T1 was determined. Subsequently, the expression of EPB41L4A-AS2, miR-103a and RUNX1T1 was up- or downregulated by exogenous transfection in SK-OV-3 cells to investigate their roles in the proliferation, migration, colony formation and invasion of OC cells. Further, the tumour formation ability of nude mice was tested in vivo. EPB41L4A-AS2 was poorly expressed in OC tissues and cells, and microarray data revealed upregulation of miR-103a and downregulation of RUNX1T1 in OC. RUNX1T1 was a target gene of miR-103a and the RNA crosstalk among EPB41L4A-AS2, miR-103a and RUNX1T1 was determined. Subsequently, the expression of EPB41L4A-AS2 was upregulated in SK-OV-3 cells to investigate their roles in the proliferation, migration, colony formation and invasion of OC cells. Further, the tumour formation ability of nude mice was tested in vivo. EPB41L4A-AS2 was poorly expressed in OC tissues and cells, and microarray data revealed upregulation of miR-103a and downregulation of RUNX1T1 in OC. RUNX1T1 was a target gene of miR-103a and EPB41L4A-AS2 bound to miR-103a. Moreover, EPB41L4A-AS2 increased RUNX1T1 expression by decreasing miR-103a expression. EPB41L4A-AS2-overexpressing SK-OV-3 cells exhibited inhibited proliferation, migration, colony formation and invasion, which was rescued by overexpression of miR-103a or silencing of RUNX1T1. Besides, overexpressed EPB41L4A-AS2 repressed tumour formation in vivo. Altogether, the current study demonstrates that overexpressed EPB41L4A-AS2 can potentially bind to miR-103a to promote the expression of RUNX1T1, thereby inhibiting OC, highlighting the potential of EPB41L4A-AS2 as a target for OC.

KEYWORDS
colony formation, invasion, long non-coding RNA EPB41L4A-AS2, microRNA-103a, migration, ovarian cancer, proliferation, Runt-related transcription factor 1

1 | INTRODUCTION

Ovarian cancer (OC), a heterogeneous cancer with a key heritable component, is difficult to diagnose at an early stage, and about 70% of women with OC are diagnosed at stages III/IV, of whom only about 30% will survive over 5 years (Jones, Kamara, Karlan, Pharoah, & Gayther, 2017). In 2017, there was a notably upward trend in the incidence of OC in Asia; the distribution was closely related to regional differences and varied particularly by income level (Coburn, Bray, Sherman, & Trabert, 2017). The pathogenesis and origin of OC are still poorly understood, and efforts made to detect OC at an early stage and to explore new therapeutic approaches have been largely unsuccessful (Kurman & Shih Ie, 2010). A variety of long non-coding RNAs (lncRNAs), such as nuclear enriched abundant transcript 1 and metastasis-associated lung adenocarcinoma transcript 1, have been suggested to play a critical role in the progression of OC (Chai, Liu, Zhang, & Liu, 2016; Lei, Xue, Zhang, & Lin, 2017). Therefore, it is necessary to explore the effect of lncRNAs from the perspective of OC diagnosis and treatment.

LncRNAs play important roles in tumour initiation, growth and metastasis as they are involved in the regulation of chromatin organization, transcription and post-transcriptional processing by
co-functioning with protein molecules, DNA, RNA and/or their combinations (Yang, Lu, & Yuan, 2014a,b). It was reported that high expression of EPB41L4A-AS2, a novel lncRNA, contributes to favourable disease outcomes in breast cancers with a suppressive role in the proliferation of tumour cells (Xu et al., 2016). Using online analysis software in the present study, binding sites were found between the EPB41L4A-AS2 gene sequence and the sequence of microRNA-103a (miR-103a). Defined as a type of small non-coding RNA molecule, miRNAs can inhibit the expression of their target genes in a sequence-dependent manner, thereby functioning in cellular processes of cancers (Woźniak, Sztiller-Sikorska, & Czyź, 2015; Zhou & Rigoutsos, 2014). One study found that when miR-103a-3p or miR-107 was sequestered, their 13 target genes were upregulated, thereby changing the proliferation, migration and invasion of bladder cancer cells (Zhong, Lv, & Chen, 2016). Also, miR-103a-3p was demonstrated to be involved in the proliferation, migration and invasion process of OC cells (Bignotti et al., 2016). The gene Runt-related transcription factor 1 (RUNX1T1) is a putative target of miR-103a in the present study as demonstrated by the RNA22 website. Low RUNX1T1 expression was observed in primary pancreatic endocrine neoplasms (Nasir et al., 2011). Moreover, it was reported in a previous study that RUNX1T1 played a tumour suppressor role during OC (Yeh et al., 2011). Based on the aforementioned information, we hypothesized that there is an interaction between EPB41L4A-AS2, miR-103a and RUNX1T1 and that this has a function in the occurrence and development of OC. Therefore, our purpose in the present study was to investigate how EPB41L4A-AS2, miR-103a and RUNX1T1 interact with each other, and the role their interaction plays in the progression of OC.

