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# CD24$^+$ ovary cancer cells exhibit an invasive mesenchymal phenotype

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ABSTRACT

We recently reported that the subset of CD24$^+$ cells in ovarian cancer possesses various cancer stem cell properties. In this study, we further show that this subpopulation of ovarian cancer cells exhibits an epithelial–mesenchymal transition (EMT) phenotype, high invasive capacity, and CXCR4/SDF-1-mediated chemotactic migration. We evaluated CD24 expression in various ovarian cancer cell lines by flow cytometric analysis. CAOV3 and a primary ovarian cancer cell line Clone 4 were sorted into CD24$^+$ and CD24$^-$ subpopulations by FACS and Western blot, cell invasion, adhesion, and *in vitro* chemotaxis assays were performed with these two subpopulations. We also assessed the effects of shRNA depletion of CD24 in CAOV3 and Clone 4 cells by Western blot and cell invasion assays. CD24 expression in ovarian cancer cell lines correlated with aggressive histologic subtypes of epithelial ovarian cancer. The CD24$^+$ subpopulation was also more invasive than the CD24$^-$ subpopulation and showed higher CXCR4/SDF-1-mediated chemotactic migration. CD24$^+$ cells exhibited an EMT phenotype as characterized by loss of E-cadherin expression and gain of vimentin, Twist, and Snail1 expression. In addition, CD24$^+$ cells stimulated cell attachment to fibronectin through the activation of β1 integrin. Depletion of CD24 expression by CD24 shRNA efficiently suppressed cell invasion and induced downregulation of CXCR4 as well as loss of the EMT phenotype. In conclusion, CD24 expression in ovarian cancer may be related to tumor aggressiveness, in particular cell invasion and chemotactic migration. Therefore, CD24 may be a good candidate for a therapeutic target for ovarian cancer.



## 1. Introduction

Cancer stem cells (CSCs) resemble cells that have undergone epithelial–mesenchymal transition (EMT) with regard to their invasiveness and motility [1–3]. Since CSCs of an epithelial malignancy were first described as CD44$^+$CD24$^{low/-}$ cells in breast cancer [4], CSCs have been identified in many other human malignancies using the markers CD24, CD44, CD133, and epithelial cell adhesion molecule (EpCAM) [5–10].

CD24 has emerged as a novel oncogene and a promoter of metastasis [11]. Various studies have identified CD24 as a marker for poor prognosis in several tumor types, including ovarian cancer [12–20]. In previous studies, we confirmed the biological role of CD24 as an ovarian CSC marker. We first documented that CD24$^+$ cells preferentially express stemness genes that play an essential role in self-renewal, proliferative capacity, and fate determination [21]. Subsequently, we showed that the phenotypes of side population cells in heterogeneous clones based on intratumoral spatial difference were closely related to CD24$^+$, CD117$^+$, and CD117$^+$/CD24$^+$ fractions [22]. A recent study also reported that the CD44$^+$CD24$^+$EpCAM$^+$ phenotype was associated with stem/progenitor cells in human ovarian cancer cell lines [23]. Based on these findings, CD24$^+$ cells are highly likely to be involved in tumor progression, drug resistance, and clonal asynchronous evolution that affect intratumoral heterogeneity.

Chemokine signaling plays a critical role in the regulation of tumor progression and metastasis in many types of cancer [24]. Recent reports have shown that stromal cell-derived factor-1 (SDF-1) and its receptor, C-X-C chemokine receptor type 4 (CXCR4), promote tumor development and metastasis [25–28]. A previous study also reported that the chemokine CXCL12 exerts multiple biological effects in ovarian cancer, including stimulation

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of cell migration and invasion through the extracellular matrix [29]. In addition, a recent study reported that CSCs in breast carcinoma lose CD24 expression and have enhanced association between CXCR4 and lipid rafts [30]. Based on observations that CD24 increases the growth of breast tumor cells by augmenting CXCR4 responsiveness, it has been suggested that CXCR4 may be associated with CSCs in breast cancer. However, no linkage between CSC development and chemokine effects during progression of ovarian cancer has been documented.

