Enrichment of ovarian cancer stem-like cells is associated with epithelial to mesenchymal transition through an miRNA-activated AKT pathway

X. Luo^{*a}, Z. Dong^{*a}, Y. Chen^{*}, L. Yang^{*} and D. Lai^{*}

^{*}The International Peace Maternity and Child Health Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, 200030, China

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Abstract

Objectives: Evidence has indicated that ovarian epithelial cancer-type cells under serum-free culture conditions can form spheroid cells and exhibit characteristics expected of cancer stem-like cells (CSCs). However, the mechanism by which differentiated ovarian cancer cells acquire stem-cell properties during CSC enrichment has needed to be elucidated. Recent studies have demonstrated that induction of epithelial to mesenchymal transition (EMT) can generate CSCs and be associated with tumour aggressiveness and metastasis.

Materials and methods: Ovarian epithelial cancer cell lines, SKOV3 and HO8920, were cultured for spheroid cells and adherent cells. CSC enrichment was investigated using MTT assay, flow cytometry and qRT-PCR and expression level of PI3K/AKT pathway components was analysed by western blotting.

Results: Compared to adherent cells, the spheroid cells expressed mesenchymal markers highly and exhibited significantly more motility; we also observed increases in phosphate AKT1 levels in the spheroid cells. Moreover, transfection of miR-20a or miR-200c led to corresponding reduction in endogenous PTEN protein, while AKT1 and phosphate AKT1 levels were upregulated in miRNAs-transfected cells. Finally, PI3K/AKT pathway inhibitor LY294002 reduced expressions of mesenchymal markers and stem-cell gene activity in spheroid cells, enhancing sensitivity of spheroid cells to paclitaxel treatment.

Conclusions: Our findings demonstrate that EMT contributed to enrichment of ovarian CSCs *in vitro*, making EMT targeting in epithelial ovarian cancer a novel therapeutic option.

Introduction

Epithelial ovarian cancer (EOC), the most common and fatal gynaecological malignancy, is associated with 5-year survival rate of only 30% (1); although approximately 80% of patients initially respond to surgery and chemotherapy, it often recurs within 5 years (2,3). Recently, the theory of cancer stem-like cells (CSCs) has been proposed to explain heterogeneity and carcinogenesis of EOC (4). CSCs are purported to be a fraction of cells that have the ability to self-renew and differentiate. They have the capacity to not only be the origin of cancers but also have chemoresistance (5–7) and lead to cancer recurrence. Thus, it is imperative to discover any involvement of CSCs in EOC; this would have significant therapeutic implications.

Epithelial to mesenchymal transition (EMT) is a process by which epithelial cells break cell–cell contact as well as cell–extracellular matrix connections and switch from epithelial phenotype to mesenchymal phenotype. Such cells acquire migratory and invasive properties that allow them to transfer to other locations (8). Some research has demonstrated that CSCs can be induced by EMT. Epithelial cells undergo a specific series of events during the EMT process and properties of natural epithelial cells, such as polarized organization and cell–cell junctions, are replaced by abilities unique to CSCs, such as novel self-renewal and differentiation (9). Previous reports have suggested that the EMT process may be regulated by a number of factors, including hypoxia (10), small GTPases (11) and activation of the TGF- β signalling pathway (12).

MicroRNAs (miRNAs) are endogenous small non-coding RNA molecules that regulate target mRNA

Correspondence: L. Dongmei, The International Peace Maternity and Child Health Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200030, China. Tel.: +86 21 64070434 27701; Fax: +86 21 64074642; E-mail: laidongmei@hotmail.com

^aThese authors contributed equally to this work.

expression (13), and dysregulation of miRNAs has been described in various diseases including cancer (14). Some miRNAs, such as miR-93 (15) or miR-214 (16), are up-regulated or downregulated in human ovarian cancer cells by targeting phosphatase and tensin homolog (PTEN), a tumour suppressor. Suppression of PTEN may induce phosphorylation of AKT1, a serine-threonine protein kinase activated by a phosphoinositide 3-kinase (PI3K)-dependent process (17). Moreover, the PI3K/AKT pathway has been considered relevant to maintenance of both EMT and CSC characteristics (18,19).