2 | METHODS

2.1 | Ethics approval

The study protocol was approved by the Ethics Committee and Experimental Animal Ethics of Jining No. 1 People’s Hospital (approval reference number: 201201005 (human) and 201806004 (animal)). Informed written consent was obtained from each patient prior to the study. All experiments involving human specimens in the present study were conducted in strict accordance with the Declaration of Helsinki (Clinical trial: ChiCTR1900025687). The animal experiments strictly adhered to the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, 8th edition (2011), and conformed to the principles and regulations as described in the editorial by Grundy (2015). Every effort was made to minimize the pain, suffering and discomfort of the experimental animals.

2.2 | Microarray-based gene expression profiling

With OC as a key word, the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) was used to search the OC-related miRNA microarray data GSE83693 (Nam et al., 2016) and gene microarray data GSE18520 (Mok et al., 2009), both of which consisted of normal samples and OC samples. A differential analysis was performed using the R language. Standardization and difference analysis of microarray data were conducted using the limma package (http://master.bioconductor.org/packages/release/bioc/html/limma.html; Ritchie et al., 2015) with the conditions of an absolute fold change (FC) value of greater than 1.5 and adjusted P-value < 0.05 (the P-value was corrected by the false discovery rate). After that, a heatmap in relation to differentially expressed genes (DEGs) was plotted using the pheatmap package (https://cran.r-project.org/web/packages/pheatmap/index.html). The subcellular localization of differentially expressed IncRNAs was confirmed in the LncATLAS database (http://lncatlas.crg.eu/; Mas-Ponte et al., 2017), and then miRNAs that may bind to IncRNAs were predicted using the RNA22 database (https://cm.jefferson.edu/rna22/; Miranda et al., 2006). The jvenn package (http://jvenn.toulouse.inra.fr/app/example.html) was subsequently used to compare the predicted miRNAs and the differentially expressed miRNAs in the microarray data GSE83693 to screen differentially expressed miRNAs (Bardou, Mariette, Escudie, Djemiel, & Klop, 2014). Then the potential target genes regulated by the screened miRNA were predicted using RNA22, mirDIP (http://ophid.utoronto.ca/mirDIP/; Tokar et al., 2018), TargetScan (http://www.targetscan.org/vert_71/; Agarwal, Bell, Nam, & Bartel, 2015), miRDB (http://www.mirdb.org/; Liu & Wang, 2019), miRWalk (http://mirwalk.umm.uni-heidelberg.de/; Sticht, De La Torre, Parveen, & Gretz, 2018) and DIANA (http://diana.imis.athena-innovation.gr/DianaTools/index.php?=microT_CDS/index; Paraskevopoulou et al., 2013). Then the predicted target genes were compared with DEGs in microarray data GSE18520 in an attempt to screen DEGs.

2.3 | Study subjects

Human OC tissues and matched paracancerous tissues were collected from 126 patients admitted at Jining No. 1 People’s Hospital from February 2012 to February 2015. None of patients received antitumour treatment such as radiotherapy or chemotherapy before operation. The resected cancerous tissues were pathologically confirmed as OC, while the paracancerous tissues were pathologically confirmed to contain no cancer cells (Yang et al., 2018a, 2018b). The
TABLE 1  Primer sequences for RT-qPCR

| Primer sequence   | Forward (5’-3’) | Reverse (5’-3’) |
|-------------------|----------------|----------------|
| EPB41L4A-AS2      | GTCGCGATTAGGGAGACAC | TGCTACCCCAGCTAACAAAGC-3 |
| RUNX1T1           | GAAAGCCCACGACATGATCAC | CAGCCACTGAGGTTTCACTC |
| miR-103a          | AAGAGAATTCGAGGTATTC | GCAAGGTCGAGGTTATTC |
| U6                | TCGTGTCTTCGCCAGC | AAAATATGGAAGGCTTCAAG |
| β-Actin           | GTGGAATCAGCAAGGAGGAGT | ATCCGTAGTCAAGGCGCAA |

miR-103a, microRNA-103a; RT-qPCR, reverse transcription quantitative polymerase chain reaction; RUNX1T1, runt-related transcription factor 1.

collected fresh tissues were temporarily stored in at −80°C in an ultra-low-temperature freezer, and long-term preserved in liquid nitrogen.

2.4  Follow-up study

All patients with OC were followed up by means of telephone or subsequent visit before February 2018. The overall survival (OS) was recorded, which was defined as starting from randomization until the patients died for any reason. Up to the deadline, the follow-up period was 36 months, and 14 out of 126 patients were lost in the follow-up with a follow-up rate of 88.89%.

