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RESEARCH ARTICLE

Thrombomodulin mediates the progression of epithelial ovarian cancer cells

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Abstract Thrombomodulin (TM), a natural anticoagulation factor, maintains circulation homeostasis in endothelial cells. TM has additional roles in modulating inflammation, thrombosis, and carcinogenesis. However, there is little information on the role of TM in the progression and metastasis of ovarian cancer. RNA silencing and cDNA expression vectors were used to manipulate target gene expression in ovarian cancer cells. Cell growth and migration were evaluated by an MTT assay, a wound-healing migration assay, a transwell migration assay, and a biosensor system. In this study, we found that TM silencing may enhance the growth rate of cells. The migratory ability of ovarian cancer cells was enhanced dramatically after

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TM silencing. TM overexpression in ovarian cells suppressed the proliferation and migration capability. Furthermore, we found that skov-3 cells treated with TM shRNA expressed high levels of fibronectin and vimentin and that the expression of these markers correlated positively with their migratory ability. Our results demonstrate that TM expression may regulate cell growth and migration in ovarian cancer cells. This finding suggests that TM may be a novel prognostic and therapeutic target for ovarian cancer.

Keywords Thrombomodulin · Ovarian cancer · Migration

Abbreviations

TM	Thrombomodulin
EMT	Epithelial-mesenchymal transition
EOC	Epithelial ovarian cancer

Introduction

Among the common gynecological cancers, ovarian cancer has the highest mortality worldwide [1]. Although the 5-year survival rate of stage I patients is greater than 90 %, it drops to 40 % in advanced stages [2]. Unfortunately, early diagnosis is a major difficulty in ovarian cancer due to the lack of specific symptoms and appropriate tumor markers [3, 4]. As a result, most ovarian cancers are diagnosed in later stages. In addition, even with surgical resection and combination chemotherapy, such as treatment with a platinum compound and a taxane, patients with late-stage ovarian cancer still suffer high recurrence rates [5]. Because invasive activity is involved in the malignant progression of cancer cells, understanding the mechanism of ovarian cancer cell migration will be helpful in the development of targeted therapies and prognostic markers to treat and detect advanced and recurrent ovarian cancer cases.

Ovarian cancer is a heterogeneous disease that is divided into three different subtypes: epithelial carcinomas, stromal carcinomas, and germ cell tumors [6]. Among these subtypes, epithelial ovarian cancer (EOC) makes up approximately 85 % to 95 % of all ovarian cancer cases [7, 8]. The invasive activity of epithelial tumor cells is based on collective cell migration or single-cell migration, such as mesenchymal-type movement [9]. This phenotypic conversion, termed the epithelial-mesenchymal transition (EMT), plays an important role in cancer metastasis. The loss of E-cadherin and increased expression of N-cadherin, fibronectin, and vimentin together modulate EMT in epithelial tumor cells and induce cell migration [10]. Although the expression levels of E- and N-cadherin, as well as those of fibronectin and vimentin, serve as biomarkers to identify the induction of EMT in cancer cells, the molecular signaling pathways modulating the expression of these markers remain unclear, particularly in EOC cells.

Thrombomodulin (TM), a natural anticoagulation factor that maintains circulation homeostasis in endothelial cells [11, 12], was recently found to have additional roles in modulating inflammation, thrombosis, and carcinogenesis [13, 14]. TM expression is detected in many tumor cells. Low or absent TM expression is associated with poor prognosis in some cancer types, including lung, oral, breast, and colorectal cancers [15–18]. Clinically, TM serves as a positive marker of mesothelioma and is widely used to discriminate between mesotheliomas and lung adenocarcinomas [19, 20]. Furthermore, Attanoos et al. have suggested that TM is also useful as a biomarker for differentiating between peritoneal epithelioid mesotheliomas and ovarian serous carcinomas [21]. In hepatocellular carcinoma (HCC) cells, the knockdown of TM decreases E-cadherin expression and enhances cell migration [22]. The loss of TM expression on tumor cells may correlate with the advanced metastases of tumor cells by modulating E-cadherin-related pathways.

TM expression and activation in tumor cells may serve as important modulators of EMT progression. Current literature indicates that TM reactivity is observed in approximately 4 % to 35 % of EOCs, most of which show weak to moderate reactivity [21, 23, 24]. However, it is unclear whether the loss of TM expression in EOC cells promotes the activation of EMT and invasive activity of EOC cells. The aim of the present study, therefore, was to clarify the role of TM in EMT progression in EOC cells and to investigate the invasive properties of these tumor cells.