Previously, we have reported that ovarian epithelial cancer cells can form spheroid cells and exhibit properties associated with CSCs, in a serum-free culture system (20,21). Here, we reveal that EMT participates in enrichment of ovarian CSCs *in vitro*, which may be achieved through PI3K/AKT pathway activated *via* miRNA-mediated PTEN down-regulation.

Materials and methods

Ovarian CSC culture and transfection

Human ovarian epithelial cancer cell lines, SKOV3 and HO8910, were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences. SKOV3 was derived from ascites of a 64-year-old Caucasian female, whereas HO8910 was derived from ascites of a Chinese patient, both SKOV3 and HO8910 being derived from poorly differentiated ovarian serous adenocarcinomas. In this study, lines were grown as adherent cells in regular plates at 37 °C, in a humidified environment containing 5% CO₂ using McCoy's 5A medium (Sigma, St. Louis, MO, USA) supplemented with 10% FBS (Hyclone, Logan, UT, USA), 100 units/ml penicillin and 100 µg/ml streptomycin. Spheroid cells were suspended in DMEM/F12 supplemented with 10 ng/ml basic fibroblast growth factor (bFGF; Invitrogen, Carlsbad, CA, USA) and 10% knockout serum (Gibco, Grand Island, NY, USA) for 7 days in dishes coated with 1.2% poly-Hema (Sigma).

SKOV3 and HO8910 cells were seeded into 24-well plates before transfection. When cell density reached 90%, they were transfected by either pSciencer 4.1 cmv puro, pSciencer 4.1 cmv puro-pre-miR-20a or pSciencer 4.1 cmv puro-pre-miR-200c *via* lipofectamine^{TM} 2000, according to the manufacturer's protocol (Invitrogen).

RNA extraction and real-time PCR for mRNA/miRNA analysis

Total RNA was isolated from cells using TRIzol Reagent (Takara, Dalian, CN). For mRNA detection, RNAs were reverse transcribed according to the manu-

facturer's protocol (Toyobo, Osaka, Honshu, Japan). For miRNA detection, all RNAs were manually polyadenylated then reverse transcribed using an anchor primer as previously described (22). Expressions of both mRNA and miRNA were analysed using SYBR Green qRT-PCR (Takara). Each experiment was repeated three times; primers of mRNA/miRNA are shown in Table S1.

MicroRNA expression vector

With human genomic DNA used as PCR template, pre-miR-20a or pre-miR-200c (flanking upstream and downstream 30–50 nt) was amplified and inserted into the pSilencer 4.1 cmv puro expressing vector through *Bam*HI and *Hind*III (Fig. 4b). Sequences of primers used were as follows: pre-miR-20a F, GCG GGA TCC CTG ATG GTG GCC TGC TAT TT; pre-miR-20a R, GCG AAG CTT ACC GAT CCC AAC CTG TGT AG; pre-miR-200c F, GCG GGA TCC TGG GTA AAT CGG TGT GTG TC; and pre-miR-200c R, GCG AAG CTT AAC AAG AAC CAC CCC TTG TG.

Drug resistance assessment

Spheroid cells were isolated by centrifugation (230 g for 5 min), after adherent cells were released from their substrate by trypsinization. Cells were seeded on to 96-well plates, using 100 µl dilutions per well. Three separate wells contained only medium to provide negative controls for absorbance readings. Paclitaxel (20 µM) was added to each well. After 48 and 72 h, proliferation was analysed by MTT assay according to the manufacturer's protocol (KeyGEN, Nanjin, China).