2.5  Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The total RNA from OC tissues was extracted by Trizol (cat. no. 16096020, Thermo Fisher Scientific Inc., Waltham, MA, USA), and 5 μg RNA was reverse transcribed into cDNA according to the instructions of the cDNA kit (K1622; Fermentas Inc., Burlington, Ontario, Canada). With cDNA as a template, RT-qPCR was conducted according to the instructions provided by TaqMan Gene Expression Assays protocol (Applied Biosystems/Thermo Fisher Scientific). U6 served as an internal control for miR-103a, and β-actin for other genes. Three duplicate wells were set for each RT-qPCR. Primer sequences are shown in Table 1. Quantification of relative gene expression was calculated by the 2−ΔΔCT method (Livak & Schmittgen, 2001).

2.6  Western blot analysis

The total protein of tissues or cells was lysed with lysis buffer containing phenylmethanesulfonyl fluoride on ice for 30 min and centrifuged at 11,800 g at 4°C for 15 min. The supernatant was transferred into a new Ependorf tube, and the protein concentration was estimated using a bicinchoninic acid kit (Thermo Fisher Scientific). Subsequently, 30 μg total protein was separated using PAGE and then transferred onto a polyvinylidene fluoride membrane (GE Healthcare, Chicago, IL, USA). The membrane was then blocked with 5% skim milk powder at room temperature for 1 h, and subsequently incubated overnight at 4°C with rabbit polyclonal antibodies (Abcam Inc., Cambridge, UK) to RUNX1T1 (ab124269, 1: 2000) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ab9485, 1: 2000). Next, the membrane was washed 3 times (10 min/time) with phosphate-buffered saline–Tween-20 (PBST) and incubated with horseradish peroxidase-labelled secondary goat anti-rabbit antibody to immunoglobulin G (lgG; ab6721, 1: 2000, Abcam) for 1 h at room temperature. After three PBST washes (10 min each time), the membrane was scanned and developed under an optical illuminometer (GE Healthcare Life Sciences, Piscataway, NJ, USA). The relative protein expression was analysed using Image Pro Plus 6.0 software (Media Cybernetics, Inc., Silver Spring, MD, USA).

2.7  Cell treatment

Normal human ovarian epithelial cell line HOSEpiC (cat. no. 7310, ScienCell Research Laboratories, Carlsbad, CA, USA) was cultured in ovarian epithelial cell medium (cat. no. 7311) in vitro. Four OC cell lines, HO-8910, OV-90, OVCAR-3 and SK-OV-3 (cat. no. ZQ0069, ZQ0073, ZQ0365 and ZQ0074, Zhong qiao Xin zhou Biotechnology Co., Ltd, Shanghai, China), were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), streptomycin (100 mg ml−1) and penicillin (100 U ml−1) at 37°C with 5% CO2. Upon reaching 85% confluence, the cells were subcultured.

Subsequently, the cells were transfected with plasmids for overexpression (oe)-EPB41L4A-AS2, oe-EPB41L4A-AS2 + miR-103a mimic and oe-EPB41L4A-AS2 + silencing (si)-RUNX1T1, with oe-NC, oe-EPB41L4A-AS2 + mimc NC and oe-EPB41L4A-AS2 + si-NC as control, respectively. After being trypsinized, the OC cells (2 × 106 cells ml−1) were seeded into a 12-well plate with 1 ml cell suspension in each well. Then, the cells were cultured for 24 h and transfected with the aforementioned plasmids in accordance with the instructions for Lipofectamine 2000 (Thermo Fisher Scientific) for 4 h. Finally, the original transfection solution was renewed with normal cell culture medium, and the cells were further cultured for subsequent experiments.

2.8  Cell counting kit-8 (CCK-8) assay

The transfected cells were detached and resuspended with the concentration adjusted to 1 × 105 cells ml−1. Subsequently, the cells were seeded into a 96-well plate at a density of 100 μl per well and cultured overnight. Then, the cell viability was determined in accordance with the instructions of a CCK-8 kit (Beyotime Biotechnology Co., Ltd, Shanghai, China) at the 24th, 48th, 72nd, and 96th hour post-seeding, during which 10 μl CCK-8 solution was added for a 4 h incubation. After that, the optical density (OD) value at 450 nm was measured with a microplate reader, and the growth curve was plotted.
2.9 | Colony formation assay

The cells in the logarithmic growth phase were dissociated to create a single cell suspension and counted. A total of 1000 cells were seeded into 60 mm culture dishes and then cultured with 5% CO₂, during which the culture medium was renewed every 3 d. After 14 d, the medium was discarded, and the cells were washed 3 times with PBS, fixed with methanol for 15 min, and stained with crystal violet for 15 min. Finally, the cell colonies formed containing over 50 cells was counted under a microscope (Wang et al., 2019a).

2.10 | Transwell assay

Cell migration experiments were firstly conducted. In brief, the OC cells in logarithmic growth phase were starved for 24 h and then subjected to detachment, centrifugation and resuspension at a final concentration of 2 x 10⁵ cells ml⁻¹. Then, 0.2 ml suspension was added to the apical chamber, while 700 µl pre-cooled DMEM containing 10% FBS was added to the basolateral chamber. Subsequently, the cells were cultured at 37°C with 5% CO₂. After 24 h, the cells on the apical chamber and the basement membrane were wiped off using a wet cotton swab, fixed for 30 min using methanol, and stained for 20 min with 0.1% crystal violet. Finally, the cells were observed and photographed under an inverted microscope with five fields of view (×200) randomly selected to count the number of transmembrane cells.

