Original Article

Tumor Biology

CXCL11 mediates TWIST1-induced angiogenesis in epithelial ovarian cancer

Tumor Biology
May 2017: 1–10

DOI: 10.1177/1010428317706226
journals.sagepub.com/home/tub
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Abstract

To investigate the role of TWIST1 in tumor angiogenesis in epithelial ovarian cancer and to identify key molecules involved in angiogenesis. TWIST1 small interfering RNA was transfected into A2780 cells, while a complementary DNA vector was transfected into non-malignant human ovarian surface epithelial cells to generate a TWIST1-overexpressing cell line. To evaluate how this affects angiogenesis, human umbilical vein endothelial cell tube formation assays were performed using the control and transfected cell lines. An antibody-based cytokine array was used to identify the molecules involved in TWIST1-mediated angiogenesis. After knockdown of TWIST1 via transfection of TWIST1 small interfering RNA into A2780 cells, the number of tubes formed by human umbilical vein endothelial cells significantly decreased in a tube formation assay. In a cytokine array, TWIST1 downregulation did not significantly decrease the secretion of the common pro-angiogenic factor, vascular endothelial growth factor, but instead inhibited the expression of the CXC chemokine ligand 11, which was confirmed by both an enzyme-linked immunosorbent assay and western blotting. In contrast, TWIST1 overexpression resulted in increased secretion of CXC chemokine ligand 11. Conversely, CXC chemokine ligand 11 downregulation did not inhibit the expression of TWIST1. Furthermore, the ability of TWIST1-expressing A2780 cells to induce angiogenesis was found to be inhibited after CXC chemokine ligand 11 knockdown in a tube formation assay. TWIST1 plays an important role in angiogenesis in epithelial ovarian cancer and is mediated by a novel pro-angiogenic factor, CXC chemokine ligand 11. Downregulation of CXC chemokine ligand 11 can inhibit tumor angiogenesis, suggesting that anti–CXC chemokine ligand 11 therapy may offer an alternative treatment strategy for TWIST1-positive ovarian cancer.

Keywords

TWIST, CXC chemokine ligand 11, ovarian cancer, epithelial–mesenchymal transition

Date received: 27 November 2016; accepted: 20 March 2017

Introduction

Epithelial ovarian cancer (EOC) is the leading cause of death in women with gynecologic malignancies. In 2012, ovarian cancer was the seventh most frequent cancer in women and the eight most common cause of death from cancer worldwide.[1] In the United States, an estimated 22,280 new cases of ovarian cancer and 14,240 deaths are expected in 2016.[2] In Korea, 1332 patients were newly diagnosed with ovarian cancer in 1999, and the number increased steadily to 2236 in 2013.[3]

Recent advances in medical technology have improved the diagnosis and treatment of various cancers, but EOC

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remains the most lethal disease among female genital tract malignancies, and its survival rate has not significantly increased. In Korea, the 5-year survival rate of ovarian cancer was 58.7% between 1993 and 1995 and 62.0% between 2009 and 2013, respectively, with only a 3.3% improvement.[3] The prognosis of early-stage disease is significantly better than late-stage disease, with the 5-year survival rate varying from 80%–93% (stage I or II) to less than 30% (stage III or IV).[4] However, 80% of all cases of EOC are late stage at diagnosis and have a high rate of recurrence due to the propensity of EOC for intraperitoneal metastasis.

EOC has several unique characteristics: one of which is the ability to co-express epithelial and mesenchymal determinants. Epithelial–mesenchymal transition (EMT) is a physiological process that converts epithelial cells into mesenchymal-like cells through disruption of cell–cell junctions and loss of apical-basolateral polarity, enabling cells to migrate and invade.[5] Studies have shown that EMT is often activated in cancer and plays critical roles in cancer invasion and metastasis. In addition to regulating cell motility, EMT of mammary tumor epithelial cells also promotes tumor angiogenesis.[6] Angiogenesis refers to the process of forming new blood vessels from pre-existing ones and is required for tumor growth and metastasis of all solid tumors.

The hallmark of EMT is the loss of epithelial markers expression (e.g. E-cadherin and catenin) and neoexpression of mesenchymal markers (e.g. N-cadherin, vimentin, and SMS actin). Several repressors of E-cadherin have been described, such as the zinc finger protein Snail, Slug, and Smad-interacting protein 1 (SIP-1: ZEB-2), and recently, TWIST has been accepted as a critical factor that induces loss of E-cadherin. In humans, the TWIST gene is a basic helix-loop-helix transcription factor that is located on chromosome 7p21.2. As a major regulator of mesenchymal phenotypes, TWIST is expressed in mesodermal and cranial neural crest cells during embryogenesis.[7]

The exact mechanism of TWIST-stimulated cancer progression has not been clearly identified. However, it has been suggested that TWIST may be a potential oncogene that inhibits apoptosis and may be responsible for chemoresistance.[8] Moreover, TWIST is thought to promote angiogenesis in several tumors, including prostate and bladder cancer.[9] In a clinical study of 97 patients with hepatocellular carcinoma (HCC), the investigators showed that TWIST1 expression was upregulated, affecting HCC angiogenesis.[10]

However, few studies have examined the role of TWIST in EOC. In particular, no study has yet evaluated the effect of TWIST expression on EOC angiogenesis. Furthermore, the mechanism by which TWIST affects angiogenesis remains unclear. To date, most studies have suggested that the key factor for TWIST-induced angiogenesis is vascular endothelial growth factor (VEGF).[11,12] VEGF is the most potent angiogenic factor that promotes growth, proliferation, and migration of endothelial cells. However, several reports have noted that TWIST could promote angiogenesis without an accompanying increase in VEGF secretion.[13]

Therefore, this study was designed to determine the role of TWIST in angiogenesis of EOC and to identify the key molecules that mediate TWIST-related angiogenesis. In this study, we first measured the basal expression level of TWIST1 in several EOC cell lines and analyzed the effect of TWIST1 expression on angiogenesis by silencing TWIST1 using small interfering RNA (siRNA) and by overexpressing TWIST1 using a complementary DNA (cDNA) vector.