Materials and methods

Chemicals, reagents, and cell cultures

Triton X-100, Tris–HCl, neomycin, Trypan Blue, EDTA, ribonuclease-A, and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Anti-TM and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The ovarian cell line (skov-3) was grown in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY, USA) containing 2 mM L-glutamine and 1.5 g/l sodium bicarbonate and supplemented with 10 % fetal bovine serum (Gibco BRL) and 2 % penicillin–streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin). The cells were cultured in a humidified incubator at 37 °C, 5 % CO_2.

Manipulation of TM expression

Knockdown of TM expression in ovarian cells The expression of TM was knocked down in ovarian cells using MISSION shRNA clones from Sigma Chemical Co. (St. Louis, MO, USA). The parental vector (pLKO.1-puro) may be used for either transient transfection or stable selection via

puromycin resistance. The target sequence for the human TM mRNA (NM_000361) was 5′-CTTGCTCATAGGCATCTC CATC-3′. The MISSION Non-target shRNA Control Vector (SHC002) was used as a scrambled control. The sequence of scrambled shRNA was 5′-CAACAAGATGAAGAGCACC AA-3′. The transfection protocol has been described previously [25–28]. In brief, 1×10^5 cells were washed twice with phosphate buffered saline (PBS) and mixed with 0.5 μg plasmid. We applied one pulse for 20 ms under a fixed voltage of 1.4 kV on a Neon pipette-type microporator (Invitrogen Life Technologies, Grand Island, NY, USA) [29, 30].

TM overexpression in ovarian cells The full-length TM cDNA was generated by polymerase chain reaction (PCR) and subcloned into the pCDNA3-topo-TM expression vector (Invitrogen Life Technologies, Grand Island, NY, USA). The full-length sequence was obtained by automatic sequencing (Applied Biosystems, Foster City, CA, USA). The pCDNA3topo-TM plasmid was transfected into skov-3 cells and selected by neomycin to obtain stably transfected cells. The TM expression levels were confirmed by real-time quantitative PCR and Western blotting as previously described [22, 29, 30].

Protein extraction and Western blot analysis

Protein abundance was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot as previously described. The cells were washed with cold PBS and lysed using cell lysis buffer containing protease inhibitors (Complete Protease Inhibitor Tablets, Boehringer Mannheim, Indianapolis, IN, USA). Equal amounts of proteins were separated by 10 % SDS-PAGE under reducing conditions and electrotransferred onto PVDF membranes (Bio-Rad Laboratories). The membranes were subsequently blotted with anti-TM or anti-actin antibodies, probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000), and visualized with enhanced chemiluminescence reagent (Amersham, Piscataway, NJ, USA) using a VersaDoc 5000 imaging system (Bio-Rad Laboratories) [27, 28].

RT-PCR and quantitative RT-PCR analysis

Total RNA was extracted from cells using the TRIZOL reagent according to the manufacturer's instructions (Invitrogen Life Technologies). cDNA was amplified from 2 μg of total RNA in a final volume of 20 μL using Moloney murine leukemia virus (M-MLV) reverse transcriptase at 37 °C for 90 min. For validation, real-time RT-PCR was performed using the Power SYBR Green Real-Time RT-PCR system and ABI StepOne™ detection system (Applied Biosystems, Foster City, CA, USA). The primers for real-time PCR were described in previous studies [27, 28]. The quantitative RT-PCR reaction was carried out using ABI SYBR Green Master Mix. Thermal cycling was performed in an ABI StepOne™ instrument (Applied Biosystems). The quantitative PCR was performed at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Cell proliferative ability

Cell proliferative ability was determined by an MTT assay and xCELLigence biosensor system. Cells were plated at 2×10^4 cells per well in 24-well plates and incubated overnight at 37 °C in 5 % CO_2. At different time intervals, the medium was aspirated. The remaining cells were incubated with 0.25 mg/ml of MTT for 1 h. MTT was extracted with DMSO, and the absorbance at 550 nm was measured using a spectrophotometer (GE Healthcare, Piscataway, NJ, USA). Experiments were conducted using the RTCA DP instrument (AECE Biosciences Inc. San Diego, CA) and growth curves were constructed using 16-well plates (E-plate 16, AECE Biosciences Inc.). Cells were seeded in an E-plate 16 at 10,000 cells/well in medium. The plate was then monitored once every 30 s for 4 h and once every half hour thereafter.