Cell invasion experiments were then conducted. In brief, extracellular matrix (ECM) Matrigel was placed at 4°C overnight at 4°C, and the next day, the Matrigel was diluted at a ratio of 1:9 using serum-free medium to a final concentration of 1 mg ml⁻¹ (all the pipettes and chambers were pre-cooled on ice for 30 min in advance). A total of 40 µl ECM Matrigel was added to the polycarbonate membrane in the apical chamber of a 24-well Transwell chamber, incubated for 5 h in an incubator at 37°C with 5% CO₂ to polymerize the ECM Matrigel. Then, 70 µl pure DMEM was added to each chamber for incubation at 37°C for 30 min to rehydrate the Matrigel. After starvation for 24 h, the cells were subjected to detachment, centrifugation and resuspension in FBS-free DMEM at a final concentration of 2.5 x 10⁵ cells ml⁻¹. In the next step, 0.2 ml suspension was added to the apical chamber in which the basement membrane had been hydrated, and to the basolateral chamber, 700 µl pre-cooled DMEM with 10% FBS was added. Thereafter, the cells were cultured at 37°C with saturated humidity and 5% CO₂ for 24 h. Subsequently, wet cotton swabs were used to wipe off the cells on the chamber and basement membrane. The chamber was fixed with methanol for 30 min, stained for 20 min using 0.1% crystal violet and dried in an inverted position. Finally, the invasive cells were counted in five randomly selected visual fields with ×200 magnification under an inverted microscope.

2.11 | Fluorescence in situ hybridization

The subcellular localization of EPB41L4A-AS2 in OC lines was identified by fluorescence in situ hybridization (FISH). According to the instructions of Ribo™ IncRNA FISH probe Mix (Red) (Guangzhou RiboBio Co., Ltd, Guangzhou, China), OC cells were seeded onto the coverslips, which were placed into a six-well culture plate in advance, followed by 1 day of culture to ensure 80% confluence. Then, the slides were fixed at room temperature using 1 ml 4% paraformaldehyde and treated with 2 µg ml⁻¹ proteinase K, glycine and acetic acid reagent. Subsequently, the cells were incubated with 250 µl prehybridization solution for 1 h at 42°C and hybridized with 250 µl hybridization solution containing probes (300 ng ml⁻¹) overnight at 42°C. Subsequently, 4′-6-diamidino-2-phenylindole (DAPI; 1: 800) diluted by PBST was added to stain the nucleus for 5 min. Then the cells were sealed with anti-fluorescent quencher after being washed 3 times with PBST (3 min each time). Finally, five different visual fields were selected to photograph cells under a ×400 fluorescence microscope (Olympus Optical Co., Ltd, Tokyo, Japan).

2.12 | RNA immunoprecipitation assay

The binding of miR-103a, EPB41L4A-AS2 and RUNX1T1 was detected by RNA immunoprecipitation (RIP) kit (Millipore, Billerica, MA, USA). The OC cells were washed with pre-cooled PBS, centrifuged and the supernatant discarded. Next, an equal volume of lysis buffer (PO013B, Beyotime Biotechnology Co.) was used to lyse the cells in an ice bath for 5 min, and the supernatant was obtained after centrifugation at 22,000 g for 10 min at 4°C. Part of the cell extracts was collected as input and the rest was incubated with the antibody for co-precipitation. In specific terms, 50 µl magnetic beads in each co-precipitation reaction system were resuspended in 100 µl RIP wash buffer. Then 5 µg antibody was added for the incubation depending on the grouping to form a magnetic bead–antibody complex. Subsequently, the magnetic bead–antibody complex was washed and then resuspended in 900 µl RIP wash buffer, followed by overnight incubation with 100 µl cell extract at 4°C. Next, the magnetic bead–protein complex was collected from the sample on a magnetic stand. The antibodies used in the experiment included anti-Argonaute2 (AgO2; ab32381, 1: 50, Abcam) for 30 min at room temperature, and IgG (1: 100, ab109489, Abcam), which was taken as a negative control (NC). Finally, RNA was extracted from the sample and input by proteinase K for subsequent RT-qPCR detection.

2.13 | Dual luciferase reporter gene assay

To construct luciferase reporter vector, RUNX1T1-3′-untranslated region (3′UTR) fragment and EPB41L4A-AS2 cDNA fragment containing the binding site of miR-103a were inserted into pGL3 plasmid. By point mutation, RUNX1T1-3′UTR-mutant (MUT) fragments (RUNX1T1-MUT sequence: 5′-UGCGUAGGACAG UGGUACGAAUU-3′) and EPB41L4A-AS2-MUT fragments were constructed and inserted into pGL3 plasmid. The insertion sequence was validated by sequencing. The recombinant vectors of pGL3-EPB41L4A-AS2-wild-type (WT), pGL3-EPB41L4A-AS2-MUT, pGL3-RUNX1T1-3′UTR-WT, pGL3-RUNX1T1-3′UTR-MUT and the Renilla reference plasmid were co-transfected into HEK293T cells with
EPB41L4A-AS2 upregulates RUNX1T1 by overexpressed EPB41L4A-AS2 inhibits the proliferation, migration and invasion of OC cells.