Materials and methods

Cell lines and cell culture

Non-malignant human ovarian surface epithelial (HOSE) cells and five human EOC cell lines (SKOV3, OVCAR3, A2780, CA-OV3, and ES-2) were used in this study. HOSE, CA-OV3, and ES-2 were purchased from ScienCell Research Laboratories (San Diego, CA, USA) and SKOV3, OVCAR3, and A2780 came from one of our laboratories (Korea University Medical Center, Guro Hospital, Seoul, South Korea). All cell lines were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS; Seralab, West Sussex, UK), 300 μg/mL L-glutamine, and 1% penicillin–streptomycin (Gibco, Paisley, UK). Cells were grown on 100 mm plates at 37°C and 5% CO_2. The culture medium was changed every 48 h. Growth and morphology of the cells were routinely monitored.

Cell transfections and siRNA-mediated gene knockdown

HOSE cells expressing TWIST1 were established by transfecting TWIST1 cDNA (#EX-U1219-M29; GeneCopoeia, Rockville, MD, USA) into the cells. Transient transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, HOSE cells were plated on six-well plates 1 day before transfection, such that they were 70%–90% confluent at the time of transfection. On the day of transfection, 10 μg of each plasmid and 12 μL of Lipofectamine 2000 were incubated separately in 150 μL of Opti-MEM I Reduced-Serum Medium (Invitrogen). After 5 min of incubation at room temperature, the diluted plasmids and Lipofectamine 2000 were combined and incubated for an additional 20 min at room temperature. The DNA-Lipofectamine 2000 complexes were then added to each well, and the cells were incubated for 48 h at 37°C in a 5% CO_2 incubator. Transfection efficiencies were determined by observing green fluorescent protein (GFP)-expressing cells under fluorescence microscopy. TWIST1 expression was verified in the cell lines, HOSE/cont

(transfected with a non-specific control sequence) and HOSE/TWIST1 (transfected with TWIST1), by reverse transcription polymerase chain reaction (RT-PCR).

For gene knockdown, siRNAs targeting human TWIST1 and human CXC chemokine ligand 11 (CXCL11) were purchased from Bioneer (Daejeon, South Korea). The following double-stranded RNA oligonucleotides specific for the TWIST1 coding region were used: siRNA1—5′-CUGAACAGUUGUUUGUGUU-3′ (sense) and 5′-AACACAAACAACUGUUCAG-3′ (antisense); siRNA2—5′-GGACCCAUGGUAAAAUGCA-3′ (sense) and 5′-UGCAUUUUACCAUGGGUCC-3′ (antisense). In addition, siRNA targeting CXCL11 included the following sequences: siRNA1—5′-CUGGUUACCAUCGGAGUUU-3′ (sense) and 5′-AAACUCCGAUGGUAACCAG-3′ (antisense); siRNA2—5′-CAAGCCCUUAUAAGUCAAA-3′ (sense) and 5′-UUUGACUUAUAAGGGCUUG-3′ (antisense).

Transient siRNA transfections were performed using Lipofectamine RNAiMax Reagent (Invitrogen). After transfected cells were cultured for 48 h at 37°C in a 5% CO_2 incubator, TWIST1 downregulation was verified by RT-PCR and western blot analysis. The transfected A2780 cells were named A2780/cont (transfected with a control vector), A2780/siTWIST1 (transfected with siRNA for TWIST1), and A2780/siCXCL11 (transfected with siRNA for CXCL11).

RT-PCR

Total RNA was extracted from HOSE cells and five human EOC cell lines using TRIzol reagent (Invitrogen) following the manufacturer's instructions. For the synthesis of first-strand cDNA, 2 μg of total RNA after DNase (RQ1 DNase; Promega, Madison, WI, USA) treatment was reverse transcribed into cDNA using a cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). The cDNA samples were then amplified by PCR using 2.5 units of Taq DNA polymerase (Roche Applied Science, Penzberg, Upper Bavaria, Germany). The PCR amplification was performed with an initial denaturation for 4 min at 95°C, followed by 35 thermal cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1.5 min with a final extension at 72°C for 7 min. The PCR products were visualized on a 1.0% agarose gel containing 5 mg/mL ethidium bromide and then analyzed using a gel documentation system (Ultra-Violet Product Limited, Upland, CA, USA).

Real-time quantitative PCR

The transcription levels for each transcript were quantified using the Applied Biosystems 7300 PCR system (Applied Biosystems) and TaqMan Universal PCR Master Mix (Applied Biosystems). The TaqMan probes and oligonucleotide primers were obtained from Bioneer. The relative quantities of TWIST1 messenger RNA (mRNA) from all samples were normalized to an internal control, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The threshold cycle (Ct) value and the slope of a standard curve for PCR amplification efficiency for each set of primers were determined using SDS 1.4 software (Applied Biosystems). The ΔCt method was used by taking the difference (ΔCt) between the Ct of GAPDH and the Ct of TWIST1 and computing $2^{-\text{Average }\Delta\Delta Ct}$. Human umbilical vein endothelial cells (HUVECs) were used as the calibrator cell line, and the fold change was defined as the ratio of the level of transcripts in a sample over that in HUVEC. The following oligonucleotides were used as primers (Bioneer): human TWIST1: 5′-GGAGTCCGCAGTCTTACGAG-3′ (forward), 3′-TCTGGAGGACCTGGTAGAGG-5′ (reverse), human GAPDH: 5′-GGTGGTCTCCTCTGACTTCAACA-3′ (forward) and 3′-GTTGCTGTAGCCAAATTCGTTGT-5′ (reverse).