β1 integrin expressed on metastatic ovarian cancer cells affects cell adhesion to the fibronectin secreted by mesothelial cells [31]. A more recent study reported that α5β1 integrin functions as a receptor for fibronectin whereas αvβ3 integrin functions as a receptor for vitronectin during the initial phase of adhesion [32]. In addition, CD24 indirectly stimulates cell adhesion to fibronectin, collagens I and IV, and laminin through the activation of α3β1and α4β1 integrin activity [33]. However, the properties of the $CD24^+$ subpopulation in ovarian cancer with respect to invasion, migration, and adhesion to the extracellular matrix have not been described.

Accordingly, the aim of the current study was to investigate invasion, adhesion, expression of EMT-related molecules, and chemokine sensitivity in $CD24^+$ and $CD24^-$ ovarian cancer cells. In addition, we evaluated CXCR4 expression in $CD24^+$ ovarian CSCs and whether CXCR4 is associated with $CD24^+$ as an ovarian CSC marker.

## 2. Materials and methods

### 2.1. Isolation of cells from human ovarian tumors and primary cell culture

The Institutional Review Board of Severance Hospital at Yonsei University College of Medicine approved this study. A tumor tissue specimen categorized as mucinous cytoadenocarcinoma was obtained from an ovarian cancer patient during surgery [21]. Clone 4 was suspended in Iscove's modified Dulbecco's medium (Gibco BRL) containing 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco BRL), 100 μg/ml gentamicin (Gibco BRL), 2.5 μg/ml amphotericin (Sigma–Aldrich), and 20% fetal bovine serum (FBS, Gibco BRL). Cells were maintained at 37 °C in a humidified atmosphere of 5% $CO_2$.

### 2.2. Cell culture

Human ovarian cancer cell lines were obtained from the American Type Culture Collection (CAOV3 and OV-90) or the Korean Cell Line Bank (SKOV3). Cells were maintained in RPMI-1640 (Invitrogen) supplemented with 10% heat-inactivated FBS and 100 units/ml penicillin–streptomycin at 37 °C in a humidified atmosphere of 5% $CO_2$. Human immortalized ovarian surface epithelial cells (IOSE) were obtained from Dr. Michael Birrer (Massachusetts General Hospital Cancer Center, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA; IOSE80NIH) and grown in a 1:1 mixture of Medium 199 (Gibco BRL) and Molecular cell developmental biology 105 (Sigma–Aldrich) supplemented with 5% FBS [34].

### 2.3. Flow cytometry and fluorescence-activated cell sorting (FACS)

Dissociated cells were counted and transferred to a 5-ml tube, washed twice with Hank's buffered salt solution (HBSS; Gibco BRL) containing 2% heat-inactivated FBS, and resuspended in HBSS/2% FBS. Fc receptor blocking reagent (MiltenyiBiotec) was added and the solution was incubated at 4 °C for 10 min, washed once, and resuspended in HBSS/2% FBS. Antibodies were added to the cell suspension and the solution was incubated for 20 min on ice in the dark. After two washes, cells were resuspended in HBSS/2% FBS and the suspension was filtered through 70-mm nylon mesh. Samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences). Cell sorting was performed on a BD FACSAria™ cell sorter (BD Biosciences). Anti-CD24-phycoerythrin was purchased from BD Pharmingen. BD FACSDiva software was used to analyze the data.

### 2.4. RNA extraction and conventional reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using an RNeasy Protect Mini Kit (Qiagen) according to the manufacturer's protocol. The SuperScript III Reverse Transcriptase kit (Invitrogen) was used to synthesize cDNA. Polymerase chain reactions (PCRs) were performed with HotStarTaq DNA polymerase (Qiagen) and the following conditions: denaturation at 95 °C for 15 min, and 28 cycles of 95 °C for 40 s, 57.5 °C (for CD24) or 60 °C (for *β-actin*) for 1 min, and 72 °C for 1 min, with a final extension for 10 min at 72 °C. Expression levels of *β-actin* were assessed as an internal control in all reactions. The following primers were used for PCR: forward primer for CD24 5′-ACCCAGCATCCTGCTAGAC-3′, reverse primer for CD24 5′-CTTAAGAGTAGAGATGCAGAA-3′; forward primer for *β-actin* 5′-GAATTCATGTTTGAGACCTTCAA-3′, reverse primer for *β-actin* 5′-CCGGATCCATCTCTTGCTCGAAGTCCA-3′.