Transwell invasion and migration assays *in vitro*

Invasion assays were performed in triplicate using 8.0 µm transwell invasion chambers (Corning, NY, USA) coated with Matrigel (100 µg per filter) (BD, Franklin Lakes, NJ, USA) as described in the manufacturer's instructions. Cells (1×10^5 per well; 200 µl/chamber) in serum-free media were seeded into top chambers. Complete medium (600 µl) was added to lower chambers. Following 48-h incubation, cells that had invaded through the surface of the membrane were fixed in methanol and stained with crystal violet. Cells that did not invade into the lower chamber were scraped from the top of the transwell plate with a cotton swab. Invading cells from three random microscope fields per filter were selected for cell counting. Procedure for the migration assay was similar to that for the invasion assay, differing in that for the migration assay, transwell chambers were not coated with Matrigel.

and 5×10^4 cells per well were seeded into top chambers and incubated for 16 h.

Soft-agar colony formation test

Pre-warmed $2 \times$ DMEM/F12 medium (500 μ l) containing 20% FBS and 500 μ l of 1.2% agar solution were mixed, transferred into each well of 6-well plates and incubated at 4 °C for 30 min to allow the bottom agar layer to solidify. Cell suspensions (500 cells in 500 μ l of $2 \times$ DMEM/F12) were mixed with 500 μ l of 0.6% agar and placed on bottom agar layers. After top agar layers solidified, plates were incubated for 14 days at 37 °C and 5% CO₂. Cell colonies were counted for 20 horizons and photographed using microscopy (LEICA, Wetzlar, HE, Germany; MDI3000).

Immunofluorescence

Cells were seeded into 48-well plates and washed in phosphate-buffered saline (PBS). They were then fixed using 4% paraformaldehyde and subsequently permeabilized with 0.2% Triton-X 100 in PBS. After incubation in 3% BSA/PBS for 1 h at room temperature, samples were incubated with primary antibodies against Twist (Abcam, Cambridge, UK; 1:100) and vimentin (Epitomics, Burlingame, CA, USA; 1:100) in 3% BSA overnight at 4 °C. Cells were then washed in PBS and incubated with anti-rabbit or anti-mouse FITC-labelled secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:100). Finally, cells were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) for 5 min and photographed by microscopy (LEICA; MDI3000).

Western blotting

Protein was extracted from cell lysates using 10% SDS and western blots were obtained using standard protocols. Antibodies used were against PTEN (A2B1) (sc-7974; Santa Cruz Biotechnology, 1:200), AKT1 (C-term; Epitomics, 1:500), phospho-AKT1 (pS473; Epitomics, 1:500), beta-actin (PTG, 1:2000), and peroxidase-conjugated anti-rabbit IgG secondary antibodies were used (Santa Cruz Biotechnology, 1:5000). Chemiluminescence was performed using the Western lighting ECL kit (Beyotime, Shanghai, China).

Cell cycle analysis

Cells were washed in PBS then fixed in 70% ethanol at 4 °C for more than 18 h. Fixed cells were treated with

RNaseA and stained with propidium iodide (PI) according to the manufacturer's protocol (KeyGEN). Each sample was analysed in triplicate. Ratio of cells at each stage of the cell cycle was analysed by flow cytometry (Beckmen, Brea, CA, USA).

Statistical analysis

Data are presented as mean $\pm$ SEM; for relative quantification of gene expression, differences were calculated as $2^{-\Delta\Delta C_t}$ and Student's *t*-test (two-tailed) was used to compare two groups. *P* < 0.05 was considered statistically significant.

Results

Spheroid cells of epithelial cancer cell lines displayed stem-like cell properties

Ovarian epithelial cancer cell lines, SKOV3 and HO8910, were cultured in serum-free medium under non-adherent conditions for 7 days. Stem-cell markers were detected by qRT-PCR. Consistent with our previous reports (20,21), Oct4, Nanog and ABCG2 were expressed at higher levels by spheroid cells than by adherent cells (Fig. 1aV&VI). Stem cells spend a substantial part of their life span in a non-active state so that they are not killed by drugs designed to target proliferating cells (23,24). Thus, we used flow cytometry to compare cell cycles between spheroid and adherent cells. We found that 79.5% and 70.2% of the SKOV3 and HO8910 spheroid cells, respectively, and 68.3% and 58.9% of the SKOV3 and HO8910 adherent cells, respectively, were in G0/G1 phase (Fig. 1b).