Western blot analysis

Cell lysates were prepared from EOC lines cultured on six-well plates (performed in triplicate) in 80 μL of lysis buffer per well (Pro-Prep Protein Extraction Solution; iNtRON Biotechnology, Seoul, South Korea). The lysis buffer included protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.7 μg/mL pepstatin A, 0.5 μg/mL leupeptin, and 2 μg/mL aprotinin). Lysates were centrifuged at 13,000 r/min for 10 min at 4°C, and the protein concentration in the supernatants was determined using a Bradford dye binding assay (Bio-Rad Laboratories, Hercules, CA, USA). A volume of 20 mg of protein from each sample was subjected to a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins in the SDS gels were transferred to a nitrocellulose membrane (Amersham Hybond-ECL; Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were washed in Tris-buffered saline (TBS) with 0.1% Tween 20 and then blocked in 2.5% membrane-blocking agent in TBS with 0.1% Tween 20 buffer for 1 h at room temperature. Membranes were incubated overnight at 4°C with primary antibodies against the TWIST1 protein (Abcam, Cambridge, MA, USA), CXCL11 (Abcam), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were washed three times with TBS, with 0.1% Tween 20 and then incubated for 1 h at room temperature with horseradish peroxidase–conjugated secondary goat anti-mouse IgG antibodies (Bio-Rad Laboratories). Membranes were washed, and visualization of the secondary antibody was performed using enhanced chemiluminescence (ECL) western blotting detection reagents (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Blots were developed on Amersham Hyperfilm, and all signal intensities were normalized to that of β-actin.

Cytokine array and enzyme-linked immunosorbent assay

The production of angiogenesis-related factors was determined using a human cytokine array (C-Series 1000; RayBiotech, Norcross, GA, USA), which can detect the expression of 43 angiogenic factors in two membranes. The array was conducted according to the manufacturer's protocols using 48-h cell-conditioned media that were controlled for cell number and media volume. Membranes from the cytokine array kit were incubated with 1 mL of the concentrated conditioned media at room temperature for 2 h. The membranes were then washed three times with wash buffer I and twice with wash buffer II, both at room temperature and for 5 min for each wash, and assayed by chemiluminescence. To verify the results, the same samples and concentrated conditioned media were assayed with enzyme-linked immunosorbent assay (ELISA). Commercially available Sandwich ELISA kits (RayBiotech) were used to evaluate the protein levels of CXCL11 in the conditioned media derived from A2780 cells.

In vitro tube formation assay

Umbilical cords were obtained, with consent, from uncomplicated normotensive pregnancies delivered by cesarean section. HUVECs were isolated according to a previously published method.[14] Cells were cultured in Endothelial Basal Medium-2 (Lonza, Walkersville, MD, USA) according to the manufacturer's instructions. HUVECs were used for these studies after three to four passages. Cultures were tested and shown to be free of mycoplasma contamination. HUVECs were characterized by their homogeneous and typical cobblestone morphology as observed with electron microscopy. All cells were maintained in a 5% CO_2 atmosphere at 37°C. For all functional and biological assays, cells with >95% viability were used at 80%–90% confluence.

HOSE and A2780 cells were cultured in six-well plates with fresh complete medium for 24 h, and 1 mL of conditioned medium was collected. For tube formation assays, the 48-well plates were coated with Matrigel (100 µL per well; Corning, Corning, NY, USA) and maintained in a 5% CO_2 atmosphere at 37°C for 30 min. Then, 2×10^4 HUVECs were suspended in 1 mL conditioned medium and applied to the pre-coated 48-well plates. After incubation at 37°C for another 7 h, images were captured under a microscope and the number of tubular structures formed in the Matrigel was counted by hand at ×100 magnification in three random fields.

Statistical analysis

All experiments were performed in at least triplicate, and the data were compiled from three separate experiments. The results of multiple observations were calculated as the mean ± standard deviation (SD). Student's t tests and one-way analysis of variance (ANOVA) were performed to analyze differences among groups using SPSS for Windows version 20.0 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered to be statistically significant.

Results

Basal expression of TWIST1

We first analyzed the basal expression of TWIST1 in five human EOC cell lines (SKOV3, OVCAR3, A2780, CA-OV3, and ES-2), HOSE cells, and HUVECs (Figure 1). TWIST1 expression varied in the different EOC cell lines, but A2780 and ES-2 cells showed higher expression levels than that in other EOC cell lines based on both RT-PCR (Figure 1(a)) and western blot analysis (Figure 1(b)). By quantitative PCR (qPCR; Figure 1(c)), the difference in relative quantities of TWIST1 mRNA among the cell lines was statistically significant ($p < 0.001$). Because A2780 cells showed the highest TWIST1 expression, A2780 was selected as the cell line for siRNA-mediated depletion of TWIST1. In contrast, HOSE cells and HUVECs expressed low levels of TWIST1 mRNA, so HOSE cells were used for induction of TWIST1 overexpression by cDNA transfection.

Silencing of TWIST1 by TWIST1-siRNA in A2780 cells

To evaluate the role of TWIST1 in EOC, we first established a TWIST1-silenced EOC cell line. Either TWIST1-siRNA or a non-specific control sequence (control) siRNA was transfected into A2780 cells. After 48 h of transfection, the levels of TWIST1 mRNA and protein were measured by RT-PCR and western blot analysis, respectively. The TWIST1 mRNA level significantly decreased in A2780/siTWIST1 cells compared to that in A2780/cont cells (Supplemental Figure 1(a)). TWIST1 protein expression also decreased in A2780/siTWIST1 cells (Supplemental Figure 1(b)). These results suggest that both TWIST1-siRNA1 and TWIST1-siRNA2 could effectively suppress TWIST1 expression at both the mRNA and protein levels in A2780 cells.

