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EpCAM is up-regulated by EGF via ERK1/2 signaling and suppresses human epithelial ovarian cancer cell migration

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ABSTRACT

Although epithelial cell adhesion molecule (EpCAM) is overexpressed in human epithelial ovarian cancer (EOC), some contradictory results have been reported regarding the correlation between EpCAM overexpression and patient survival. In addition to this controversy, the function and regulation of EpCAM in EOC remain largely unknown. Here, we show that epidermal growth factor (EGF) up-regulates EpCAM expression by activating ERK1/2 signaling in a human EOC cell line, SKOV3. Additionally, EpCAM overexpression suppresses not only basal but also EGF-stimulated SKOV3 cell migration, whereas EpCAM knockdown increases both basal and EGF-stimulated cell migration in another human EOC cell line, OVCAR4. This study demonstrates the regulation of EpCAM and its role in mediating the effects of EGF on human EOC cell migration.

1. Introduction

Epithelial cell adhesion molecule (EpCAM), which does not structurally resemble any of the major families of adhesion molecules, is a type I transmembrane glycoprotein that was first identified as a tumor-associated antigen in human colon carcinomas [1,2]. In adults, EpCAM is normally localized at the basolateral membrane of simple, pseudostratified and transitional epithelial cells of most organs and glands, with the highest expression in the colon. It is not expressed in non-epithelial tissue and is not detected in squamous stratified epithelial cells and some specific epithelial cell types, such as epidermal keratinocytes, hepatocytes, thymic cortical epithelial cells and myopethelial cells [3,4]. The biological function of EpCAM was first identified in mouse fibroblast cells by showing its ability to mediate calcium-independent hemophilic cell–cell adhesion [5]. Subsequent studies in the same experimental model have shown that the overexpression of EpCAM reduces E-cadherin-mediated cell–cell adhesion and that this inhibitory effect is due to disruption of the binding between α-catenin and F-actin [6,7].

In addition to mediating cell adhesion, accumulating evidence shows that EpCAM also regulates multiple cellular functions, such as proliferation, migration, invasion and differentiation, suggesting important roles in the regulation of cancer development and progression [8]. It has been shown that EpCAM is only expressed in human epithelium-derived cancers, with frequent overexpression [9,10]. In breast and pancreatic cancers, the expression levels of EpCAM positively correlate with a poor prognosis; in contrast, high expression levels of EpCAM in renal and thyroid cancer patients results in a better prognosis. Interestingly, the correlation between EpCAM overexpression and patient survival remains controversial in esophageal and colorectal cancer. These results indicate that EpCAM can act as either a tumor promoter or suppressor in human cancers depending on the type of cancer and the tumor microenvironment [11]. In human epithelial ovarian cancer (EOC), EpCAM is overexpressed consistently across all stages in four histological subtypes, including high-grade serous carcinomas, the most frequent histological subtype of EOC [12]. In addition, both metastatic and recurrent EOC exhibit higher expression levels of EpCAM than primary EOC [13]. However, the factors that cause the overexpression of EpCAM in EOC remain unclear. A recent study shows that EpCAM overexpression is significantly associated with a favorable overall patient survival and a higher response to platinum-based chemotherapy [14], though these results are contrary to a previous study [15]. Regardless of the association between EpCAM expression levels and EOC patient survival, the exact

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roles of EpCAM during EOC progression and the molecular and cellular mechanisms of its functions remain poorly defined.

The lethality of ovarian cancer is due primarily to the fact that the majority of patients present with disseminated disease. It has been shown that EpCAM knockdown decreases cell migration and invasion in human breast cancer cells [16,17]. Our previous studies have demonstrated that epidermal growth factor (EGF) can induce EOC cell migration and invasion by down-regulating the expression of the cell–cell adhesion molecule E-cadherin [18–22]. However, whether EGF can regulate EpCAM expression and the role of EpCAM in regulating human EOC cell migration remain unknown. Therefore, the present study was designed to examine the effect of EGF on EpCAM expression and define EpCAM's role in the regulation of cell migration in human EOC cells.