To examine resistance of EOC cells to chemotherapy, both adherent and spheroid cells were cultured in 20 μ M paclitaxel. MTT assay showed that spheroid cells had significantly higher resistance to paclitaxel compared to adherent cells, in both SKOV3 and HO8910 lines (Fig. 1c). These results suggest that both SKOV3 and HO8910 cells can form spheroid cells in a serum-free suspension culture system, that exhibits stem-like cell properties.

To assess potential for spheroid cells to contribute to malignancy, we performed a colony-forming assay frequently utilized for *in vitro* evaluation of malignant transformation and tumorigenesis. Both adherent and spheroid cells were disassociated and subsequently seeded in soft agar at identical concentrations. As shown in Fig. 1d, spheroid cells generated significantly more as well as larger colonies than did adherent cells, indicating that the spheroid cells possessed higher oncogenic potential.

Figure 1. Ovarian epithelial cancer spheroid cells have the properties of CSCs. (a): I) SKOV3 and III) HO8910 cells grown as adherent cells in McCoy's 5A media supplemented with 10% FBS. II and IV) These cell lines also formed spheroid cells, as seen suspended in the dishes coated by 1.2% poly-Hema and containing serum-free medium for 7 days. V and VI) ABCG2, Oct4 and Nanog were detected by qRT-PCR using 18S rRNA as a reference gene ($*P < 0.05$). (b) Cell cycle distribution analysis of SKOV3 and HO8910 cells in different culture systems was performed by flow cytometry. The percentages of cells in the G0/G1 phase were 79.5% (SKOV3) and 70.2% (HO8910) of spheroid cells, and 68.3% (SKOV3) and 58.9% (HO8910) of adherent cells. (c) The spheroid cells were more drug-resistant compared with the adherent cells after 48 and 72 h of treatment with paclitaxel (20 μ M). (d) Spheroid cells form more and larger colonies than adherent cells in soft agar. I) Representative images of the colonies derived from the adherent and spheroid cells. II) Corresponding quantitative data depicting the colony number/field ($**P < 0.01$).

Enrichment of CSCs was accompanied by EMT

Evidence has shown that induction of EMT can generate CSCs (9). To demonstrate biological changes during CSC enrichment, we examined EMT markers in adherent and spheroid cells. As shown in Fig. 2a,

mesenchymal markers were expressed more by spheroid cells of both SKOV3 and HO8910 lines, while epithelial markers CK19 and E-cadherin did not change significantly. Consistent with these observations, immunofluorescence staining of mesenchymal markers Twist and vimentin was greater in spheroid cells (Fig. 2b).

Figure 2. The expression of mesenchymal markers increased in ovarian epithelial cancer spheroid cells. (a) As shown by qRT-PCR, SKOV3 or HO8910 spheroid cells, under stem cell-selective conditions, exhibited higher expression of mesenchymal markers compared with SKOV3 or HO8910-adherent cells under differentiating condition ($*P < 0.05$). (b) Representative immunofluorescent staining of Twist and vimentin in SKOV3 or HO8910 cells. Nuclei were stained with DAPI.

Increasing evidence indicates that cancer cell invasion and metastasis could be promoted through aberrant activation of EMT (25). The transwell invasion assay showed that spheroid cells had significant elevation in their invasive ability (Fig. 3a) and transwell migration assay was further used to assess their motility. After 16 h, spheroid cells moved more quickly than adherent cells (Fig. 3b). These results suggested that spheroid formation may be associated with acquisition of the EMT phenotype.