TWIST1 knockdown inhibits angiogenesis in A2780 cells

To determine the effects of TWIST1 silencing on ovarian cancer cell angiogenesis, we used tube formation assay (Figure 2(a) and (b)). The results showed that the mean number of complete tubular structures formed by HUVECs was significantly lower in conditioned medium from A2780/siTWIST1 cells (6.7 ± 3.5 in A2780/TWIST1-siRNA1 and 7.3 ± 2.1 in A2780/TWIST1-siRNA2)

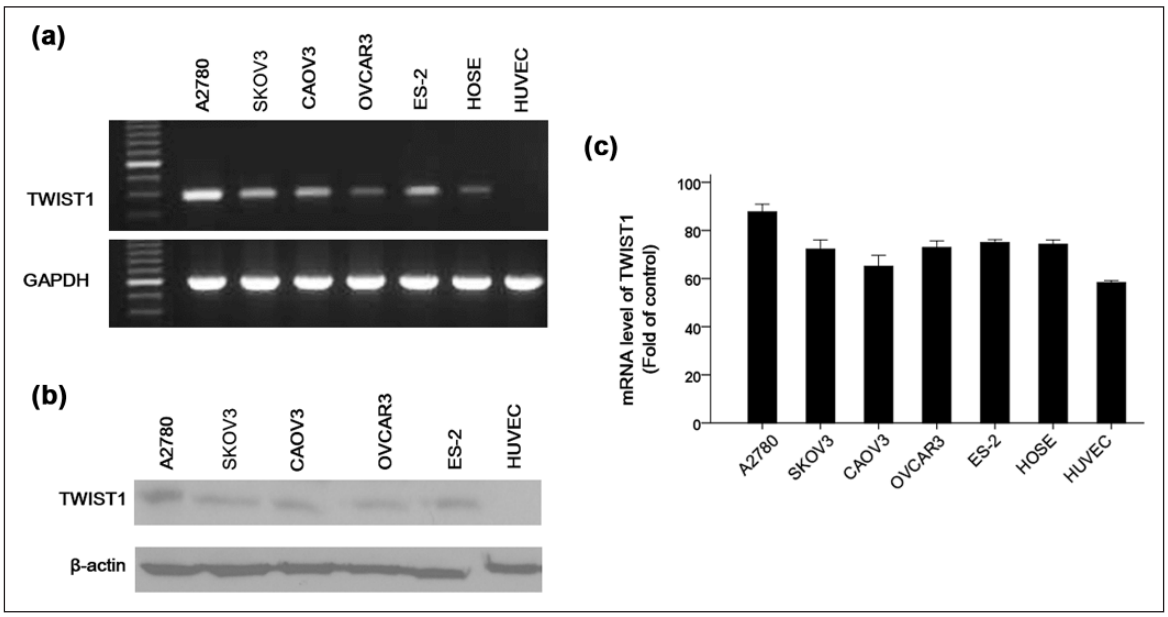

Figure 1. Basal expression of TWIST1. The expression of TWIST1 was analyzed by (a) RT-PCR, (b) western blotting, and (c) real-time qPCR. A2780 cells showed the highest expression of TWIST1 at the protein and mRNA levels. HOSE cells are non-malignant human ovarian surface epithelial cells and expressed lower levels of TWIST1 than A2780 cells. (c) By real-time qPCR, the difference in relative quantities of TWIST1 mRNA among the cell lines was statistically significant (one-way ANOVA, $p < 0.001$).

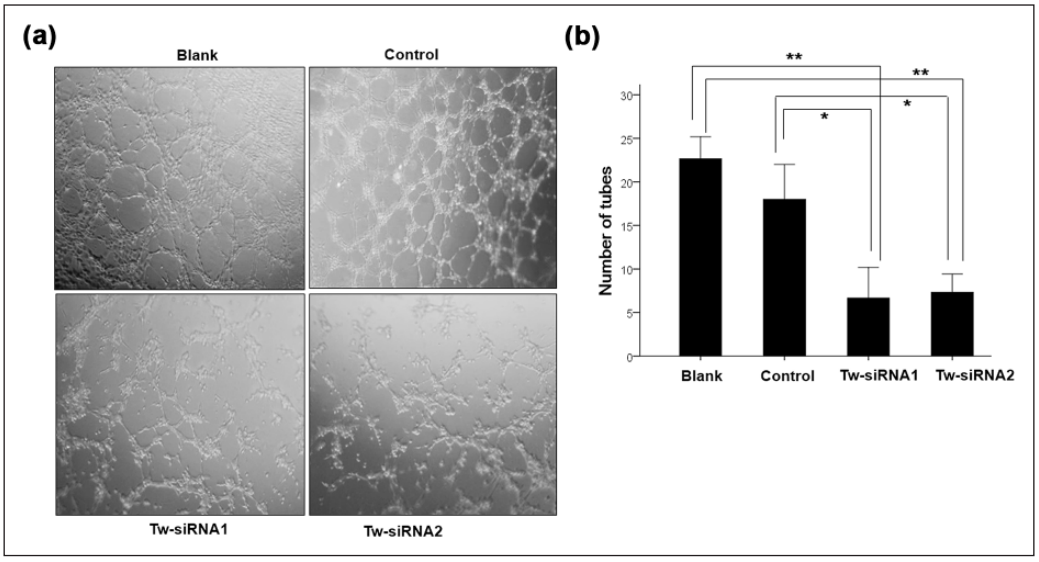

Figure 2. TWIST1 downregulation inhibits angiogenesis in ovarian cancer cells. (a) Capillary-like tubes were observed and photographed (magnification: ×100). TWIST1 knockdown in A2780 cells inhibited HUVEC tube formation. (b) The numbers of tubes formed per field were counted in three random fields of view for A2780/blank, A2780/cont, A2780/TWIST1-siRNA1, and A2780/TWIST1-siRNA2 cells (22.7 ± 2.5, 18.0 ± 4.0, 6.7 ± 3.5, and 7.3 ± 2.1, respectively). Data are presented as the mean ± SD (*$p < 0.05$; **$p < 0.01$).

compared to that in the corresponding control (22.7 ± 2.5 in A2780/blank and 18.0 ± 4.0 in A2780/cont; $p < 0.05$ for all comparisons). These data indicate that knockdown of TWIST1 significantly inhibits the angiogenic capacity of the EOC cells.