### 2.5. Western blot analysis

Equal amounts of cell extract were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Invitrogen). Blots were blocked with 5% non-fat dry milk freshly dissolved in 1× phosphate buffered saline with Tween 20 (PBS-T). Blots were probed with antibodies specific for CD24, fusin (CXCR4), E-cadherin, vimentin, Twist, Snail1, β1 integrin (Santa Cruz Biotechnology), or *β-actin* (Cell Signaling Technology) at a 1:1000 dilution, followed by incubation with peroxidase-labeled secondary antibodies. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection kit (Santa Cruz Biotechnology).

### 2.6. Cell invasion assays

Cell invasion was assessed using 6.5-mm transwell chambers with a pore size of 8 μm (Corning). Transwell upper chambers were pre-coated with 50 μg/chamber of solubilized basement membrane in the form of Matrigel (BD Biosciences). RPMI-1640/10% FBS was added to the bottom chamber. Ovarian cancer cells ($5 \times 10^4$ per chamber) were seeded in serum-containing media in the upper well of the transwell chambers and incubated for 24 h at 37 °C in 5% $CO_2$. The noninvasive cells in the upper chamber were removed and the invasive cells in the lower chamber (below the filter surface) were fixed in 70% ethanol, stained with 0.1 mg/ml crystal violet solution (Sigma–Aldrich), and counted under a microscope (20× magnification). Cells from at least five fields were counted per chamber.

### 2.7. Cell chemotaxis assays

Cell chemotaxis assays were performed in 6.5-mm transwell chambers with a pore size of 8 μm (Corning). Cells were resuspended in serum-free media and added to the top well of transwell chambers ($5 \times 10^5$ per chamber) that contained serum-free media in both compartments. Serially diluted recombinant SDF-1 (R&D Systems) was added to the lower chambers and CAOV3 and Clone 4 cells were allowed to migrate from the upper to lower chamber

for a maximum of 24 h. After this time, cells that had not migrated were removed from the upper side of the filter dividing the chambers with cotton swabs, and cells that had migrated to the lower surface of the filters were fixed in 70% ethanol and stained with 0.1 mg/ml crystal violet solution. Cells in at least five random fields were counted per chamber.

### 2.8. CD24 mRNA depletion by plasmid transfection of shRNA

We used SureSilencing shRNA plasmid for human CD24 (KH02365P for puromycin resistance), as well as a scrambled sequence negative control plasmid (Qiagen). The shRNA target sequences were 5′-CATGATTGGTGAGCAGAAGTT-3′ for CD24 and 5′-GGAATCTCATTCGATGCATAC-3′ for the negative control. Cells were seeded and transfected using the Attractene Transfection Reagent (Qiagen) according to the manufacturer's protocol. For selection of stable transfectants, 5 μg/ml puromycin was added 48 h after transfection, and selection was continued for 72 h.

For invasion experiments, a fixed number of cells were seeded in invasion chambers after puromycin selection and the number of invasive cells was counted 72 h post-transfection.

### 2.9. Statistical analysis

Analysis of variance was performed using GraphPad Prism 5 (GraphPad Software). Continuous variables are expressed as mean ± standard deviation. Student's *t*-test was used for analyses, and results were considered statistically significant if the hypothesis was rejected with a *P*-value <0.05.

## 3. Results

### 3.1. Expression of CD24 correlates with histological aggressiveness of ovarian cancer

To evaluate the surface expression patterns of CD24 among ovarian cancer cell lines of different histologic types, we characterized one primary ovarian cancer cell line (Clone 4, mucinous [21]), three ovarian cancer cell lines (CAOV3, serous; OV-90 and SKOV-3, papillary serous), and one normal ovarian cell line (IOSE80NIH [34]) by flow cytometric analysis (Fig. 1A and Supplementary Table S1). CD24 expression levels correlated with the aggressiveness of ovarian cancer cell lines according to histologic type, compared with IOSE80NIH ($P < 0.01$).

### 3.2. CD24$^+$ cells have a highly invasive phenotype and exhibit an EMT molecular signature

To investigate the correlation between CD24 expression and the invasiveness of ovarian cancer, we isolated CD24$^+$ and CD24$^-$ subpopulations from CAOV3 and Clone 4 cells by FACS (Supplementary Fig. S1A). We then immediately analyzed CD24 expression by conventional RT-PCR to confirm the purity of the CD24$^+$ and CD24$^-$ cell populations (Supplementary Fig. S1B). We selected CAOV3 and Clone 4 for sorting into CD24$^+$ and CD24$^-$ subpopulations because CAOV3 moderately expressed CD24 (Fig. 1A and Supplementary Table S1) and Clone 4 showed CSC-like characteristics in our previous study [21].