2. Materials and methods

2.1. Cell culture

The SKOV3 ovarian cancer cell line was obtained from the American Type Culture Collection (Manassas, VA). The OVCAR4 ovarian cancer cell line was kindly provided by Dr. T.C. Hamilton (Fox Chase Cancer Center, Philadelphia, PA). Cells were grown in a 1:1 (v/v) mixture of M199/MCDB105 medium (Sigma–Aldrich, Oakville, ON) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT). The cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

2.2. Antibodies and reagents

Monoclonal anti-EpCAM and polyclonal anti-actin and anti-EGFR antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-phospho-ERK1/2 (Thr202/Tyr204) and polyclonal anti-ERK1/2, anti-phospho-Akt (Ser473) and anti-Akt antibodies were obtained from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Bio-Rad Laboratories (Hercules, CA). Human epidermal growth factor (EGF), AG1478 and LY294002 were obtained from Sigma. U0126 was obtained from Calbiochem (San Diego, CA).

2.3. Small interfering RNA (siRNA) transfection and protein overexpression

For endogenous EGFR or EpCAM knockdown, cells were transfected with 50 nM ON-TARGET*plus* SMARTpool ERFR or EpCAM siRNA (Dharmacon Research, Inc., Lafayette, CO) using Lipofectamine RNAiMAX (Invitrogen, Burlington, ON). siCONTROL Non-targeting siRNA (Dharmacon) was used as a transfection control. For EpCAM overexpression, the pReceiver-M02 vector and pReceiver-M02-EpCAM were transfected into cells using Lipofectamine LTX (Invitrogen). The empty pReceiver-M02 vector and human EpCAM-containing pReceiver-M02 vector were purchased from GeneCopoeia (Rockville, MD).

2.4. Western blotting

Cells were lysed in lysis buffer (Cell Signaling Technology, Danvers, MA), and the protein concentrations were determined using a DC protein assay kit with BSA as the standard (Bio-Rad Laboratories). Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes. After blocking with Tris-buffered saline (TBS) containing 5% non-fat dry milk for 1 h, the membranes were incubated overnight at 4 °C with primary antibodies, followed by incubation with the HRP-conjugated secondary antibody. Immunoreactive bands were detected with an enhanced chemiluminescent substrate (Pierce, Rockford, IL).

2.5. Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed with 3 μg RNA, random primers and M-MLV reverse transcriptase (Promega, Madison, WI). The primers used for SYBR Green RT-qPCR were as follows: EpCAM, 5′-CGC AGC TCA GGA AGA ATG TG-3′ (sense) and 5′-TGA AGT ACA CTG GCA TTG ACG-3′ (antisense); and GAPDH, 5′-GAG TCA ACG GAT TTG GTC GT-3′ (sense) and 5′-GAC AAG CTT CCC GTT CTC AG-3′ (antisense). RT-qPCR was performed using an Applied Biosystems 7300 Real-Time PCR System (Perkin–Elmer) equipped with a 96-well optical reaction plate. All RT-qPCR results represent the mean from at least three independent experiments conducted in triplicate. The relative quantification of mRNA levels was performed by the comparative Ct method using GAPDH as the reference gene and the formula $2^{\Delta\Delta Ct}$.

2.6. Migration assay

Cell culture inserts (24-well, pore size 8 μm; BD Biosciences, Mississauga, ON) were used for migration assays. The cell culture inserts were seeded with 1×10^5 cells in 250 μl of medium supplemented with 0.1% FBS. Medium with 10% FBS (750 μl) was added to the lower chamber and served as a chemotactic agent. After incubation for 12 (for SKOV3 cells) or 24 (for OVCAR4 cells) hours, non-migrating cells were removed from the upper side of the membrane, and the cells on the lower side of the membrane were fixed with cold methanol and air dried. The cell nuclei were stained with Hoechst 33258 and counted by epifluorescence microscopy using Northern Eclipse 6.0 software (Empix Imaging, Mississauga, ON). Each individual experiment was performed in triplicate (i.e., three inserts), and five microscopic fields were counted per insert.