TWIST1 knockdown induces CXCL11 downregulation

To determine the molecules involved in TWIST1-mediated angiogenesis in EOC, we examined the factors secreted

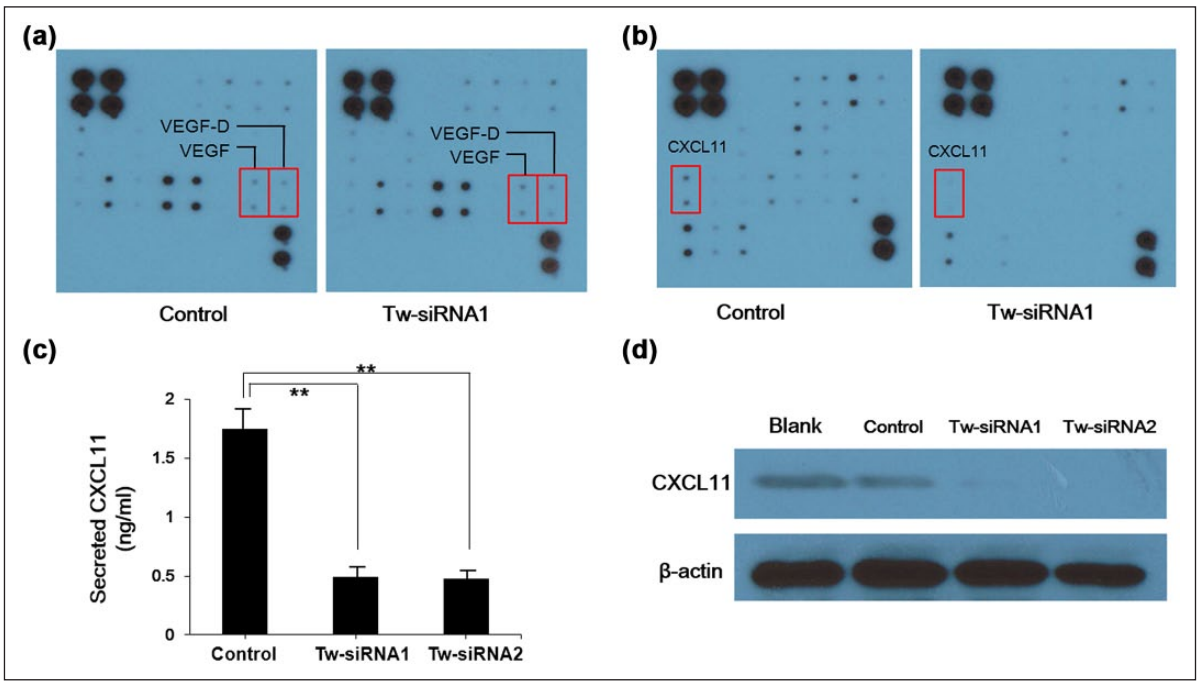

Figure 3. TWIST1 knockdown downregulates CXCL11. A cytokine expression array shows (a) 20 cytokines on membrane 1 and (b) 23 cytokines on membrane 2 secreted by A2780/cont and A2780/TWIST1-siRNA1 cells. TWIST1 knockdown did not affect VEGF levels, but CXCL11 expression was lower in the conditioned media from A2780/siTWIST1 cells. This result was validated by (c) ELISA and (d) western blot analysis (**$p < 0.01$).

from TWIST1-expressing or TWIST1-silenced cells that affected the proliferation of endothelial cells. A cytokine array that could detect the expression of 43 angiogenic factors in two membranes was used. The secreted levels of common pro-angiogenic factors, such as VEGF and fibroblast growth factor, were not significantly altered by TWIST1 knockdown in the cytokine array (Figure 3(a)). However, CXCL11 levels decreased in the conditioned media from A2780/siTWIST1 cells (Figure 3(b)). To verify this result, we performed ELISA and found that depletion of TWIST1 decreased the secretion levels of the CXCL11 protein from A2780 cells (1.75 ± 0.19 ng/mL for the control, 0.49 ± 0.08 ng/mL for TWIST1-siRNA1, and 0.48 ± 0.07 ng/mL for TWIST1-siRNA2; $p < 0.01$ for all comparisons; Figure 3(c)). Furthermore, western blot analysis revealed that CXCL11 protein levels in A2780/siTWIST1 cells were lower than those in A2780/cont cells (Figure 3(d)), suggesting that downregulation of TWIST1 may inhibit angiogenesis via CXCL11 in EOC.

Upregulation of TWIST1 by transfecting TWIST1 cDNA in HOSE cells

To confirm the interaction between TWIST1 and CXCL11, we first established HOSE cells expressing TWIST1. Either a plasmid vector expressing TWIST1 or an empty vector (control) was transfected into HOSE cells. After 48 h of transfection, samples were tested by RT-PCR (Figure 4(a)). In addition, high transfection efficiency was confirmed by fluorescence microscopy (Figure 4(b)).

TWIST1 upregulation induces CXCL11 overexpression

In our previous experiments (Figure 1), the basal expression of TWIST1 was shown to be lower in HOSE cells than in A2780 cells. Similarly, the levels of secreted CXCL11 were also lower in conditioned media from HOSE/cont than that in the conditioned media from A2780/cont in a cytokine array (Figure 4(c)). However, secreted CXCL11 protein levels increased after TWIST1 overexpression resulting from transfection with TWIST1 cDNA in HOSE cells. Taken together, these data indicate that TWIST1 is involved in the regulation of CXCL11 expression.