PI3K/AKT pathway activation was induced by miR-20a and miR-200c involved in CSC enrichment

Previous studies have suggested that miRNAs are critical regulators of EMT progress. To gain insight into participation of miRNAs in enrichment of CSCs, expressions of 28 miRNAs reportedly associated with ovarian cancer, were tested in both adherent and spheroid cells of ovarian cancers. Expression levels of 6 miRNAs (miR-140, miR-20a, miR-200c, miR-376c, miR-9 and

Figure 3. The ovarian spheroid cells exhibit greater cell motility. (a, b) The invasion and migratory abilities of the adherent and spheroid cells were both analysed by transwell assay. The spheroid cells showed greater invasion and migratory capabilities compared with the adherent cells. (a): I) Representative images of invading adherent and spheroid cells. Corresponding quantitative data are shown in II. (b): I) Representative images of migrating adherent and spheroid cells. Corresponding quantitative data are shown in II. Corresponding quantitative data depicting the cell number/field (** $P < 0.01$).

miR-98) were found to be significantly higher in spheroid cells of both SKOV3 and HO8910 lines. (Tables S2 and S3).

As the PI3K/AKT pathway is an important mediator of EMT (9), we looked for PTEN, AKT1 and phosphate AKT1 expression in both adherent and spheroid cells. Compared to adherent cells, higher phospho-AKT1 AKT1 levels were detected through western blotting of spheroid cells; simultaneously, PTEN levels were found to be lower in spheroid cells (Fig. 4a). miRNAs that corresponded to upstream PI3K/AKT pathway gene *PTEN* were analysed, revealing that *PTEN* might be the target gene of miR-20a and miR-200c (Fig. 4b). To confirm whether *PTEN* was directly regulated by miR-20a and miR-200c, a miR-20a or a miR-200c expression vector was transfected into both SKOV3 and HO8910 cells. Expression of both miRNAs and *PTEN* was detected by qRT-PCR after 48 h transfection. Results showed that, along with increasing miR-20a or miR-

200c expression, expression of PTEN reduced (Fig. 4c). Western blot analysis also revealed that upregulated miR-20a or miR-200c in cells corresponded with a reduction in endogenous PTEN protein, while AKT1 and phospho-AKT1 levels were also upregulated in miRNAs-transfected cells (Fig. 4d). Thus it seems that miR-20a and miR-200c might mediate the PI3K/AKT pathway during enrichment of ovarian CSCs.

PI3K/AKT pathway inhibitor LY294002 blocked ovarian CSC enrichment by mediating the EMT process

To demonstrate whether the PI3K/AKT pathway played an important role in EMT during ovarian CSC enrichment, LY294002 (Beyotime), a PI3K-specific inhibitor, was used to treat spheroid cells. After 7 days treatment, LY294002 had an obvious effect in reducing protein expressions of AKT1 and phospho-AKT1 in both SKOV3 and HO8910 spheroid cells (Fig. 5a). Addition-

Figure 4. The AKT pathway was activated by miRNA-mediated PTEN gene downregulation. (a) PTEN, AKT1 and phospho-AKT1 expression in adherent and spheroid cells of SKOV3 and HO8910 lines. Phospho-AKT1 was activated in spheroid cells and PTEN was downregulated. (b) Sequence matching between PTEN 3'-UTR and miRNA was bioinformatically predicted. Pre-miR-20a and pre-miR-200c were amplified and inserted into the expression vector. pSilencer 4.1 cmv puro by *Bam*HI and *Hind*III. (c) SKOV3 and HO8910 cells were transfected with a miR-20a or miR-200c expression vector. PTEN mRNA expression was downregulated by miR-20a and miR-200c. (d) PTEN, AKT1 and phospho-AKT1 expression were analysed in SKOV3 cells that were transfected with a miR-20a or miR-200c expression vector. Both AKT1 and phospho-AKT1 were upregulated in miR-20a- and miR-200c-transfected cells, while PTEN was downregulated (* $P < 0.05$, ** $P < 0.01$).

ally, qRT-PCR showed that, compared to non-treated cells, the treated cells exhibited significantly lower expression of mesenchymal markers (Fig. 5b). Simultaneously, expression levels of Nanog, Oct4 and ABCG2 were significantly lower in LY294002-treated cells (Fig. 5c).