2.7. Statistical analysis

The results are presented as the mean ± SEM of at least three independent experiments. The results were analyzed by a one-way ANOVA and Tukey's multiple comparison test using PRISM software. Significant differences were defined as $p < 0.05$.

3. Results

3.1. EGF up-regulates EpCAM in human EOC cells

To examine whether EGF treatment can up-regulate EpCAM expression, a human EOC cell line, SKOV3, was treated with 100 ng/mL EGF for different time periods, and the EpCAM mRNA levels were then examined. As shown in Fig. 1A, treatment with EGF for 9 h did not affect the mRNA levels of EpCAM, whereas after 24 h of EGF treatment, the EpCAM mRNA levels were significantly up-regulated. EGF treatment for 24 and 48 h resulted in comparable effects on the up-regulation of EpCAM mRNA levels. The stimulatory effect of EGF on EpCAM protein levels was further confirmed by western blot analyses (Fig. 1B). To confirm the involvement of EGFR in EGF-up-regulated EpCAM expression, the EGF receptor (EGFR)-specific inhibitor AG1478 was used to block the activation of EGFR. The western blot analyses showed that AG1478 abolished the EGF-induced up-regulation of EpCAM protein levels (Fig. 2A). Given the off-target effects of pharmacological inhibitors, an siRNA-mediated knockdown approach was also used to further confirm

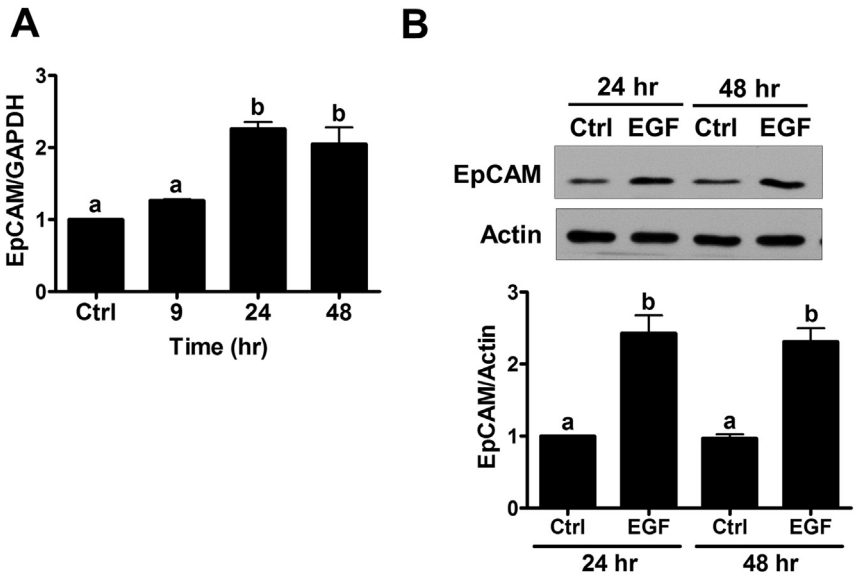

Fig. 1. EGF up-regulates EpCAM expression in SKOV3 cells. A, Cells were treated with vehicle control (Ctrl) or 100 ng/mL EGF. EpCAM mRNA levels were analyzed at different time-points by RT-qPCR. B, Cells were treated with 100 ng/mL for 24 and 48 h. EpCAM protein levels were analyzed by western blotting. The results are expressed as the mean ± SEM from at least three independent experiments. Values without a common letter are significantly different ($P < 0.05$).

that EGFR is required for EGF-induced EpCAM up-regulation. Transfection with an EGFR specific siRNA significantly knocked down the endogenous EGFR protein levels. In addition, the knockdown of EGFR abolished the EGF-induced up-regulation of EpCAM protein levels (Fig. 2B). It is well characterized that upon ligand binding, EGFR rapidly undergoes lysosomal degradation [23], and our results showed that treatment with EGF for 24 h down-regulated the EGFR protein levels in SKOV3 cells, in agreement with this process (Fig. 2B).