Silencing of CXCL11 by CXCL11-siRNA in TWIST1-expressing A2780 cells

CXCL11, also known as a T cell α chemoattractant (I-TAC), is an interferon (IFN)-inducible chemokine. Recently, it has been suggested that CXCL11 plays a major role in tumor progression by mediating pro-angiogenic effects. As shown in Figures 2 and 3, the downregulation of TWIST1 inhibited both angiogenesis and CXCL11 expression in ovarian cancer. Therefore, to determine whether CXCL11 mediates TWIST1-induced angiogenesis, we first knocked down the

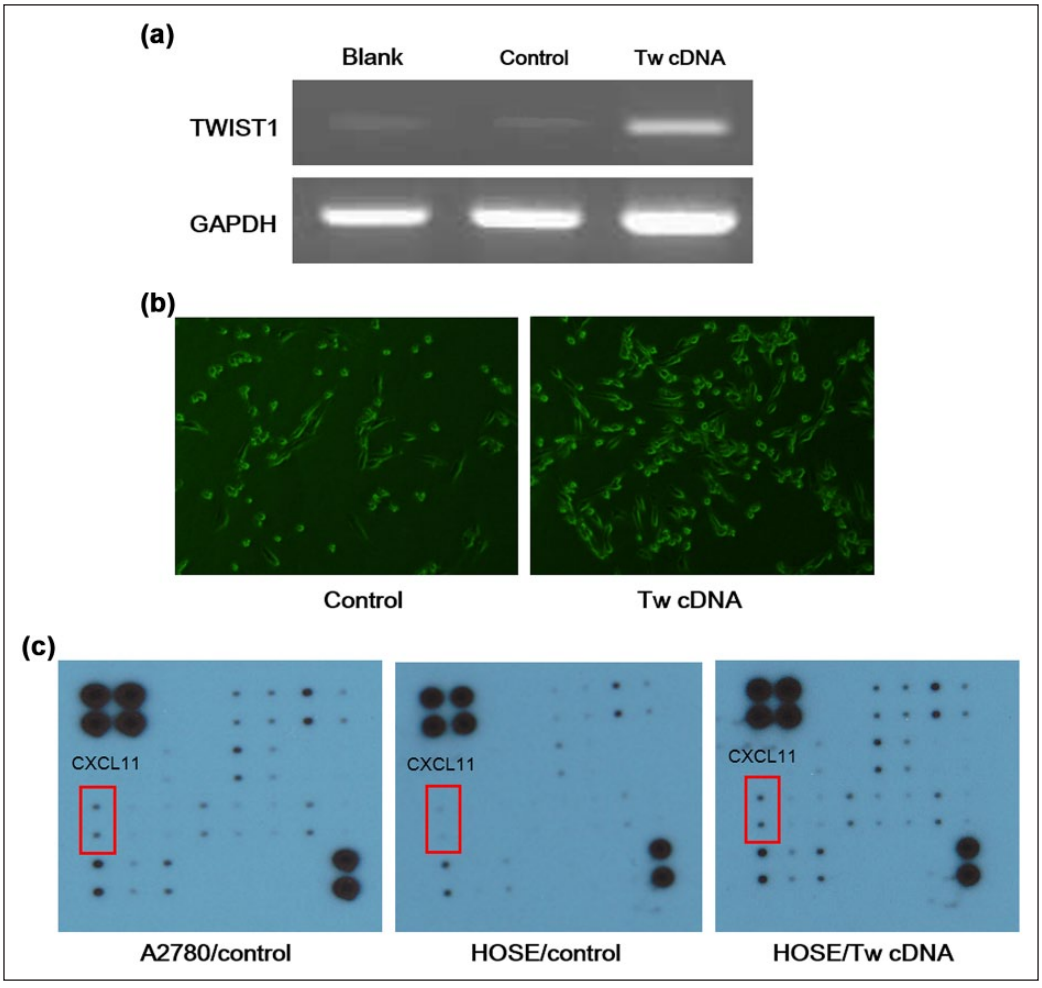

Figure 4. TWIST1 upregulation induces CXCL11 overexpression. (a) HOSE cells were transfected with a TWIST1 cDNA plasmid and analyzed by RT-PCR 48 h after transfection. (b) Cell transfection efficiency was observed using fluorescence microscopy. Green fluorescent protein (GFP)-positive HOSE cells transfected with either empty vector (control) or TWIST1 cDNA are shown (magnification: ×200). (c) A cytokine array revealed that the secreted level of CXCL11 was lower in conditioned media from HOSE/cont than that of A2780/cont cells. However, TWIST1 upregulation by cDNA transfection of HOSE cells induced CXCL11 expression, indicating that TWIST1 is sufficient to induce CXCL11 expression.

CXCL11 gene. A2780 cells were transfected with a vector control, CXCL11-siRNA1, or CXCL11-siRNA2 and cultured in medium for 48 h. Successful depletion of CXCL11 was verified by western blot analysis (Figure 5(a)).

CXCL11 is a downstream effector that mediates TWIST1-induced angiogenesis

After A2780 cells were transfected with CXCL11-siRNA, the conditioned medium was collected to test whether CXCL11 downregulation could inhibit the ability of TWIST1-expressing A2780 cells to induce endothelial cell tube formation. As shown in Figure 5(b), HUVECs had a limited ability to form tubes when the conditioned medium from A2780/siCXCL11 cells was used. The mean number of complete tubular structures formed by HUVECs was 50.3 ± 6.2 in A2780/blank-conditioned medium, 42.0 ± 8.1 in A2780/cont-conditioned medium, 12.0 ± 2.4 in A2780/CXCL11-siRNA1-conditioned medium, and 15.5 ± 1.9 in A2780/CXCL11-siRNA2-conditioned medium ($p < 0.01$ for all comparisons between A2780/cont and A2780/CXCL11-siRNA; Figure 5(c)). These data indicate that the ability of TWIST1-expressing A2780 cells to induce angiogenesis is inhibited by CXCL11 knockdown.