RESULTS

2.14 Tumour xenograft in nude mice

A total of 20 female BALB/c nude mice (aged 3–4 weeks, weighing 14 ± 2 g) were purchased from Hui Ao Biotechnology Co., Ltd (Beijing, China). All mice were housed in an environment with constant temperature of (25–27°C) and humidity (45%–50%) with free access to water and food. These mice were then respectively injected with OC cells expressing oe-NC and oe-EPB41L4A-AS2 (10 mice for each injection). Subsequently, stably transfected cell lines were constructed, and the cells were adjusted to a concentration of 1 × 10⁷ cells ml⁻¹. Then 20 µl cell suspension was subcutaneously inoculated into nude mice, after which the growth of tumours was observed and recorded every day after photographing. The volume of tumours was also recorded at an interval of 6 d, followed by plotting a growth curve based on a formula of \((a \times b^2)/2\) (where \(a\) represents the longest diameter of tumours and \(b\) represents the shortest diameter of tumours) (Zhu, Ma, & Zhang, 2017). After 30 days, the nude mice were killed by excessive inhalation of CO₂, and the xenograft tumours were extracted and weighed for subsequent RT-qPCR and western blot analysis.

2.15 Statistical analysis

SPSS 21.0 statistical software (IBM Corp., Armonk, NY, USA) was used for data analysis. Measurement data were expressed as mean ± standard deviation. Student’s paired t test was used for comparisons between cancer and paracancerous tissues, while an unpaired t test was used for the comparisons between two groups. One-way analysis of variance (ANOVA) was used to analyse data among multiple groups, followed by Tukey’s post hoc test. Repeated measurement ANOVA was utilized for comparisons of data at different time points, followed by a Bonferroni post hoc test. The effect of EPB41L4A-AS2 on the OS of OC patients was analysed by Kaplan–Meier method (the log-rank test). Counting data were analysed by a chi-square test. The difference was regarded as statistically significant when \(P < 0.05\).

3 RESULTS

3.1 EPB41L4A-AS2 is poorly expressed in OC tissues and cells

Initially, the R language was used to perform differential analysis of OC microarray data. From microarray data GSE18520, 1260 DEGs were screened as they met the conditions of an absolute FC value of greater than 1.5 and adjusted P-value < 0.05. A heatmap for the top 25 DEGs was plotted (Figure 1a), in which we noted that EPB41L4A-AS2 was expressed at a low level in OC. Besides, EPB41L4A-AS2 was identified as suppressing the progression of breast cancer (Pang et al., 2019), hepatocellular carcinoma (Wang, Wang, Shi, & Zhai, 2019b) and non-small cell lung cancer (Shu, Li, Chen, Zhu, & Yu, 2018). RT-qPCR was conducted to detect the expression of EPB41L4A-AS2 in 126 OC tissues and paracancerous tissues, the results of which documented that EPB41L4A-AS2 was poorly expressed in OC tissues compared with in paracancerous tissues (\(P < 0.05\); Figure 1b). These specimens were divided into the EPB41L4A-AS2-high group (63 cases) and the EPB41L4A-AS2-low group (63 cases) by taking the median expression of EPB41L4A-AS2 in 126 OC specimens of 2.169 as the cut-off point. As shown in Table 2, the expression of EPB41L4A-AS2 was closely related with tumour–node–metastasis (TNM) staging, Fuhrman grade and tumour size. The relationship between EPB41L4A-AS2 expression and the OS of patients with OC was analysed by the Kaplan–Meier method, and the results demonstrated that the OS of OC patients in the EPB41L4A-AS2-low group was lower than that in patients in the EPB41L4A-AS2-high group (Figure 1c, log-rank \(P < 0.05\)). RT-qPCR was conducted to detect the expression of EPB41L4A-AS2 in human normal ovarian epithelial cell line HOSEpiC and four OC cell lines (HO-8910, OV-90, OVCAR-3 and SK-OV-3). Compared with HOSEpiC cells, EPB41L4A-AS2 was downregulated in the four OC cell lines (\(P < 0.05\)), among which EPB41L4A-AS2 presented the lowest expression in SK-OV-3 cell lines (Figure 1d). Therefore, SK-OV-3 cells were selected for subsequent experiments.