Determination of the migratory abilities of cells

Transwell migration assay In vitro cell migration assays were performed using BD Falcon cell culture inserts (BD Biosciences). An aliquot of 5×10^4 cells was suspended in 500-μL serum-free DMEM and seeded into the upper part of each chamber. The lower compartments were filled with 1 mL of DMEM supplemented with 10 % FCS. After incubation for 24 h at 37 °C in 5 % CO_2, the non-migrating cells were removed from the upper surface of the membrane by scrubbing. Cells on the reverse side were stained with 0.1 % crystal violet, and migrating cells were counted under a microscope at ×100 magnification.

xCELLigence biosensor system Experiments were carried out using an RTCA DP instrument (Roche Diagnostics GmbH, Germany) in a humidified incubator maintained at 37 °C in 5 % CO_2. Cell migration was assessed using specially designed 16-well plates (CIM-plate 16, Roche Diagnostics GmbH) with 8-μm pores. These plates are similar to conventional transwell plates, with the microelectrodes located on the underside of the membrane of the upper chamber. Medium supplemented with 10 % FCS was added to the lower chamber, and cells were seeded into the upper chamber at 20,000 cells/well in serum-free medium. The CIM-plate 16 was monitored every 10 s for 40 min and once every hour thereafter. Data analysis was carried out using the RTCA software 1.2 supplied with the instrument.

Wound-healing migration assay The wound-healing migration assay was performed using an ibidi Culture-Insert

system (ibidi GmbH, Martinsried, Germany) [31, 32]. In brief, 70 μL of cell suspension (7×10^5 cells/ml) was seeded into each well and incubated at 37 °C in 5 % CO_2 for 24 h. After the cells attached, the Culture-Insert was gently removed using sterile tweezers. The culture medium was replaced, and the migration of the cells was monitored under a microscope.

Statistical analysis

All experiments were repeated a minimum of three times. The data presented in some figures are from a representative experiment that was quantitatively similar to the replicate experiments. The Student's *t* test (two-tailed) was used for statistical comparisons between two groups of data sets.

Results

TM silencing increased the proliferation of skov-3 cells

We used TM-specific shRNA to silence TM expression in skov-3 cells and obtained stably transfected cells after 2 weeks of antibiotic selection. As shown in Fig. 1a, TM expression was reduced by over 85 % at both the transcriptional and