In addition, the protein levels of TWIST1 in A2780 cells transfected with either a control vector or CXCL11-siRNA were analyzed by western blotting (Figure 5(d)). The results showed that CXCL11 downregulation did not affect TWIST1 expression. Conversely, TWIST1 induced CXCL11 expression, and TWIST1 knockdown inhibited CXCL11 expression as shown in Figures 3 and 4. These data indicate that TWIST1 is an upstream regulator of CXCL11 expression.

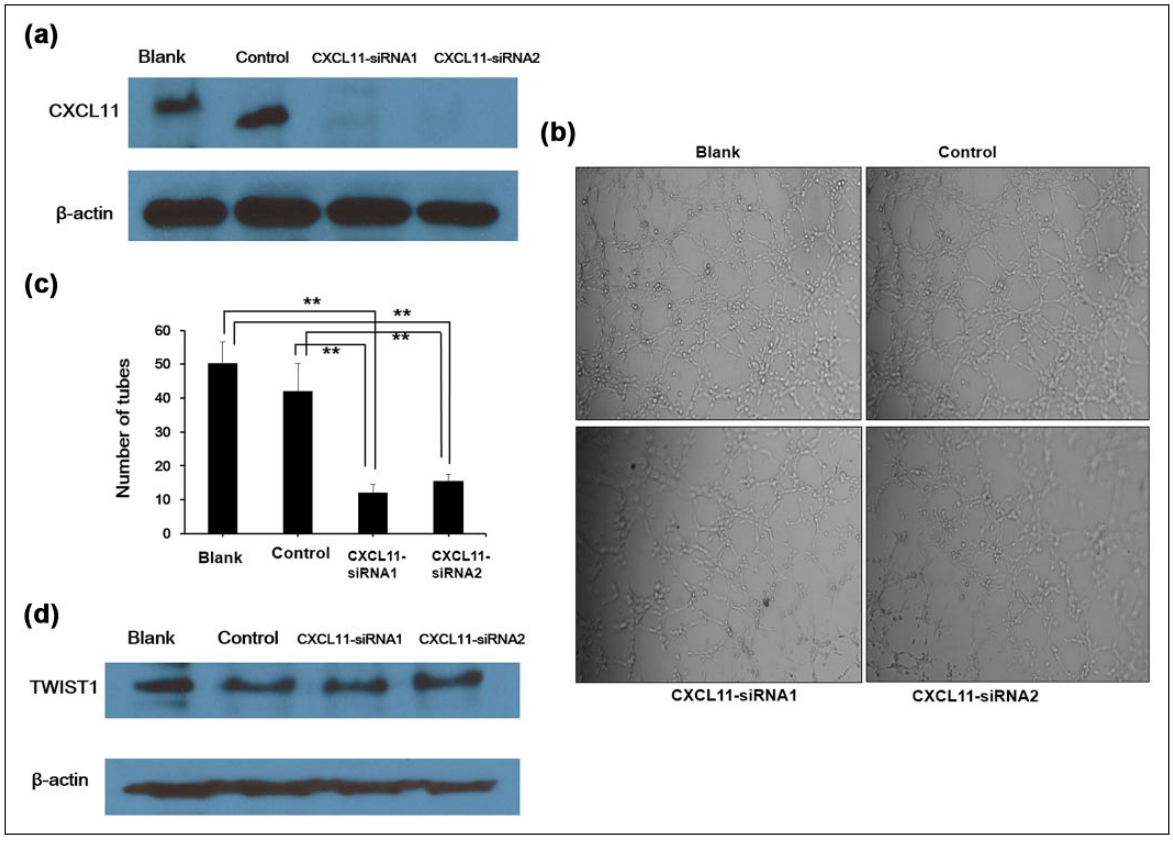

Figure 5. CXCL11 is a downstream effector that mediates TWIST1-induced angiogenesis. (a) A2780 cells were transfected with a vector control, CXCL11-siRNA1, or CXCL11-siRNA2 and cultured in medium for 48 h. The CXCL11 downregulation was verified by western blot analysis. (b) Capillary-like tubes were observed and photographed (magnification: ×100). CXCL11 knockdown in A2780 cells inhibited HUVEC tube formation. (c) The number of tubes was 50.3 ± 6.2 in conditioned medium from A2780/blank, 42.0 ± 8.1 from A2780/cont (transfected with vector control), 12.0 ± 2.4 from A2780/CXCL11-siRNA1, and 15.5 ± 1.9 from A2780/CXCL11-siRNA2 (**$p < 0.01$). Data are presented as the mean ± SD. (d) TWIST1 protein levels in A2780/siCXCL11 were analyzed by western blotting. CXCL11 downregulation did not affect TWIST1 expression.

Discussion

In this study, we showed that A2780 cells expressed the highest level of TWIST1 among the five EOC cell lines. TWIST1 knockdown inhibited angiogenesis in A2780 cells based on the tube formation assay. Furthermore, TWIST1 knockdown inhibited CXCL11 expression while TWIST1 upregulation induced CXCL11 expression. The ability of TWIST1-expressing A2780 cells to induce angiogenesis was inhibited by CXCL11 knockdown, but CXCL11 downregulation did not affect TWIST1 expression. On the basis of these results, we propose that CXCL11 is a downstream molecule that mediates TWIST1-induced angiogenesis.