It has been shown that CSCs are relatively quiescent, remaining predominantly in G0/G1 phases of the cell cycle, which is critical for maintaining and protecting their stem-cell properties. Compared to that of adherent cells, percentages of spheroid cells in G0/G1 was higher (79.5% of SKOV3 or 70.2% of HO8910 spheroid cells versus 68.3% of SKOV3 or 58.9% of HO8910 adherent cells, Fig. 1b). However, after LY294002 treatment, proportions of spheroid cells in G0/G1 decreased (from 76.8% to 63.9% of SKOV3 cells and from 71.3% to 64.9% of HO8910 cells) (Fig. 5d). We further examined effects of LY294002 on sensitivity of spheroid cells to paclitaxel, a drug commonly used to treat ovarian cancer in patients. As shown in Fig. 5e, LY294002 seemed to

sensitize spheroid cells to the chemotherapeutic drug. A significant difference in mean cell survival after 48 and 72 h treatment was noted between cells treated with paclitaxel plus LY294002 versus paclitaxel alone. The soft-agar colony formation test showed that spheroid cells without LY294002 treatment generated more and larger colonies compared to LY294002-treated spheroid cells (Fig. 5f). These results suggest that PI3K inhibitor may block enrichment of ovarian CSCs and may sensitize spheroid cells to chemotherapeutic drugs.

Discussion

There is an increasing amount of evidence that cancer cells from both ovarian cancer cell lines and primary ovary tumour samples can survive and grow in serum-free suspensions, form non-adherent spheroids and display remarkable stem-like properties (26,27). As spheroid cells can produce consistently large numbers of CSCs, it may represent an *in vitro* model suitable for

Figure 5. PI3K inhibitor LY294002 blocked EMT during ovarian epithelial cancer stem-like cells enrichment through the AKT pathway. (a) AKT1 and phospho-AKT1 expression in SKOV3 and HO8910 spheroid cells were blocked due to treatment with PI3K inhibitor LY294002. (b) Results from qRT-PCR showed that the expression of mesenchymal markers was significantly lower ($*P < 0.05$) and the expression of epithelial markers CK19 or E-cadherin changed little in spheroid cells treated with PI3K inhibitor LY294002. (c) LY294002 treatment decreased the mRNA expression of stem-cell markers, including Oct4, Nanog and ABCG2 in spheroid cells. (d) Cell cycle distribution analysis of SKOV3 and HO8910 spheroid cells treated with or without LY294002 was performed by flow cytometry. The percentage spheroid cells in the G0/G1 phase declined after treatment with LY294002 (from 76.8% to 63.9% of SKOV3 cells or from 71.3% to 64.9% of HO8910 cells). (e) PI3K inhibitor LY294002 enhanced the sensitivity of spheroid cells to paclitaxel. The SKOV3 or HO8910 spheroid cells were treated with LY294002 and paclitaxel as indicated. The cell survival rate was determined by MTT assay. (f) Spheroid cells without LY294002 treatment form more and larger colonies compared with LY294002 treated in soft agar. I) Representative images of the colonies derived from the spheroid cells with or without LY294002 treatment. II) Corresponding quantitative data depicting the colony number/field ($*P < 0.05$).

understanding biology of ovarian cancer-initiating cells. However, mechanisms by which differentiated ovarian cancer cells acquire stem-cell properties *in vitro* has not been elucidated previously.

Recently, a new concept, referred to as EMT, describes that some tumour cells acquire new characteristics by losing epithelial differentiation and changing into a mesenchymal phenotype during cancer progression (28). Some researchers have considered EMT to be a prerequisite for tumour infiltration and metastasis. Additionally, increasing lines of evidence indicate that ovarian carcinogenesis is intrinsically linked to EMT (29,30). Recently, Mani *et al.* have reported that immortalized human mammary epithelial cells (HMLEs) can acquire mesenchymal traits and properties of stem cells through EMT (9).