To determine whether CD24$^+$ ovarian cancer cells show increased invasion, we compared the invasive capabilities of CD24$^+$ and CD24$^-$ populations. The CD24$^+$ population showed markedly increased invasiveness compared with the CD24$^-$ cells: at 24 h

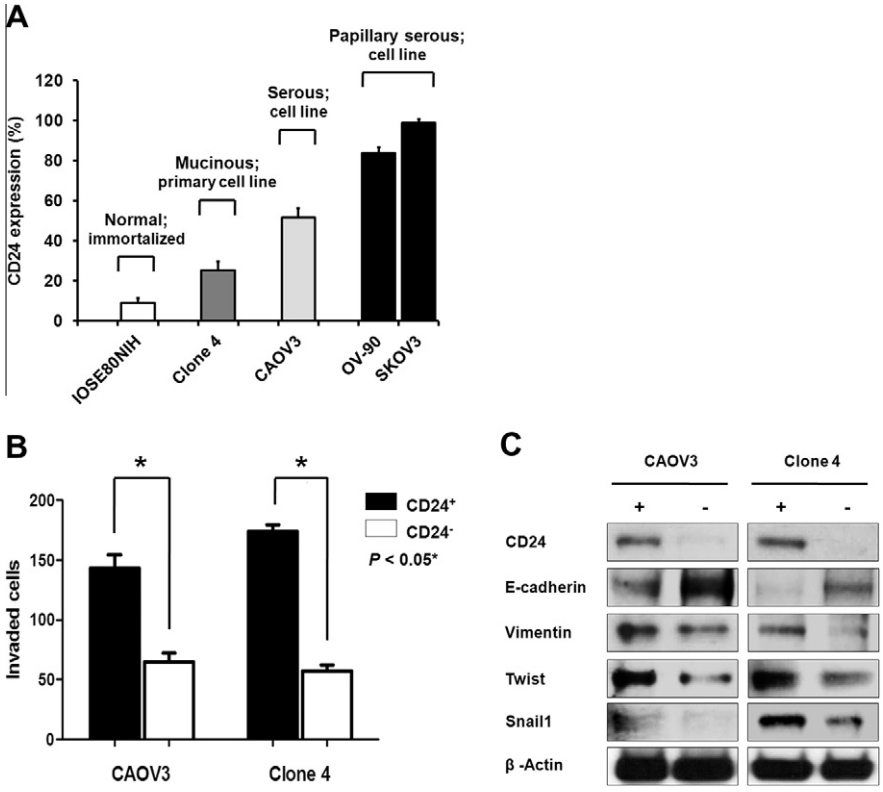


**Fig. 1.** CD24$^+$ cells possess an invasive mesenchymal phenotype. (A) Flow cytometric analysis of CD24 expression patterns in various ovarian cell lines. Data are represented as mean ± SD of three independent experiments performed in triplicate. (B) Cell invasion assays in CD24$^+$ and CD24$^-$ subpopulations of CAOV3 and Clone 4 cells. CD24$^+$ and CD24$^-$ cells were analyzed for invasive ability using transwell invasion. Cells were added to invasion chambers 48 h after transfection and the invaded cells were counted 24 h after seeding. Columns, means from three independent experiments, each performed in triplicate; error bars, SD. * indicates *P*-values <0.05. (C) Expression of EMT markers in CD24$^+$ and CD24$^-$ subpopulations of CAOV3 and Clone 4 cells. β-Actin served as a loading control.

after seeding under monoculture conditions $CD24^+$ cells demonstrated approximately 2.2- to 3.1-fold greater invasion than $CD24^-$ cells (Fig. 1B; $P < 0.05$).

We next investigated the expression patterns of EMT markers in $CD24^+$ and $CD24^-$ populations by Western blot analysis. $CD24^+$ cells of both cell lines showed reduced expression of E-cadherin and elevated expression of vimentin, Twist, and Snail1 compared with $CD24^-$ cells (Fig. 1C).

### 3.3. $CD24^+$ cells showed high expression of CXCR4 and exhibited increased chemotactic migration in response to SDF-1

The expression level of CXCR4, the receptor for the chemokine SDF-1 and an important mediator in cell migration, in $CD24^+$ and $CD24^-$ populations was examined by Western blot analysis. Both cell lines showed higher expression of CXCR4 in $CD24^+$ cells compared with $CD24^-$ cells (Fig. 2A).