In mammals, there are two types of TWIST-like proteins, TWIST1 and TWIST2, which share a high degree of structural homology. To date, several studies have reported that overexpression of TWIST is present in many types of neoplasms, including glioma, gastric cancer, prostatic cancer, and breast cancer.[11,15] In ovarian cancer, a literature review has shown that the rate of tumors overexpressing TWIST protein was as high as 40%.[9] Researchers evaluated 108 cases of human ovarian tissues and demonstrated that TWIST expression was higher in ovarian cancer tissues than in benign and borderline ovarian tumors.[16] In our study, the TWIST1 mRNA and protein levels were higher in A2780 and ES-2 cells than HOSE cells and HUVECs.

In recent years, TWIST has been recognized as a crucial regulator of EMT and implicated in a variety of tumor processes, such as inhibition of apoptosis, drug resistance of the tumor, tumor invasion, and peritoneal metastasis.[17] In 2004, a study demonstrated that TWIST expression was associated with cellular resistance to paclitaxel in human ovarian cancer cell lines.[18] In a study of a series of clinical ovarian carcinomas of all histological types, TWIST expression was reported to be associated with poor survival outcomes and was found to be an independent prognostic factor in multivariate analysis.[19]

Most of the literature on TWIST has focused on the role of this mesenchymal marker in EMT and tumor metastasis; but recently, it has been shown that TWIST may have a key role in angiogenesis, which is required for tumor growth. Our observations in EOC cell lines support this

idea. In this study, we showed that TWIST1 knockdown by siRNA inhibited angiogenesis in A2780 cells. Angiogenesis is a hallmark of the initiation and early growth of primary epithelial cancers. TWIST-induced angiogenesis may not depend on only a single mechanism but rather on various pro-angiogenic factors, including VEGF, matrix metalloproteinase (MMP), and angiopoietins, which have been reported to be positively regulated by TWIST in various tumors.[10,12,20] Notably, several studies suggested that the main mediator in TWIST-induced angiogenesis is the pro-angiogenic factor VEGF.[11,12] For example, a study of breast cancer demonstrated a fourfold increase in VEGF in a cancer cell line overexpressing TWIST compared with the control cell line.[12] In addition, Niu et al.[21] observed a significant positive correlation between TWIST protein expression and VEGF, suggesting that TWIST promotes angiogenesis via VEGF upregulation in human HCC tissues. However, another report showed that TWIST1 expression did not increase the secretion of VEGF but rather recruited macrophages through chemoattractant CCL2 induction to promote angiogenesis and tumor progression.[13]

Consistent with these results, we showed that TWIST1 knockdown inhibited the expression of CXCL11 rather than VEGF in the cytokine assay. To examine the interaction between TWIST1 and CXCL11 in greater detail, we performed ELISA and western blot assays and also established a TWIST1-overexpressing A2780 cell line. Our data demonstrate that TWIST1 is sufficient to regulate CXCL11 expression in ovarian cancer. In addition, TWIST1-induced angiogenesis is mediated by CXCL11. This result was surprising because previous studies have suggested that VEGF is the most potent angiogenic factor involved in the TWIST1-induced angiogenesis. Currently, few studies have been reported on the role of chemoattractants (or chemokines) in EOC. The TWIST1-CXCL11-angiogenesis axis in ovarian cancer is a novel finding.

CXCL11 is principally produced by macrophages and mediates recruitment of T cells, natural killer cells, monocytes, and macrophages, predominantly through their cognate G-protein coupled receptor, CXCR3.[22] Accumulating studies have found that CXCL11–CXCR3 binding is implicated not only in multiple physiological activities, including maturation of T cells and vasculogenesis, but also in diverse pathological conditions, such as immune-related diseases, infections, tumor progression, and metastasis.[23] Interestingly, however, some investigators have reported that stimulation of tumor growth after exposure to CXCL11 was not accompanied by stimulation of tumor angiogenesis.[24] As a potential explanation for this result, a receptor-dependent action of CXCL11 in tumor angiogenesis is of great interest. Namely, signaling through CXCR3 has been found to have anti-angiogenic effects, while signaling through the second receptor CXCR7 is most likely to be pro-angiogenic.[24,25] In this study, we demonstrated that CXCL11 is a downstream effector of TWIST1 and is positively correlated with tumor angiogenesis. Therefore, we speculate that TWIST1-induced angiogenesis in ovarian cancer may be ascribed to CXCL11 action via CXCR7. However, we do not provide any data regarding CXCL11 receptors, and further studies should be undertaken to identify the dominant receptors that contribute to TWIST-mediated angiogenesis in EOC.

Our study has several limitations. First, we failed to elucidate the molecular mechanism by which TWIST1 regulates CXCL11 expression in EOCs and CXCL11 induces angiogenesis. Second, we examined only the A2780 cell line for knockdown of TWIST1 because A2780 cells showed the highest basal expression of TWIST1. The present finding is consistent with a result reported by the Cancer Cell Line Encyclopedia (CCLE) project,[26] in which A2780 cells showed the second highest expression of TWIST1 mRNA among 52 types of ovarian cancer cell lines. However, it is necessary to also perform experiments in various EOC cell types to further evaluate the role of TWIST1 and CXCL11 in tumor angiogenesis. Finally, our result should be verified in tumor tissues using an orthotopic mouse model and clinical samples.

In conclusion, our findings uncover a novel function of TWIST1 in ovarian cancer. TWIST1 can regulate CXCL11, an IFN-inducible chemokine that recruits macrophages and facilitates tumor angiogenesis. Downregulation of CXCL11 can inhibit tumor angiogenesis, suggesting that anti-CXCL11 therapy may be an alternative treatment strategy for TWIST1-positive ovarian cancer. Additional studies are needed to further confirm the interaction between TWIST1 and CXCL11 in various types of cancer and to evaluate the therapeutic efficacy of anti-CXCL11 agents.

Acknowledgements

Y.J.K. wrote this paper. K.A.S. and U.S.J. participated in the laboratory procedures. K.J.M. statistically evaluated the data. T.J.K. critically revised the manuscript. J.H.H. revised the final manuscript. All authors have read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (grant number: NRF-2013 R1A1A2060431).

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