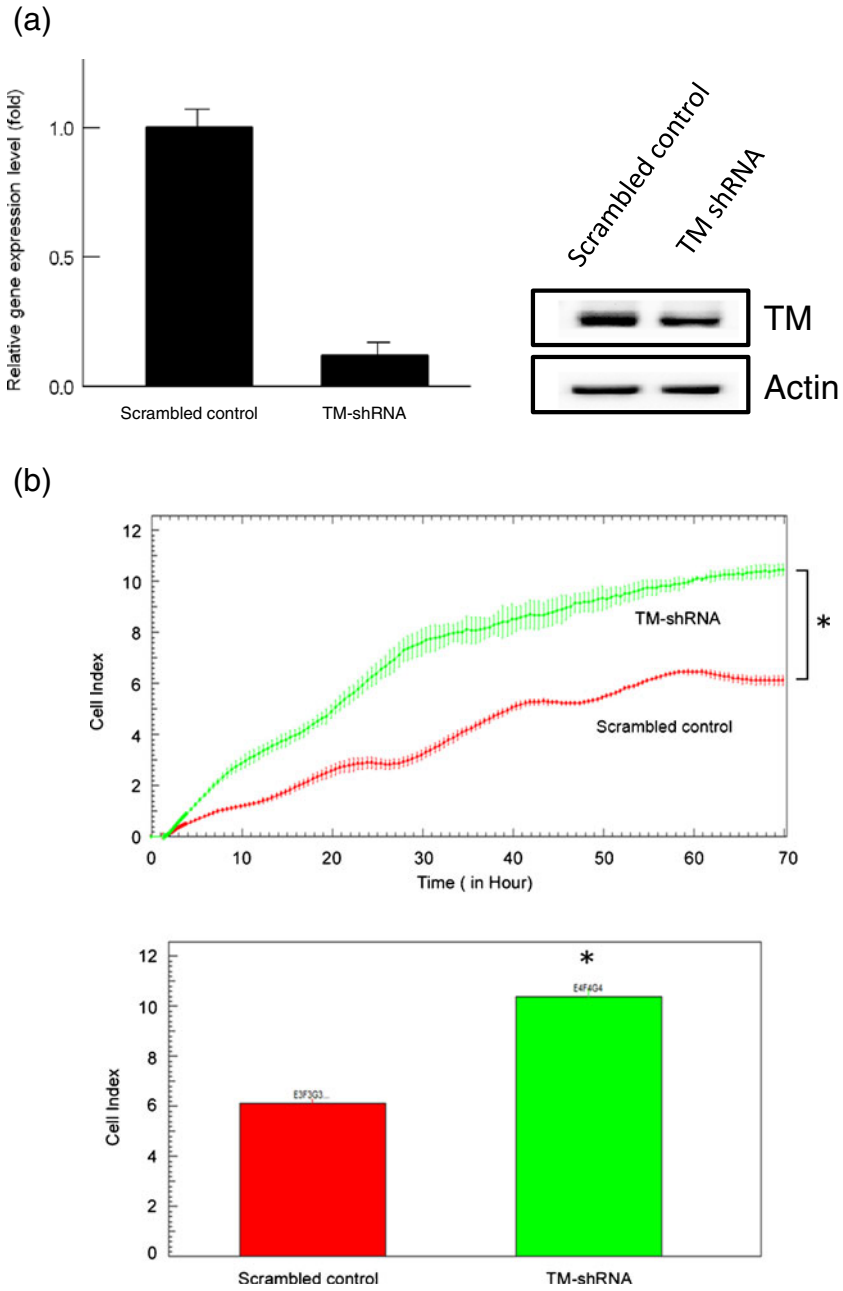

Fig. 1 Knockdown of thrombomodulin expression in ovarian cancer cells. Skov-3 cells were transfected with TM-shRNA or scrambled-shRNA (scrambled control). **a** Western blotting analysis revealed that the expression levels of TM were reduced by over 80 % in TM-shRNA skov-3 cells compared with scrambled control skov-3 cells. Total RNA was extracted from cells and analyzed by real-time quantitative PCR and Western blotting. GAPDH was used as an internal control. **b** The proliferative capacity of TM-shRNA and scrambled control skov-3 cells were determined by a biosensor system. Cell growth was reduced after TM silencing (*$p<0.05$)

translational levels in TM-shRNA-knockdown (TM-shRNA) cells compared with scrambled-shRNA-transfected (scrambled control) cells. We analyzed the cell growth in TM-shRNA cells and scrambled control cells using the xCELLigence biosensor assay system. As shown in Fig. 1b, cell growth is indicated by cell index. The growth rate of TM-shRNA skov-3 cells was higher than that of scrambled control cells. These results demonstrated that TM silencing increased the growth rate of skov-3 cells.

TM silencing increased the migratory ability of skov-3 cells

Cancer metastasis is a major problem in the management of cancer therapy. The effect of TM expression on metastasis in ovarian cancer was examined using a transwell migration assay. TM-shRNA and scrambled control cells were seeded with serum-free medium in the upper chamber of filter inserts. We found that cell migration was significantly increased after TM silencing by TM-shRNA in skov-3 cells (Fig. 2). To further verify the role of TM in migration, a wounding-healing migration assay was performed. As shown in Fig. 2c, the wound area was 71 % in scrambled control cells after 24 h. In contrast, the wound area was approximately 30 % in TM-shRNA skov-3 cells. These results indicate that silencing TM expression may enhance the migratory abilities of ovarian cancer cells.

TM overexpression suppressed proliferation in skov-3 cells

To confirm the role of TM in ovarian cancer progression, we cloned the TM gene into the expression vector pCDNA3topo and stably transfected the resulting plasmid (pCDNA3topo-TM) into skov-3 cells. The level of TM expression was determined by Western blotting. As shown in Fig. 3a, the expression levels of TM in skov-3 cells increased dramatically after transfection with pCDNA3topo-TM. In the MTT assay, we found that the cell growth rate was reduced 10 % in TM-overexpressing cells compared with vector control cells (Fig. 3b). Furthermore, we used the biosensor system to confirm that overexpression of TM in skov-3 cells decreases their proliferation (Fig. 3c).