Chemotaxis assays were performed to measure migration of $CD24^+$ and $CD24^-$ populations of CAOV3 and Clone 4 cells towards SDF-1. Both CAOV3 and Clone 4 cells migrated in response to SDF-1 in a dose-dependent manner. For both cell lines, $CD24^+$ cells were more responsive to SDF-1 than $CD24^-$ cells (Fig. 2B; $P < 0.05$).

### 3.4. Binding of $CD24^+$ cells to fibronectin is promoted by activation of $\beta 1$ integrin

We also performed cell adhesion assays to determine whether $CD24^+$ ovarian cancer cells have an altered ability to bind to extracellular matrix components. Binding of $CD24^+$ cells to fibronectin was approximately 4-fold greater than that of $CD24^-$ cells (Fig. 3A; $P < 0.05$). In contrast, binding to vitronectin was not significantly different between $CD24^-$ and $CD24^+$ cells. Using Western blot analysis, we confirmed that $CD24^+$ cells expressed β1 integrin at a markedly higher level than $CD24^-$ cells (Fig. 3B).

### 3.5. shRNA-mediated depletion of CD24 reduces the invasiveness of ovarian cancer cells by blocking the epithelial–mesenchymal transition

To assess the effect of CD24 downregulation on the invasiveness of ovarian cancer cells, CAOV3 and Clone 4 cells were transfected with CD24-specific shRNA. We confirmed that CD24 shRNA was effectively depleted at both the RNA and the protein level compared with cells transfected with negative control shRNA plasmid for CAOV3 and Clone 4 cell lines (Supplementary Fig. S2). After puromycin selection, we compared the change of cellular morphology by CD24-specific shRNA (Fig. 4A). $CD24^+$ cells showed a fibroblast-like morphology suggestive of EMT than $CD24^-$ cells. CD24 shRNA-treated cells showed a very similar morphological appearance to $CD24^-$ cells, including round and spindle cell morphology, and reduced cell–cell contacts.

To perform cell invasion assays, equal numbers of stable transfected cells were seeded in invasion chambers and the number of invasive cells was counted after 72 h. Cells in which CD24 was silenced were 3.7- to 4.6-fold less invasive than cells transfected with negative control shRNA (Fig. 4B; $P < 0.005$).

We next performed Western blot analysis to evaluate the effect of CD24 shRNA on EMT-related molecules in stably transfected CAOV3 and Clone 4 cells (Fig. 4C). Transfection with CD24 shRNA efficiently decreased CD24 and CXCR4 expression in CAOV3 and Clone 4 cells. In addition, E-cadherin expression in CAOV3 and Clone 4 cells was upregulated by transfection with CD24 shRNA treatment compared with negative control shRNA plasmid, whereas vimentin, Twist, and Snail1 expression was downregulated by CD24 shRNA treatment. These findings indicate that it may be possible to reduce cell invasion by blocking the EMT process in ovarian cancer cells.

## 4. Discussion

As we mentioned above, we first documented the possibility of CD24 as an ovarian CSC marker in our previous studies [21,22]. Herein we further made an effort to evaluate the potential that CD24 expression in ovarian cancer can promote cell invasion, induce significant amounts of EMT-associated markers, and increase SDF-1-mediated chemotactic migration through upregulation of CXCR4 protein. In addition, CD24 expression in ovarian cancer enhanced cell adhesion to fibronectin through the activation of β1 integrin.

EMT, which is associated with a key step in tumor metastasis via induction of a highly invasive phenotype, has been intensely studied [1–3]. CSCs have been found to transiently acquire stem cell-like properties as a consequence of EMT [35]. Here we showed that sorted $CD24^+$ ovarian cancer cells consistently acquired an