3.2. Activation of ERK1/2, but not PI3K/Akt, signaling is required for EGF-up-regulated EpCAM expression

To ascertain the possible signaling pathways involved in EGF-induced EpCAM expression in human EOC cells, we examined the effect of EGF on the activation of ERK1/2 and PI3K/Akt signaling pathways. Treatment with EGF increased the phosphorylation levels of ERK1/2 and Akt in SKOV3 cells. To further examine which pathway is required for the EGF-induced up-regulation of EpCAM, a specific

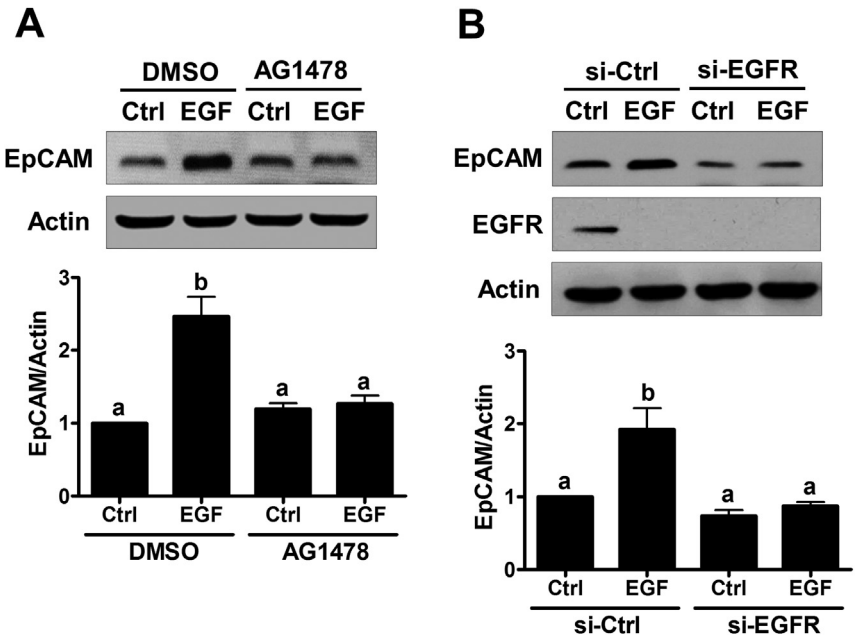

Fig. 2. EGFR is required for the EGF-induced up-regulation of EpCAM expression in SKOV3 cells. A, Cells were pretreated for 1 h with AG1478 (10 μM) and then treated with 100 ng/mL EGF for 24 h. EpCAM protein levels were analyzed by and western blotting. B, Cells were transfected for 48 h with 50 nM control siRNA (si-Ctrl) or EGFR siRNA (si-EGFR) and then treated with EGF for 24 h. Protein levels of EpCAM and EGFR were analyzed by western blotting. The results are expressed as the mean ± SEM from at least three independent experiments. Values without a common letter are significantly different ($P < 0.05$).

MEK inhibitor, U0126, and a PI3K inhibitor, LY294004, were used to block the ERK1/2 and PI3K/Akt signaling pathways, respectively. As shown in Fig. 3A, U0126 blocked EGF-induced ERK1/2 phosphorylation without affecting the phosphorylation of Akt. Similarly, LY294002 specifically blocked EGF-induced Akt phosphorylation. Western blot analyses showed that treatment with U0126 not only down-regulated the basal but also abolished the EGF-up-regulated EpCAM protein levels (Fig. 3B). Interestingly, the inhibition of PI3K/Akt signaling did not affect the basal or EGF-up-regulated EpCAM protein levels (Fig. 3B). These results indicated that EGF-up-regulated EpCAM expression in human EOC cells is mediated by the ERK1/2 signaling pathway and not PI3K/Akt signaling.