TM overexpression suppresses the migration of skov-3 cells

We further measured the migratory ability of TM-overexpressing and vector control skov-3 cells using the xCELLigence system. As shown in Fig. 4, migration was decreased dramatically in TM-overexpressing skov-3 cells compared with that in vector control cells (Fig. 4). This result indicates that TM may play a suppressive role in the migration of cancer cells.

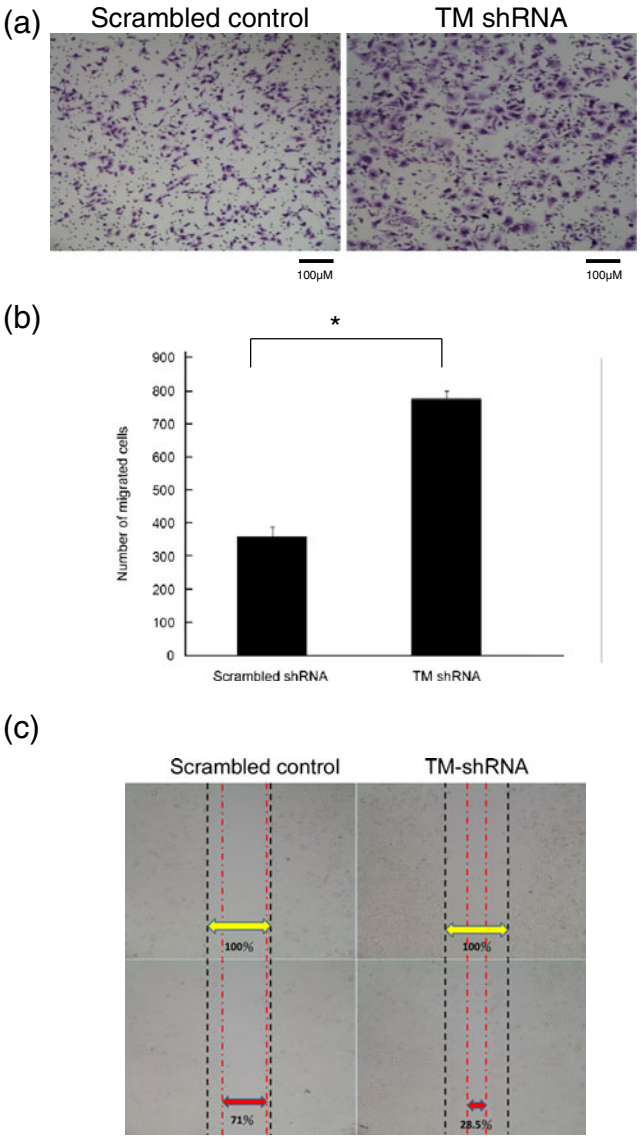

Fig. 2 Reduction of thrombomodulin expression enhances cancer cell migration. A transwell migration system was used to evaluate migratory ability. **a** The knockdown of TM expression in skov-3 cells resulted in enhanced migration in the transwell migration system. **b** Quantitation of the transwell migration assay. The *y*-axis represents the number of migrated cells. **c** A wound-healing migration assay was performed using the ibidi Culture-Insert system. The healing of the wound area was monitored under a microscope (*$p<0.05$)

TM expression modulates the expression of EMT biomarkers

Because EMT is a major pathologic event in cancer metastasis, and changes in EMT biomarker expression levels was correlated to migration [33], we further determined the EMT biomarker expression levels in scrambled control and TM-shRNA skov-3 cells. As shown in Fig. 5, TM-shRNA treatment did not affect the expression of E-cadherin or N-cadherin in the cells. However, the fibronectin and vimentin expression levels were increased in TM-shRNA skov-3 cells