3.2 Overexpressed EPB41L4A-AS2 inhibits the proliferation, migration and invasion of OC cells

The aforementioned results showed that EPB41L4A-AS2 was reduced in OC tissues and cells. To further study the role of EPB41L4A-AS2 in OC cells, the proliferation, migration and invasion of SK-OV-3 cells transfected with oe-EPB41L4A-AS2 were measured. RT-qPCR was first applied to detect the expression of EPB41L4A-AS2 after oe-EPB41L4A-AS2 transfection, the results of which showed a marked increase in relation to the expression of EPB41L4A-AS2 in cells transfected with oe-EPB41L4A-AS2 (\(P < 0.05\); Figure 2a). Subsequently, CCK-8 and colony formation assay were conducted to detect cell viability, and the results demonstrated that the viability of cells transfected with oe-EPB41L4A-AS2 was less than that of cells transfected with oe-NC (\(P < 0.05\); Figure 2b,c). The migration and invasion of OC cells were detected with a Transwell assay, the results of which showed that the migration and invasion of OC cells transfected with oe-EPB41L4A-AS2 were significantly decreased (\(P < 0.05\); Figure 2d,e). These results collectively documented that upregulated EPB41L4A-AS2 could inhibit the proliferation, migration and invasion of OC cells.

3.3 EPB41L4A-AS2 upregulates RUNX1T1 by binding to miR-103a

In order to further explore the mechanism of EPB41L4A-AS2 in OC, the subcellular localization data of EPB41L4A-AS2 was obtained using the EPB41L4A-AS2 was reduced in OC tissues and cells. To further study the role of EPB41L4A-AS2 in OC cells, the proliferation, migration and invasion of SK-OV-3 cells transfected with oe-EPB41L4A-AS2 were measured. RT-qPCR was first applied to detect the expression of EPB41L4A-AS2 after oe-EPB41L4A-AS2 transfection, the results of which showed a marked increase in relation to the expression of EPB41L4A-AS2 in cells transfected with oe-EPB41L4A-AS2 (\(P < 0.05\); Figure 2a). Subsequently, CCK-8 and colony formation assay were conducted to detect cell viability, and the results demonstrated that the viability of cells transfected with oe-EPB41L4A-AS2 was less than that of cells transfected with oe-NC (\(P < 0.05\); Figure 2b,c). The migration and invasion of OC cells were detected with a Transwell assay, the results of which showed that the migration and invasion of OC cells transfected with oe-EPB41L4A-AS2 were significantly decreased (\(P < 0.05\); Figure 2d,e). These results collectively documented that upregulated EPB41L4A-AS2 could inhibit the proliferation, migration and invasion of OC cells.
**FIGURE 1** EPB41L4A-AS2 is downregulated in OC tissues and cells. (a) Heatmap for the top 25 DEGs in microarray data GSE18520. The abscissa shows the sample number and the ordinate shows DEGs. The right upper histogram is the colour grading, and each rectangle in the panel corresponds to a sample expression value. (b) Expression of EPB41L4A-AS2 in OC tissues and paracancerous tissues measured by RT-qPCR. *P < 0.05 vs. paracancerous tissues, n = 126. (c) Relationship between the expression of EPB41L4A-AS2 and the prognosis of patients with OC analysed by Kaplan–Meier method. (d) Expression of EPB41L4A-AS2 in HOSEpiC, HO-8910, OV-90, OVCAR-3 and SK-OV-3 cell lines measured by RT-qPCR. *P < 0.05 vs. HOSEpiC cell lines. The cell experiments were repeated 3 times. Measurement data were expressed as mean ± standard deviation. A paired t test was used for comparison between cancer and paracancerous tissues, and ANOVA was used for comparisons among multiple groups, followed by Tukey’s post hoc test.
Overexpressed EPB41L4A-AS2 prevents the proliferation, migration and invasion of OC cells. SK-OV-3 cells were treated with oe-EPB41L4A-AS2 or oe-NC. (a) Expression of EPB41L4A-AS2 tested by RT-qPCR. (b,c) Cell proliferation measured by CCK-8 assay and colony formation assay. (d,e) Cell migration (d) and invasion (e) detected by Transwell assay. Scale bars: 50 μm.