3.3. *EpCAM suppresses human EOC cell migration*

To examine the function of EpCAM in basal and EGF-stimulated EOC cell migration, we applied both loss- and gain-of-function approaches. As shown in Fig. 4A, the expression of EpCAM was detected in another human EOC cell line, OVCAR4, and the levels were much higher than those in SKOV3 cells. Therefore, we overexpressed EpCAM in SKOV3 cells, which express a lower level of EpCAM. In contrast, we used an siRNA-mediated approach to knockdown endogenous EpCAM expression in OVCAR4 cells, which express a higher level of EpCAM. As shown in Fig. 4B, the overexpression of EpCAM significantly increased EpCAM protein levels in SKOV3 cells, and the transfection of EpCAM siRNA significantly down-regulated EpCAM protein levels in OVCAR4 cells (Fig. 4B). To determine the role of EpCAM in basal and EGF-stimulated cell migration, we performed a transwell migration assay. As shown in Fig. 4C, treatment with EGF increased cell migration in SKOV3 cells. In addition, the basal level of cell migration was decreased, and EGF-stimulated cell migration was significantly attenuated but still detectable in the EpCAM-overexpressing cells (Fig. 4C). Similarly, EGF increased OVCAR4 cell migration, whereas EpCAM knockdown increased the basal level and enhanced EGF-stimulated cell migration in OVCAR4 cells (Fig. 4D). Taken together, these results indicate that EpCAM acts as a suppressor of human EOC cell migration.

4. Discussion

Depending on the tumor type, the overexpression of EpCAM can be correlated with either increased or decreased overall survival [11]. It has been shown that higher EpCAM expression is detected in poorly differentiated human EOC and that EpCAM overexpression correlates with decreased overall survival; however, another study does not identify any impact of EpCAM overexpression on survival [15,24]. A very recent study shows an opposite result: EpCAM overexpression is associated with better survival [14]. These contradictory results may be due to heterogeneity in the clinical and histopathological characteristics of the patients. It is important to note that although almost all studies address the association between EpCAM and patient survival, there are only few studies to date that directly examine the roles of EpCAM in cancer development or progression. In association with the tight junction protein claudin-7, the EpCAM-claudin-7 complex increases HEK293 cell migration, proliferation and resistance to cisplatin treatment *in vitro*. *In vivo*, the EpCAM-claudin-7 complex promotes tumorigenicity and tumor cell dissemination of rat pancreatic adenocarcinoma cells [25]. Furthermore, knockdown of EpCAM decreases cancer cell growth in human breast cancer [26]. These results indicate a tumor-promoting role for EpCAM. In human EOC, knockdown of EpCAM does not affect tumor cell growth, but EpCAM overexpression does inhibit tumor cell growth [26]. In the present study, our loss- and gain-of-function experiments demonstrated that EpCAM is a suppressor of human EOC cell migration. These results indicate the tumor suppressor role of EpCAM in human EOC and support the results from a recent study [14] showing EpCAM as a good indicator of human EOC patient survival. Taken together, EpCAM can act as either a tumor promoter or suppressor, depending on the tumor type.

To date, no splicing variants of EpCAM have been reported, and mutations in EpCAM have only been identified in patients with congenital tufting enteropathy or Lynch syndrome [27–29]. Previous studies have identified several putative transcription factor binding sites in the EpCAM promoter [11]. However, little is known regarding the regulation of EpCAM. It has been shown that tumor necrosis factor-alpha down-regulates EpCAM in squamous cell carcinoma by activating NF-κB signaling [30]. In addition, p53 is able to bind to the EpCAM promoter and repress its expression in human breast cancer cells [17]. Treatment with chemotherapeutic agents, such as paclitaxel or vinorelbine tartrate, increases the cell surface expression of EpCAM in various adenocarcinoma cells [31]. Moreover, a recent study shows that EpCAM is up-regulated by

