Emodin inhibits epithelial to mesenchymal transition in epithelial ovarian cancer cells by regulation of GSK-3β/β-catenin/ZEB1 signaling pathway

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Abstract. Emodin (EMO) has been shown to possess pleiotropic anticancer capabilities in many types of cancer, including epithelial ovarian cancer (EOC). Inhibitory efficacy of EMO on EOC invasion and migration was previously observed, however, the underlying mechanisms have not been completely elucidated. The present study is aimed to explore the mechanisms. Transwell assay demonstrated that EMO significantly inhibited A2780 and SK-OV-3 cell invasion. Western blot analysis was performed to detect the expression levels of epithelial to mesenchymal transition (EMT)-related markers. We found that EMO treatment dose-dependently upregulated E-cadherin, keratin and downregulated N-cadherin, vimentin, matrix metalloproteinase-9 (MMP-9) and matrix metalloproteinase-2 (MMP-2) to repress EMT. Mechanistically, EMO could inhibit glycogen synthase kinase 3β (GSK-3β) phosphorylation, decrease total β-catenin protein levels and subsequently downregulate transcription factor zinc finger E-box binding homeobox 1 (ZEB1) expression. These effects of EMO were weakened when the cells were pretreated with SB216763, an inhibitor of GSK-3β kinase. Besides, we utilized small interfering RNA (siRNA) to downregulate ZEB1 expression. We found that treatment of ZEB1-knockdown cells with EMO, ZEB1 levels were lowest and cell invasion was weakest but ZEB1 knockdown had no effect on the expression of phospho-Ser9-GSK-3β (p-GSK-3βSer9), β-catenin. In conclusion, our results suggested that EMO inhibited EOC cell invasion by regulation of GSK-3β/β-catenin/ZEB1 signaling pathway to suppress EMT in vitro.

Introduction
Ovarian cancer is the most lethal gynecologic malignancy (1), and more than 90% is classified as epithelial ovarian cancer (EOC) (2). The majority of EOC patients are diagnosed at advanced stages and the overall 5-year survival rate is approximately 40% (3). It has been reported that invasion and metastasis process remains the leading cause of recurrence and death from EOC and the molecular mechanisms of invasiveness and the metastatic property are not clearly understood.

Accumulating evidence reveals that epithelial to mesenchymal transition (EMT), a well-recognized process by which cells from a differentiated epithelial state could convert into a dedifferentiated migratory mesenchymal phenotype, which plays an essential role in the regulation of embryogenesis, organ fibrosis, wound healing and cancer metastasis (4). The crucial events of EMT include decreasing the cell-cell adhesion molecule E-cadherin; increasing more plastic mesenchymal proteins such as vimentin, N-cadherin, and deregulating the canonical WNT/β-catenin signaling pathway (5). Emerging evidence indicates that EMT is of vital importance in obtaining invasive and migratory ability in EOC (6-8). A number of transcription factors (TFs), such as Twist, Snail, Slug, and zinc finger E-box binding homeobox 1 (ZEB1)/2, are considered as the major inducers of EMT that repress E-cadherin directly or indirectly in EOC cells (9-13). Thus, EOC patients may benefit from targeted therapies that inhibit EMT.