3.4 Overexpressed EPB41L4A-AS2 prevents the progression of OC by activating RUNX1T1 via miR-103a

The expression of RUNX1T1 was measured by RT-qPCR and western blot analysis. The results showed that compared with cells transfected with oe-NC + mimic NC, the expression of RUNX1T1 was significantly increased in cells transfected with oe-EPB41L4A-AS2 + mimic NC (P < 0.05), while there was no significant difference in cells transfected with oe-EPB41L4A-AS2 + miR-103a mimic (P > 0.05). A higher expression of RUNX1T1 occurred in cells transfected with oe-EPB41L4A-AS2 + si-NC (P < 0.05), and no significant difference was found in cells transfected with oe-EPB41L4A-AS2 + si-RUNX1T1 compared with cells transfected with oe-NC + si-NC (P > 0.05; Figure 4a,b). Next, the proliferation, migration and invasion of OC cells were detected by CCK-8, colony formation assay and Transwell...
FIGURE 3  EPB41L4A-AS2 binds to miR-103a to regulate RUNX1T1. (a) Subcellular localization of EPB41L4A-AS2 revealed by LncATLAS. (b) Subcellular localization of EPB41L4A-AS2 in SK-OV-3 cells detected by FISH (scale bar = 25 μm). (c) Comparison between miRNAs binding to EPB41L4A-AS2 predicted in RNA22 and differentially expressed miRNAs in microarray data GSE83693. (d) Comparison of target genes of miR-103a predicted by mirDIP, TargetScan, miRDB, miRWalk, RNA22 and DIANA. (e) Comparison between the target genes of miR-103a and DEGs in microarray data GSE18520. (f) Expression of RUNX1T1 in microarray data GSE18520. (g) Binding sites between EPB41L4A-AS2 and miR-103a predicted by RNA22. (h) Binding relationship between EPB41L4A-AS2 and miR-103a verified by the dual luciferase reporter gene assay. (i) Binding relationship between miR-103a and RUNX1T1 predicted by RNA pull-down. (k) Binding relationship between miR-103a and RUNX1T1 predicted by RNA pull-down. (l) Expression of miR-103a and RUNX1T1 upon oe-EPB41L4A-AS2 transfection measured by RT-qPCR. (m) Expression of miR-103a and RUNX1T1 upon miR-103a mimic transfection measured by RT-qPCR. *P < 0.05. The cell experiments were repeated 3 times. Measurement data were expressed as means ± standard deviation. Unpaired t test was used for comparisons between two groups.
Overexpressed EPB41L4A-AS2 inhibits tumour growth of OC cells in vivo

The tumour growth of nude mice was observed, with the volume and weight recorded. The volume and weight of tumour of mice injected with oe-EPB41L4A-AS2 were progressively decreased relative to mice injected with oe-NC (P < 0.05; Figure 5a–c). Then, RT-qPCR was conducted to measure the expression of EPB41L4A-AS2, miR-103a and RUNX1T1. The results showed that in mice injected with oe-EPB41L4A-AS2, the expression of EPB41L4A-AS2 and RUNX1T1 was markedly increased, while the expression of miR-103a was decreased compared with mice injected with oe-NC (Figure 5d; P < 0.05). Subsequently, the protein expression of RUNX1T1 was further detected by western blot analysis, the results of which demonstrated that protein expression was higher in mice injected with oe-EPB41L4A-AS2 than that in mice injected with oe-NC (Figure 5e; P < 0.05). The above results documented that upregulated EPB41L4A-AS2 could promote the expression of RUNX1T1 and inhibit tumorigenesis of OC cells in vivo.

4 | DISCUSSION

In terms of morbidity and mortality, OC ranks in the top eight leading cancers around the world (Coburn et al., 2017). At present, several approaches to treat OC have had a significant favourable initial response, but still many OC patients with advanced disease will show recurrence within 18 months (Jayson, Kohn, Kitchener, & Ledermann, 2014; Oza et al., 2015), thus emphasizing the urgent need for treatments contributing to the improvement of clinical outcome. In the present study, we performed different kinds of experiments to study the effects EPB41L4A-AS2 on OC and reveal the related mechanism. The results obtained from our experiments documented that EPB41L4A-AS2 expression was poor in OC tissues and cells, and EPB41L4A-AS2 overexpression impeded the proliferation, migration and invasion of OC cells by promoting the expression of RUNX1T1 via miR-103a.

Initial findings from our study revealed poor EPB41L4A-AS2 expression in OC tissues and cells. It has been demonstrated that overexpressed lncRNAs such as HAND2 antisense RNA 1 and nicotinamide nucleotide transhydrogenase-antisense RNA1 play a suppressive role in the proliferation and metastasis of OC cells (Huang et al., 2018a, 2018b, 2018c; Yang et al., 2018a, 2018b). EPB41L4A-AS2 was proven to be poorly expressed in non-small cell lung cancer (NSCLC) tissues and cells, and the apoptosis of NSCLC cells was promoted by highly expressed EPB41L4A-AS2 (Shu et al., 2018). It has been demonstrated that tumours of a higher degree and more malignant characteristics possess lower expression of EPB41L4A-AS2, and overexpressed EPB41L4A-AS2 leads to better OS of several malignant tumours including breast cancer, renal cancer and lung cancer (Xu et al., 2016). To further verify our results, we performed in vitro experiments that demonstrated that the proliferation, invasion and migration of OC can be prevented when the expression of EPB41L4A-AS2 is upregulated. These data were supported by a prior study suggesting that ectopic expression of EPB41L4A-AS2 repressed cell proliferation in lung, breast and renal cancer (Xu et al., 2016), which indicated the suppressive effects of EPB41L4A-AS2 on tumour formation. EPB41L4A-AS2 was also reported to be poorly expressed in head and neck squamous cell carcinoma (HNSCC), and played a suppressive role in the invasion and metastasis of HNSCC by suppressing the expression of transforming growth factor β receptor 1 (Huang et al., 2018a, 2018b, 2018c). This was in accordance with the results from Huang et al. that demonstrated that EPB41L4A-AS2 was poorly expressed in resistant breast cancer cells and might be regarded as a possible biomarker for docetaxel sensitivity of breast cancer (Huang et al., 2018a, 2018b, 2018c). All of these studies have verified that EPB41L4A-AS2 acts as a suppressor in the progression of OC.