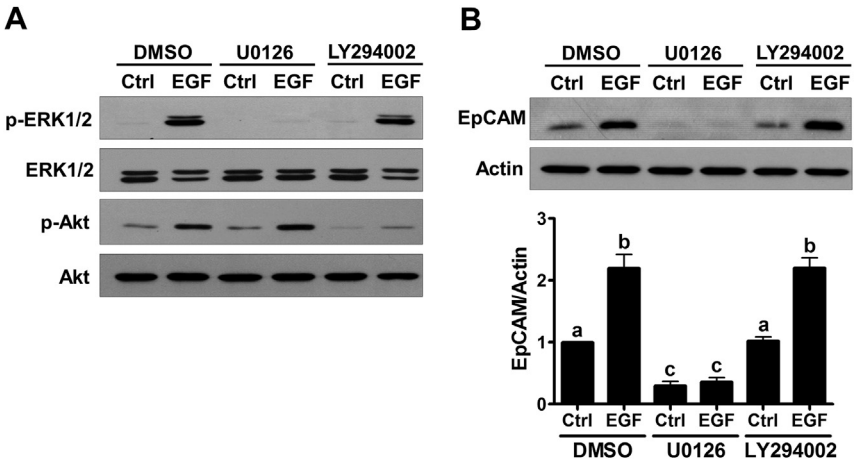

Fig. 3. The ERK1/2 signaling pathway is involved in EGF-up-regulated EpCAM expression in SKOV3. A, Cells were pretreated for 1 h with U0126 (10 μM) or LY294002 (10 μM) and then treated with 100 ng/mL EGF for 10 min. The levels of phosphorylated ERK1/2 and Akt were analyzed by western blotting. The membranes were stripped and re-probed with antibodies against total ERK1/2 and Akt. B, Cells were pretreated for 1 h with U0126 (10 μM) or LY294002 (10 μM) and then treated with 100 ng/mL EGF for 24 h. The protein levels of EpCAM were analyzed by western blotting. Values without a common letter are significantly different ($P < 0.05$).