Emodin (1, 3, 8-trihydroxy-6-methylanthraquinone; EMO) is a natural anthraquinone derivative in the roots and rhizomes of Polygonum cuspidatum and Rheum palmatum, possessing various biological activities such as anti-inflammatory, anti-bacterial, antioxidant and anticancer properties (14-17). EMO displays anticancer activities in several types of cancers, including EOC. In one study it was elucidated that EMO exerted antiproliferative and apoptosis-inducing effects in A2780 (paclitaxel-sensitive) and A2780/taxol (paclitaxel-resistant) cells by reducing the expression of anti-apoptotic molecules, such as survivin and X-linked inhibitor of apoptosis (XIAP) (18), whereas, EMO had potential to inhibit tumor invasion and metastasis of HO-8910PM cells via repression of the production of MMP-9 (19). Studies in vitro also demonstrated that EMO was effective on restraining SKOV3 and HO8910...
cell invasion (20). However, the molecular mechanisms of the anti-invasive and antimetastatic functions are unknown. In previous studies, EMO was shown to exert inhibitory effects on cell invasion and migration of colorectal and cervical cancer, and head and neck squamous cell carcinoma (HNSCC), which is associated with the inhibition of EMT and deregulation of WNT/β-catenin signaling pathway (21-23). Therefore, we speculated that EMO may inhibit the EOC cells to undergo the EMT progress by regulation of WNT/β-catenin signaling pathway, consequently weakening the invasiveness and metastatic ability.

We investigated the effects of EMO on EOC cells in vitro. In the present study, we demonstrated that EMO could inhibit EOC cells invasion and migration by suppressing EMT, demonstrated by increased epithelial markers E-cadherin and keratin and decreased mesenchymal markers vimentin and N-cadherin. One of the molecular mechanisms was that EMO was able to activate glycogen synthase kinase-3β (GSK-3β), which may targets β-catenin and then decreases its target gene ZEB1 expression.

**Materials and methods**

**Cell culture and treatment.** A2780, OVCAR-3 and SK-OV-3 cells, and human EOC cell lines were purchased from the Conservation Genetics Chinese Academy of Sciences (CAS) Cell Bank (Shanghai, China). A2780 cells were maintained in RPMI-1640 medium (Gibco by Life Technologies, Grand Island, NY, USA) supplemented with 2.0 g/l NaHCO3 and 10% fetal bovine serum (FBS) (Gibco by Life Technologies, Australia). OVCAR-3 cells were cultured in RPMI-1640 medium supplemented with 2.0 g/l NaHCO3 and 20% FBS. SK-OV-3 cells were maintained in McCoy's 5A medium (Sigma, St. Louis, MO, USA) with 2.2 g/l NaHCO3 and 10% FBS. All cell culture supplements contained 100 U/ml penicillin and 0.1 mg/ml streptomycin (Beijing Solarbio Science and Technology Co., Ltd.). Cells were kept at 37˚C in a humidified 5% CO2 incubator. EMO and inhibitor SB216763, dissolved in 100% dimethylsulfoxide (DMSO; Sigma) at 50 mM stock solution respectively, were purchased from Sigma. For EMO or SB216763 treatment, stock solution was added into culture medium at a final concentration of 0.1% DMSO (v/v). DMSO solution alone was used as a control for all experiments.

**Cell viability assay.** The Cell Counting Kit-8 (CCK-8) assay (BestBio, Shanghai, China) was used to study the influence of EMO on cell proliferation. Cells were seeded at a density of 2.5x10^3 cells/well in 100 µl medium into 96-well plates. After treatment with 20 µM EMO for 48 h, 10 µl of CCK-8 was added to each well, followed with incubation for 2 h at 37°C. Cell viability was determined by the absorbance at 450 nm in each well measured by a plate reader (Bio-Rad, Hercules, CA, USA). Each experiment was performed in quintuplicate.

**Transwell Matrigel invasion assays.** Cell invasion assay with a Matrigel-coated membrane (1:4 dilution in serum-free medium; BD Biosciences, San Jose, CA, USA) was performed using 24-well Transwell inserts with a pore diameter of 8-µm (Corning Costar, Cambridge, MA, USA). When cells were seeded into 6-well plates until treatment with EMO for 48 h when reaching confluence of 70-80%, 3x10^4 cells were re-suspended in 200 µl serum-free medium and then seeded into the upper chamber. Medium (750 µl) containing 10% FBS was added into the lower chamber. After incubation for 24 h, the cells on the upper surface of the filter were removed by wiping with a cotton swab. The invaded cells on the lower surface of the filter were fixed in methanol solution for 5 min and 3.7% formaldehyde solution for 5 min, and then stained with Giemsa (Beijing Solarbio Science and Technology Co., Ltd.). Images were captured by an Olympus IX51 inverted microscope (Olympus Optical, Melville, NY, USA) and five visual fields (magnification, x100) were counted.