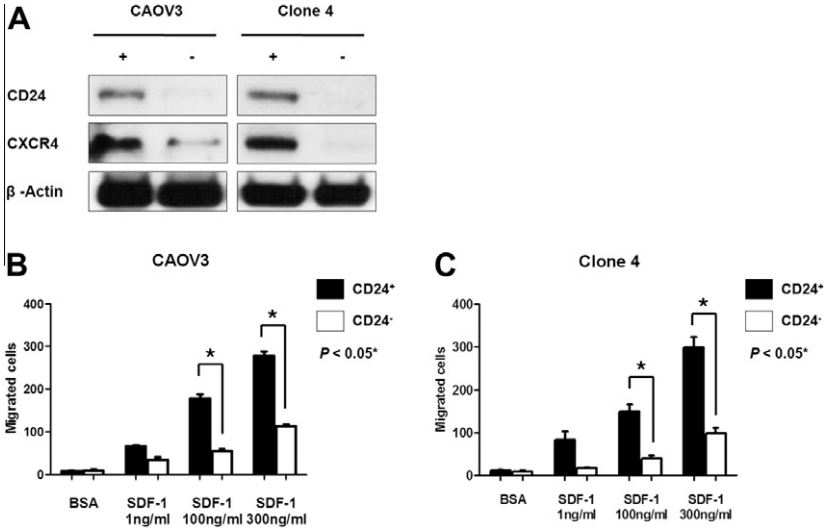


**Fig. 2.** $CD24^+$ cells showed high expression of CXCR4 and were chemotactically responsive to SDF-1 in a dose-dependent manner. (A) Expression of CXCR4 in $CD24^+$ and $CD24^-$ subpopulations of CAOV3 and Clone 4 cells. β-Actin served as a loading control. (B) Cell chemotaxis assays in $CD24^+$ and $CD24^-$ subpopulations of CAOV3 and Clone 4 cells. Columns, means from three independent experiments, each performed in triplicate; error bars, SD. * indicates *P*-values <0.05.

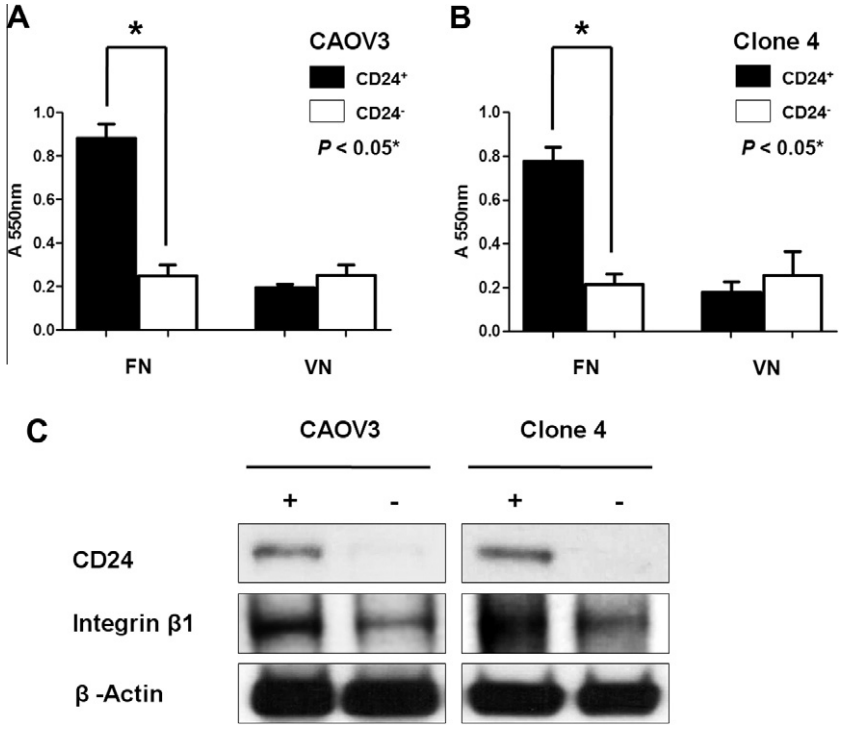


**Fig. 3.** CD24$^+$ cells bind fibronectin through the activation of β1 integrin. (A) Cell adhesion assays in CD24$^+$ and CD24$^-$ subpopulations of CAOV3 and Clone 4 cells. Columns, means from three independent experiments, each performed in triplicate; error bars, SD. $*$ indicates $P < 0.05$. (B) Expression of β1 integrin in CD24$^+$ and CD24$^-$ subpopulations of CAOV3 and Clone 4 cells. β-Actin served as a loading control.