In our present study, we report for the first time that ovarian CSC enrichment is accompanied by EMT. Using a serum-free suspension culture system, we were able to acquire ovarian CSCs from EOC cell lines; expression of mesenchymal markers in spheroid cells derived from these lines was strikingly higher. In addition, spheroid cells exhibited a significant increase in migration and invasion capabilities compared to adherent cells. Our results are consistent with current thought that EMT can enable cancer cells to disseminate from a primary tumour, promote their self-renewal capabilities, drive the invasion-metastasis cascade, and finally promote growth of micrometastases into macroscopic metastases (colonization) (31). Although EMT is characterized by increased expression of mesenchymal markers and reduced expression of epithelial markers (32), evidence has shown that carcinoma cells may not completely lose their epithelial phenotype during the EMT process (33,34). Some workers have classified EMT into three different subtypes, oncogenic EMT, classified into type III EMT, is more suitably given the name of an EMT-like process. Complete oncogenic EMT depends on both cell and microenvironmental contexts; carcinoma cells may express epithelial and mesenchymal markers simultaneously. In this study, we also found that mRNA expression levels of epithelial markers (*CK19* and *E-cadherin*) changed little during CSC enrichment, implying that the cells did not completely lose their epithelial phenotype completely (35,36).

Previous research has shown that AKT has a distinct role in regulating EMT and cell migration (19). Li *et al.* demonstrated in Hela and MCF7 cells that maintenance of EMT-associated cancer stem cell-like characters involves in activation of β -catenin and Akt pathway induced by Twist overexpression. (37). We sought to discover whether the PI3K/AKT pathway could regulate EMT-associated CSC enrichment of EOC cells.

MicroRNAs are small non-coding RNAs that may be oncogenic or else tumour-suppressors. They are involved in various physiological and pathological processes, including malignancy. Many miRNAs have been reported to have a modulatory effect on PTEN expression and its downstream PI3K/AKT signal pathway. Meng *et al.* revealed that miR-21 has a modulatory effect on PTEN expression in human hepatocellular cancer (38). Autophagy in malignant glioma cell lines can be enhanced by inhibiting the PI3K/AKT pathway by silencing of miR-21 (39). MiR-214 can enhance ovarian cancer cell survival and cisplatin resistance by interrupting the AKT pathway (16). Previous studies have also demonstrated that Twist is a regulator of miR-199a, which is responsible for differentiation of ovarian CSCs through the PTEN/AKT pathway (40). Our results showed that miR-20a and miR-200c could downregulate *PTEN*, a tumour-suppressor gene antagonist of PI3K, and facilitate activation of the PI3K/AKT pathway. Previous studies have reported that miR-20a and miR-200c can promote proliferation and invasion of human ovarian cancer cells (41,42). Interestingly, evidence has also shown that miR-20a plays important roles in maintaining self-renewal capacity of mouse bronchioalveolar stem cells (43). Our findings have revealed that miR-20a and miR-200c may regulate the PI3K/AKT pathway and promote EMT in EOC cells.

As we have previously reported, SKOV3 spheroid cells have characteristics distinct from differentiated cells, and cDNA microarray analysis showed that 3487 genes had more than 2-fold difference in expression (21). Candidate therapeutic agents that specifically target CSCs have been considered promising for eradication of cancer, however, just targeting CSC populations will be unlikely to yield durable clinical responses, as CSC populations can regenerate from non-CSCs within a tumour undergoing EMT. Thus, both bulk tumour cells and CSCs must be eliminated, potentially demanding combination chemotherapies (44). In the present study, we found that LY294002, a PI3K-specific inhibitor, could efficiently inhibit the PI3K/AKT pathway, EMT and self-renewal during CSC enrichment of EOC cells. The PI3K inhibitor also sensitized spheroid cells to paclitaxel, revealing the possibility of targeting EMT as a novel therapeutic option for ovarian cancer.

Our study provides new insights into regulation of CSC enrichment. Our results demonstrate that a highly differentiated ovarian cancer cell population might acquire the ability to convert to CSCs by EMT; thus, it is insufficient to inhibit tumour progression and metastasis by targeting the oncogenic EMT/CSC population alone. Future research should be undertaken to target both CSCs and the bulk tumour cell population effectively, to attain the best patient response.

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Conflict of interest statement

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

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. The miRNA and mRNA primer sequences.

Table S2. The expression of miRNA that was significantly higher in SKOV3 spheroid cells.

Table S3. The expression of miRNA that was significantly higher in HO8910 spheroid cells.