**Western blot analysis.** The total cellular proteins were extracted by adding appropriate RIPA lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and sodium orthovanadate, sodium fluoride, EDTA, leupeptin] complemented a protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (all from Beyotime Biotechnology) for western blot analysis. After centrifugation with 15,000 rpm at 4˚C for 15 min, the supernatants were collected, then protein concentration was determined using BCA protein assay kit according to the manufacturer's instructions and whole lysates were mixed with 5X SDS-PAGE sample loading buffer (both from Beyotime Biotechnology) at a ratio of 1:4. Samples were heated at 95°C in metal bath for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a pure nitrocellulose blotting membrane (Pall Life Sciences, Mexico). The membrane blots were probed with the appropriate primary antibody at 4°C overnight and then incubation with horseradish peroxidase-conjugated secondary antibody. Signals from the bound antibodies were detected with enhanced chemiluminescent substrate (Thermo Fisher Scientific Inc., Waltham, MA, USA). The results were quantified by densitometry, using ImageJ software (NIH, Bethesda, MD, USA). Primary antibodies against matrix metalloproteinase-9 (MMP-9), matrix metalloproteinase-2 (MMP-2), E-cadherin, keratin, N-cadherin, vimentin, ZEB1, GSK-3β, β-catenin and glyceraldehyde-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology (Danvers, MA, USA). Primary antibodies against phospho-Ser9-GSK-3β (p-GSK-3βSer9) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG), goat anti-mouse IgG and rabbit anti-goat IgG were all obtained from Zhongshan Jinqiao Biological Technology (Beijing, China).

**Small interfering RNA (siRNA) experiments.** The sequence for ZEB1 specific siRNA (Shanghai GenePharma Co., Ltd., China) was: 5'-UGAUCAGCCUCAUCUGCAAAUGCA-3'. Non-targeting scrambled siRNA was the negative control. Cells were transfected with 50 nM of siRNA using NanoFectin transfection reagent (Excelle Biotechnology, Shanghai, China) according to the manufacturer's instructions. The transfection cocktail mixing 5 µl of NanoFectin transfection reagent and 245 µl Opti-MEM medium (Gibco, Auckland, New Zealand) with 5 µl siRNA and 245 µl Opti-MEM medium were incubated for 20 min at room temperature. Then, the above
mixtures were added to the 6-well plates with cells that had been seeded overnight to reach 50-60% confluence prior to transfection process. Transfection was performed for 6 h at 37°C in a 5% CO₂ incubator and the old medium was changed with fresh complete medium for a further 12 h followed by EMO 20 µM treatment for 48 h. Then the cells were harvested for Transwell assay and western blot analysis.

Statistical analysis. All statistical analyses were performed by two-tailed Student's t-test using GraphPad Prism version 6.02 (GraphPad Software, Inc., La Jolla, CA, USA) and P<0.05 were considered as significant. The data are shown as the mean ± standard deviation (SD) from three independent assays.