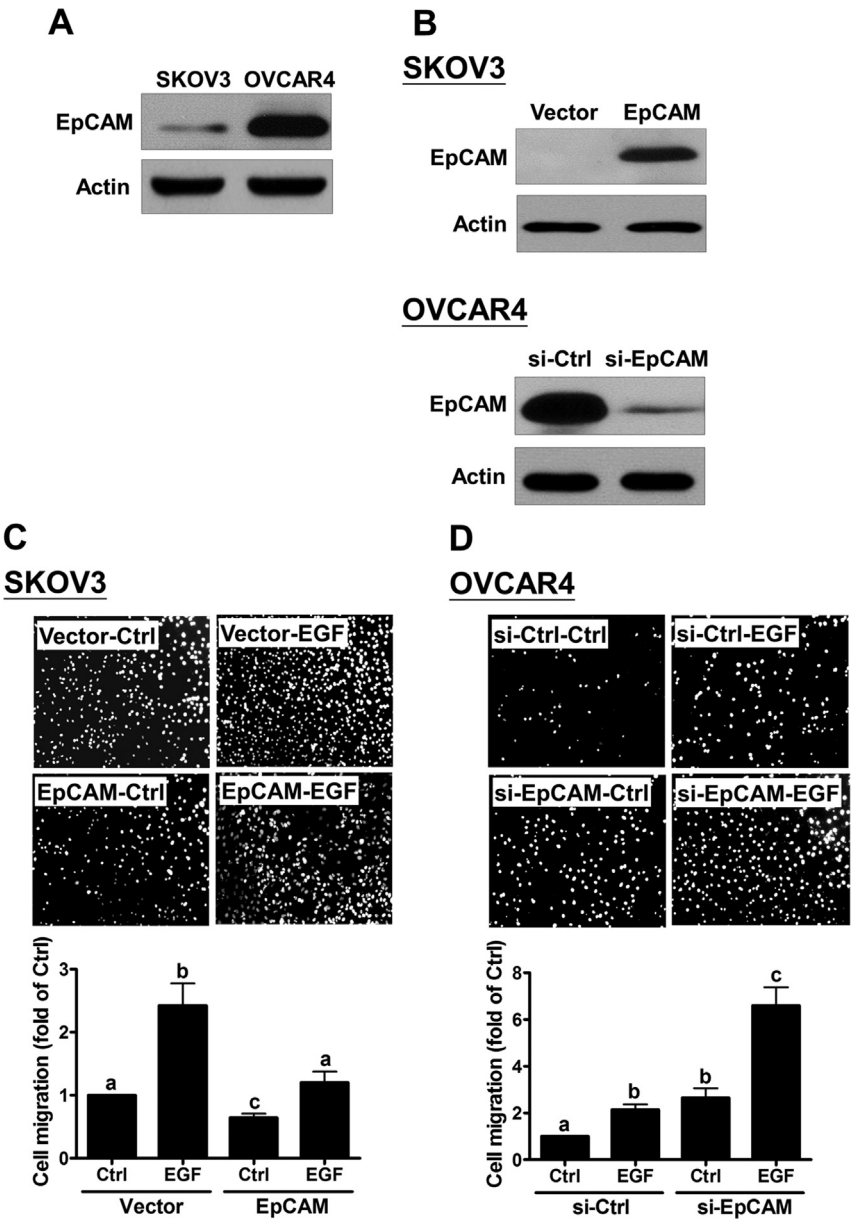

Fig. 4. EpCAM inhibits basal and EGF-stimulated cell migration. A, The basal protein levels of EpCAM in SKOV3 and OVCAR4 cell lines were analyzed by western blotting. B, SKOV3 cells were transfected with 1 μg empty vector (Vector) or vector encoding human EpCAM (EpCAM) for 48 h. OVCAR4 cells were transfected with 50 nM control siRNA (si-Ctrl) or EpCAM siRNA (si-EpCAM) for 48 h. The protein levels of EpCAM were analyzed by western blotting. C and D, After 48 h of EpCAM overexpression or knockdown, cells were treated with vehicle control (Ctrl) or 100 ng/mL EGF and then seeded into transwell inserts and cultured for an additional 12 (for SKOV3 cells) (C) or 24 (for OVCAR4 cells) (D) hours. Non-migrating cells were removed from the upper side of the filter, and the nuclei of the cells were stained with Hoechst 33258. The upper panel shows representative fluorescence images from the migration assay. The lower panel summarizes the quantitative results, which are expressed as the mean ± SEM from at least three independent experiments. Values without a common letter are significantly different ($P < 0.05$).

TGF-β1 in human breast cancer cells, which is required for TGF-β1-induced epithelial–mesenchymal transition [32]. EGF has been identified as an important factor for regulating human EOC progression, and the overexpression of EGFR is associated with a poor prognosis [33]. In the present study, our results showed that EGF treatment up-regulated EpCAM expression in human EOC cells by activating the ERK1/2, but not PI3K/Akt, signaling pathway. Our results provide another novel regulation mechanism for the regulation of EpCAM expression. A recent study identified 10 transcription factors that are associated with EpCAM expression in human EOC [34]. Therefore, future studies will be needed to delineate the transcriptional regulatory machinery of EpCAM that regulated by EGF.

To date, only a handful of studies have examined the function of EpCAM in the regulation of cell migration or invasion. It has been shown that EpCAM knockdown decreases human breast cancer cell migration and invasion [16,17]. Contradictory to these previous studies, in the present study, we showed that the overexpression of EpCAM decreased cell migration and that EpCAM knockdown increased cell migration in human EOC cells. It has been suggested that the dual functions of EpCAM in the regulation of cell adhesion and migration are dependent on the microenvironment of the tumor and the endogenous EpCAM expression levels. In tumor cells, EpCAM decreases E-cadherin-mediated cell–cell adhesion and thereby enhances cell migration and metastasis. Alternatively, EpCAM directly mediates cell–cell adhesion and thereby prevents

cell migration [8]. EpCAM has been shown to connect with the cytoskeleton by interacting with α-actinin. Moreover, the overexpression of EpCAM in EpCAM-negative thymic stromal cells induces the formation of stress fibers and cellular protrusions. However, the detail mechanisms of EpCAM-regulated cell migration require further elucidation.

In summary, our results demonstrated that treatment with EGF up-regulated the expression of EpCAM in human EOC cells. In addition, EGF induced EpCAM expression by activating ERK1/2 signaling. Moreover, we showed that EpCAM acts as a suppressor to inhibit basal as well as EGF-stimulated human EOC cell migration. This study demonstrates a novel biological function of EpCAM in human EOC, which suggests its tumor suppressor role in the regulation of human EOC progression.

Conflict of interest

The authors declare no conflict of interest.

Disclosure statement

The authors have nothing to disclose.

Acknowledgments

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