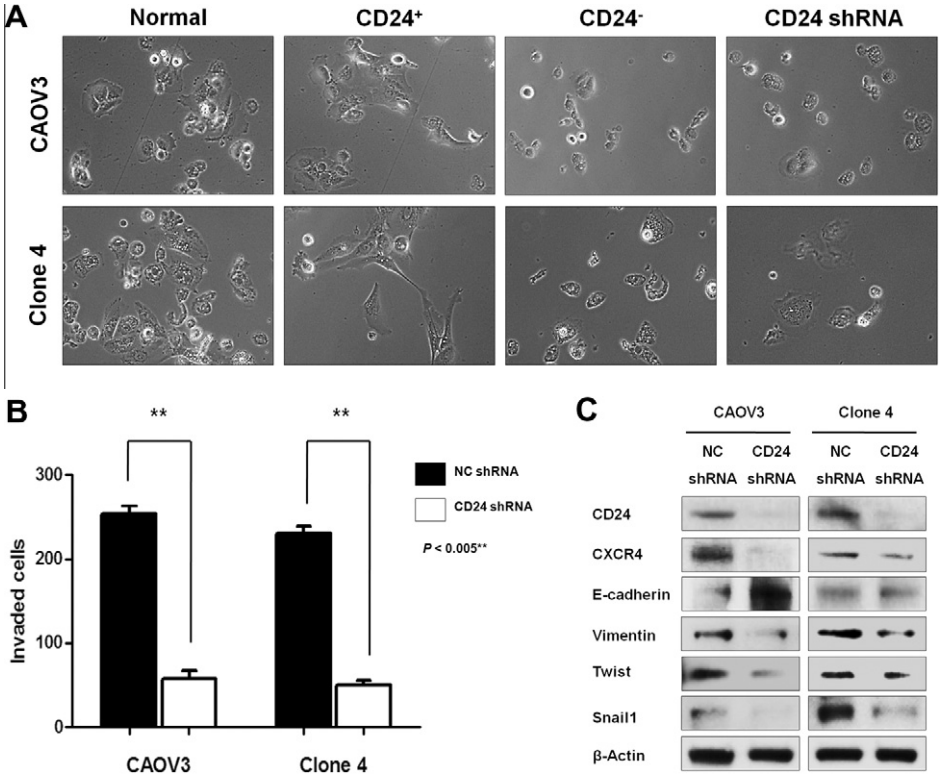


**Fig. 4.** Suppression of CD24 in ovarian cancer cells reduces their invasive properties. (A) Alteration of cellular morphology by CD24-specific shRNA (original magnification, 200×). (B) Effect of stable CD24 depletion by shRNA on cell invasion in CAOV3 and Clone 4 cells. Columns, means from three independent experiments, each performed in triplicate; error bars, SD. $**$ indicates $P < 0.005$. (C) Effect of stable CD24 depletion by shRNA on the expression of EMT makers in CAOV3 and Clone 4 cells. β-Actin served as a normalizing control.

EMT phenotype. These data indicate that $CD24^+$ cells are actively involved in the EMT process in ovarian cancer.

Recently proposed models have implicated CXCR4 in metastasis-promoting cancer cells [28] and CSCs [30]. Considering that CD24 is differentially expressed in CSCs of ovarian and breast cancer, we wondered if CXCR4 is associated with $CD24^+$ as an ovarian CSC marker or with $CD24^-$ cells as in breast cancer. We found that CXCR4 expression was associated with the ovarian CSC marker, CD24, as it was more highly expressed in $CD24^+$ cells compared to $CD24^-$ cells. $CD24^+$ cells also showed increased chemotactic migration in response to SDF-1, the ligand of CXCR4.

Integrins mediate cell adhesion and migration and activate intracellular signaling pathways that modulate cell survival and apoptosis [36]. CD24 augmented β1 integrin-dependent cell motility and stimulated transmigration and invasion across a monolayer of endothelial cells [37]. In contrast to the typical effect of integrins, αvβ3 integrin on tumor cells actually slows tumor progression [38]. In addition, CSCs express high levels of integrin, which can serve as an enrichment marker [39–41]. However, the role of integrin–ligand interactions between ovarian CSCs and the ECM has not yet been extensively investigated. Hence, we found that CD24 expression stimulated cell adhesion to fibronectin through the activation of β1 integrin.

In conclusion, all results in the present study suggest that $CD24^+$ cells are more highly invasive, adherent, and chemotactically responsive than $CD24^-$ cells in ovarian cancer. In addition, $CD24^+$ cells upregulate markers associated with EMT compared to $CD24^-$ cells and show mesenchymal phenotype. Accordingly, targeting CD24 may be useful as part of a novel therapeutic strategy based on CSCs to treat ovarian cancer.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.102.

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