Results

A2780 and SK-OV-3 cells exhibit a spindle-like mesenchymal phenotype with more invasive ability than OVCAR-3 cells. Before conducting the following experiment, we chose cells with more invasive potential and having undergone EMT. First, we compared the morphological characteristics of the human EOC cell lines A2780, OVCAR-3 and SK-OV-3. We found that the SK-OV-3 cells displayed elongated fibroblastoid morphology while the OVCAR-3 cells showed a rounded shape, typical of an epithelial cobblestone-like appearance, growing in clusters. Morphology feature of A2780 cells was between epithelial round-shaped and spindle-shaped mesenchymal form (Fig. 1A). The results above were in accordance with their relative invasive properties and the expression of markers of epithelial and mesenchymal phenotype (Fig. 1B-E). The weakest invasive activity and highest expression of E-cadherin and keratin (epithelial markers) were shown in OVCAR-3 cells; however, vimentin, N-cadherin and ZEB1 (mesenchymal markers) were rarely expressed. In contrast, the A2780 and SK-OV-3 cells exhibited more invasive ability and expressed significantly lower levels of E-cadherin and keratin and higher levels of vimentin, N-cadherin and ZEB1. Thus, we used A2780 and SK-OV-3 cells to undergo EMO treatment.

EMO suppresses the invasion property of A2780 and SK-OV-3 cells by inhibiting EMT. To confirm that EMO inhibited the invasion of ovarian cancer cells as previously reported, we performed Transwell Matrigel invasion assays. We found that the invasion activities were significantly decreased compared with the normal control group in both A2780 and SK-OV-3 cells (Fig. 2A and B). Moreover, EMO 20 µM failed to regulate cell proliferation ability (Fig. 2C). We further studied whether EMO could change the EMT phenotype. After the treatment of cells with various concentrations of EMO for 48 h, upregulation of E-cadherin and keratin and downregulation of vimentin, N-cadherin, MMP-9 and MMP-2 in a concentration-dependent manner were observed (Fig. 2D and E).

EMO inhibits WNT/β-catenin signaling pathway and the expression of ZEB1 via GSK-3β activation. To investigate the functional mechanism of EMO inhibition of EMT on A2780 and SK-OV-3 cells, we tested WNT/β-catenin signaling pathway and transcription factor ZEB1, both closely connected with EMT. The cells were treated with EMO concentrations ranging from 0 to 40 µM for 48 h. The data showed that EMO treatment dose-dependently increased levels of p-GSK-3βSer9, one of negative regulatory sites, led to the increase of GSK-3β kinase activity accordingly, occurred in the absence of any changes in total GSK-3β levels and decreased total β-catenin
protein levels (Fig. 3A and B). EMO treatment also significantly decreased ZEB1 protein levels in a concentration-dependent manner (Fig. 3A and B). To further clarify that EMO regulated the GSK-3β/β-catenin/ZEB1 pathways, cells were pretreated with SB216763, a selective GSK-3β kinase inhibitor, and we found that the effects of EMO were blocked (Fig. 3C and D). SB216763 treatment restored EMO-induced decrease of p-GSK-3βSer9, β-catenin and ZEB1 protein expression. Furthermore, EMO treatment did not decrease mesenchymal phenotype such as vimentin and slightly increased epithelial phenotype such as keratin protein levels in the presence of SB216763 compared to its absence (Fig. 3E and F).

**EMO represses EMT via ZEB1 inhibition.** To further study the mechanism of inhibition of EMT, ZEB1 siRNA was designed to evaluate the relationship between WNT/β-catenin signaling pathway and ZEB1. Either EMO treatment alone or treatment of ZEB1-knockdown cells with EMO almost equally played the same role in regulating the activity of GSK-3β and the expression of β-catenin (Fig. 4A and B). Actually, ZEB1 siRNA failed to participate in the above process. Furthermore, we investigated the effects of EMO on cell invasion followed by ZEB1 knockdown. Upon ZEB1 knockdown, A2780 and SK-OV-3 cells treated with EMO exhibited more decreased invasion capacity (Fig. 4E and F), decreased vimentin protein levels and consistently more increased keratin expression levels (Fig. 4C and D) compared to ZEB1 knockdown or EMO treatment alone. Accordingly, ZEB1 protein levels were lowest in the EMO-treated ZEB1 knockdown cells (Fig. 4C and D).