Another important finding was that overexpressed EPB41L4A-AS2 inhibited the development of OC through increasing RUNX1T1 by combining with miR-103a. In a previous study, RUNX1T1 protein
FIGURE 4 EPB41L4A-AS2 overexpression prevents the progression of OC by increasing RUNX1T1 via miR-103a. SK-OV-3 cells were treated with miR-103a mimic or si-RUNX1T1 in the presence of oe-EPB41L4A-AS2. (a) mRNA expression of RUNX1T1 measured by RT-qPCR. (b) Protein expression of RUNX1T1 protein evaluated by western blot analysis. (c) Cell viability of OC cells detected by CCK-8 assay. (d) Colony formation of OC cells detected by colony formation assay. (e,f) Migration (e) and invasion (f) of OC cells detected by Transwell assay. Scale bar = 50 μm. *P < 0.05 vs. cells transfected with oe-NC + mimic NC, #P < 0.05 vs. cells transfected with oe-NC + si-NC. The cell experiments were repeated 3 times. Measurement data are expressed as means ± SD. ANOVA was used for comparisons among multiple groups, followed by Tukey’s post hoc test. Repeated measures one-way ANOVA was used for comparisons of data at different time points, followed by a Bonferroni post hoc test.
FIGURE 5 EPB41L4A-AS2 overexpression inhibits tumorigenesis of OC cells in vivo. Nude mice were treated with OC cells harbouring oe-EPB41L4A-AS2 or oe-NC. (a–c) Xenograft tumours and quantitative analysis of tumour volume in nude mice. (d) Expression of EPB41L4A-AS2, miR-103a and RUNX1T1 in nude mice detected by RT-qPCR. (e) Protein expression of RUNX1T1 evaluated by western blot analysis. *P < 0.05. n = 10. Measurement data were expressed as means ± SD. Unpaired t-test was used for comparisons between two groups. Repeated measures one-way ANOVA was utilized for comparisons of data at different time points, followed by a Bonferroni’s post hoc test.

was verified as a promising biomarker for the diagnosis of liver metastases since it was poorly expressed in cells of well-differentiated metastatic primary pancreatic endocrine tumours and associated with non-metastatic emerges and primaries (Nasir et al., 2011). Besides, it has been verified in a previous study that the expression of RUNX1T1 is downregulated in OC cell lines, and the restoration of RUNX1T1 could inhibit the growth of OC cells (Yeh et al., 2011). Multiple investigations have reported the suppressive function of the interaction between lncRNAs, miRNAs and mRNAs in human cancers. For example, overexpressed IncRNA LINC00312 can inhibit the proliferation, migration and invasion of thyroid cancer cells by inhibiting miR-197-3p expression (Liu, Huang, Yan, Luo, & Min, 2017). The migration of glioma cells can be suppressed after overexpressed ADAM metallopeptidase with thrombospondin type 1 motif, 9 antisense RNA 2 treatment, which can be reversed by silencing the expression of DNMT1 (Yao et al., 2014). Besides, it has been documented that by targeting RUNX2, upregulated miR-103a contributes to inhibition of bone formation (Zuo et al., 2015). Another study demonstrated that a serous epithelial OC cohort was correlated with high expression of miR-103 (Kan et al., 2012). Furthermore, microarray data showed that miR-103a was overexpressed, but RUNX1T1 was diminished in OC. The highly expressed miR-103 is also present in OC cells and acts a promoter in OC cell metastasis by targeting Dicer1 (Yang et al., 2014a, 2014b). Moreover, another study also identified that miR-103a-3p exhibited high expression in OC cells and mediated proliferation, migration and invasion of OC cells (Bignotti et al., 2016). Collectively, the interaction of EPB41L4A-AS2, miR-103a and RUNX1T1 functions in the occurrence and progression of OC.

Altogether, the key findings from the experiments of the present study suggest that EPB41L4A-AS2 can play a tumour inhibitory role in OC. EPB41L4A-AS2 was downregulated in OC tissues and cells, and highly expressed EPB41L4A-AS2 could impede the proliferation, colony formation, migration and invasion of OC cells by inducing RUNX1T1 expression by combining with miR-103a. Investigation of EPB41L4A-AS2 in OC cells and its function yields a better understanding of the in-depth mechanisms and may have potentially important therapeutic implications in the treatment of OC. In the future, further experiments will be warranted on how the expression of miR-103a and RUNX1T1 affects the development of OC in vivo.

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COMPETING INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

T.S. and P.Y. designed the study. P.Y. and Y.G. collated the data, carried out data analyses and produced the initial draft of the manuscript. T.S. and Y.G. contributed to drafting the manuscript. All authors have read and approved the final submitted manuscript.
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