**Discussion**

We have validated that A2780 and SK-OV-3 cells displayed a spindle-like mesenchymal phenotype with higher invasive property than OVCAR-3 cells. Then, we showed for the first time that the inhibition of invasion and EMT by treating A2780 and SK-OV-3 cells with different concentrations of EMO. No previous studies had found the influence of EMO on GSK-3β/β-catenin-regulated EMT markers, including ZEB1 in EOC. Therefore, the present study provides the first report to explore the role of GSK-3β/β-catenin/ZEB1 pathways in repressing EMT by EMO.

We confirmed that A2780 and SK-OV-3 cells had obviously lower protein levels of E-cadherin and keratin and higher protein levels of vimentin, N-cadherin and transcription factor
ZEB1. We also showed that A2780 and SK-OV-3 cells had elongated fibroblastoid mesenchymal phenotype with more invasive ability than OVCAR-3 cells, which displayed epithelial cobblestone-like morphology in line with earlier reports (24).

In agreement with previous studies showing that exposure to EMO could inhibit EOC cells invasion and metastasis (19, 20), we further demonstrated the molecular mechanism in the present study. We showed that EMO could target the EMT process in A2780 and SK-OV-3 cells, as manifested by decreased invasive capacities and alterations in the expression of EMT-related markers, including upregulation of the expression of E-cadherin and keratin, and downregulation of the expression of vimentin, N-cadherin, MMP-9 and MMP-2. These results demonstrated that EMO alters correlation of the gene products with EMT induction or maintenance by repression of the mesenchymal phenotype of EOC cells.

Previous reviews summarized that the combined action of various signaling pathways, including WNT/β-catenin signaling pathway, make a greater contribution to the initiation and morphogenic process of the EMT (25). In particular, WNTs and their downstream effectors could regulate the important processes for tumor initiation, tumor growth, cell senescence and apoptosis, differentiation and metastasis (26). Besides, WNT signaling pathway was investigated as a potential target in the development of new drugs for ovarian cancer (27). Previous studies showed that EMO possessed the capability of inhibiting the WNT signaling pathway by downregulating T-cell factor (TCF)/lymphocyte

Figure 3. EMO inhibits the WNT/β-catenin signaling pathway and the expression of ZEB1 via GSK-3β activation. (A) A2780 and SK-OV-3 cells were exposed with various concentration of EMO for 48 h, p-GSK-3βSer9, GSK-3β, total β-catenin and ZEB1 expression levels detected by western blot analysis are shown. (B) Quantitative analyses of markers in SK-OV-3 cells shown in A; *P<0.05; **P<0.01 vs. controls. (C and E) Cells were treated with 10 μM SB216763 (GSK-3β kinase inhibitor) for 6 h prior to EMO 20 μM treatment for 48 h. Western blot analysis of p-GSK-3βSer9, GSK-3β, total β-catenin, ZEB1, keratin and vimentin protein levels are shown. (D and F) Quantitative analyses of markers in SK-OV-3 cells showed in C and E; *P<0.05; **P<0.01 vs. controls; #P<0.05; ##P<0.01. All the results are representative of three independent experiments.
enhancer factor (LEF) transcriptional activity and inducing morphological changes indicating MET accompanied by the increase in E-cadherin expression in human colorectal cancer cells (SW480 and SW620) (21). Besides, EMO inhibited cell population and migration in cervical cancer cells (SiHa and HeLa cells) and downregulated the WNT/β-catenin signaling pathway activated by TGF-β through inhibiting β-catenin in HeLa cells (22). Furthermore, in head and neck squamous cell carcinoma (HNSCC), EMO inhibited Twist1-induced invasion and EMT by inhibiting the β-catenin and Akt pathways in vitro and in vivo (23). In the present study, we demonstrated for the first time that EMO had anti-invasive effect in A2780 and SK-OV-3 cells, evidenced by the repression of EMT in vitro and these effects were mediated by decreased phosphorylation of GSK-3β on Ser9 site and total β-catenin levels and inhibition of subsequent ZEB1 expression.

It is well known that reduction of the expression of p-GSK-3βSer9 led to the increase of GSK-3β kinase activity, followed by phosphorylation of β-catenin, a multifunctional protein in WNT-mediated signal transduction, leading to ubiquitylation or proteasomal degradation. Accordingly, the non-phospho-form of β-catenin binds to TCF bind elements (TBE)-specific DNA sequences that interact with TCF/LEF TFs after it translocates into the nucleus, leading to the transcription of

Figure 4. EMO represses EMT via ZEB1 inhibition. A2780 and SK-OV-3 cells were transfected with siRNA of ZEB1 and cultured for an additional 12 h or treated with EMO 20 µM for 48 h. (A and C) Western blot analysis of ZEB1, keratin, vimentin, p-GSK-3βSer9, GSK-3β and total β-catenin protein levels are shown. (B and D) Quantitative analyses of markers in SK-OV-3 cells showed in A and C; *P<0.05; **P<0.01 vs. controls; #P<0.05; ##P<0.01. (E) Transwell Matrigel invasion assays were created for 24 h after exposure. (F) Quantification of invasive cells in E; *P<0.01 vs. controls; #P<0.05; ##P<0.01. All the results are representative of three independent experiments.
WNT-responsive genes (28). Moreover, ZEB1 is a direct target of β-catenin/TCF4 and acts also as an effector of this signaling pathway in regulating genes associated with tumor invasiveness in intestinal primary tumors and colorectal cancer cell lines with mutant APC (29). In addition, PI3K/Akt targeting GSK3β/β-catenin pathway could dynamically control the ZEB1 gene transcription in bladder cancer cells (T24-L, lung metastasis) (30). In our study, western blot analysis showed that EMO decreased the expression of p-GSK-3βSer9, total β-catenin and ZEB1, and these effects were rescued by the selective GSK-3β inhibitor, SB216763. Besides, cells treated with SB216763 alone expressed elevated β-catenin and ZEB1 protein. These results suggest that EMO inhibits EMT in EOC cells via activating GSK-3β followed by targeting β-catenin and regulating its downstream target ZEB1.

Studies have demonstrated that a number of TFs were shown to be key regulators of E-cadherin expression, including Twist, Snail, Slug, ZEB1 and ZEB2 (31-34). Among them, the ZEB1, encoded by the TCF8 gene, contributes to malignant progression of various epithelial tumors. ZEB1 plays crucial effects on the induction of EMT by inhibiting expression of E-cadherin and microRNAs, which induce an epithelial phenotype (35). The downregulation of the ZEB1 was found to be associated with the decreased colony-forming ability and the cell migration ability in the human ovarian cancer SK-OV-3 and HO8910 cells in vitro and also with the attenuated tumorigenesis of the shZEB1-SKOV3 cells in nude mice by blocking the EMT process (36). So ZEB1 may be a potential therapeutic target in future clinical trials for preventing and treating EOC metastases. In the present study, concomitant knockdown of ZEB1 expression and EMO treatment synergistically suppressed cell invasion and EMT at greater levels. These results indicate that ZEB1 is a key regulator of cell invasion and EMT in EMO-treated EOC cells. Besides, ZEB1 knockdown alone did not affect GSK-3β activity and β-catenin levels. Similarly, EMO treatment alone or treatment of ZEB1 knockdown cells with EMO almost equally decreased p-GSK-3βSer9 and β-catenin levels. The results suggest that ZEB1 is downstream of β-catenin.

In conclusion, the present study showed that the anti-invasive efficacy of EMO on EOC cells was associated with inhibition of EMT. The mechanism may be that GSK-3β activation by EMO treatment decreased expression of total β-catenin, leading to repressed WNT/β-catenin signaling pathway and sequentially the decreased ZEB1 levels. Based on our findings, we provide additional scientific evidence for the use of EMO in the treatment of EOC.

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References