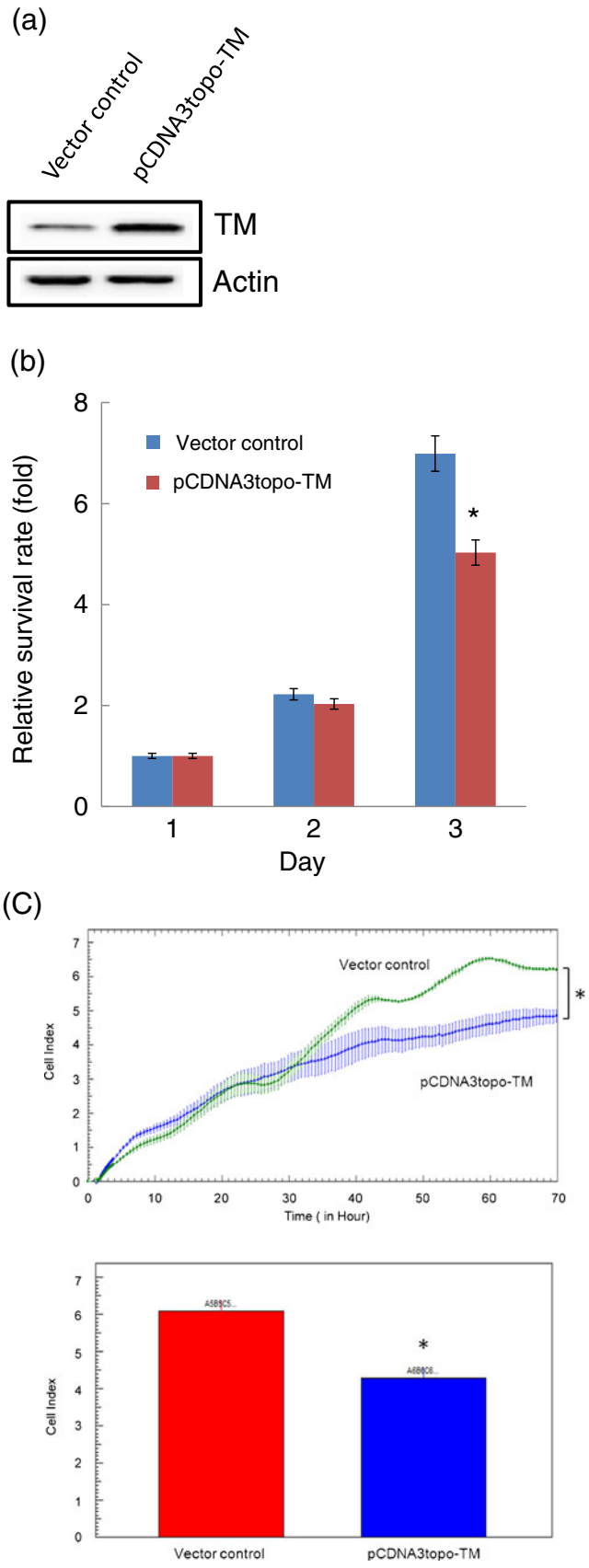

Fig. 3 Overexpression of thrombomodulin inhibits skov-3 cancer cell growth. Skov-3 cells were transfected with pCDNA3-topo or pCDNA3-topo-TM plasmids and selected by antibiotic resistance. **a** TM expression was evaluated by Western blotting. **b** The proliferation of TM-overexpressing and vector control skov-3 cells were determined by an MTT assay. **c** The growth of vector control and pCDNA3-topo-TM skov-3 cells was measured by the xCELLigence biosensor system. Proliferation was quantified using the RTCA software. The *y*-axis represents the cell index. All the experiments repeated at least three times independently (triplicated at each time; $*p<0.05$)

compared with scrambled control skov-3 cells, reflecting their enhanced migratory ability. These results suggest that TM expression may regulate migratory ability by modulating the expression of EMT biomarkers.

Discussion

Ovarian cancer is the leading cause of gynecologic cancer death in the USA. The majority of ovarian cancers (95 %) arise from epithelial cells; the remainder arise from other types of ovarian cells [34]. Our study demonstrated that knockdown of thrombomodulin (TM) enhanced the expression of fibronectin and vimentin but did not significantly change the expression of E-cadherin in epithelial ovarian cancer cells.

Recent evidence indicates that the EMT process plays a key role in tumor cell invasion and metastasis [33] and that the reduction of E-cadherin levels in tumor cells is a defining feature of EMT [35]. Clinical observations have also demonstrated that the absence of E-cadherin expression is associated with poor survival in ovarian cancer patients [36, 37]. Moreover, the activation of EMT-related transcription factors, such as snail, slug, twist-2, and zeb-2, have been found to induce chemoresistance in human ovarian cancer cells [38]. In the EMT process, TM functions as a negative regulator that maintains cell adhesion by promoting E-cadherin and β-catenin localization to cell–cell boundaries, and the loss of TM results in the disruption of cell adhesion, increased cell motility, and the transition to the mesenchymal phenotype in the human keratinocyte cell line HaCaT [22]. This pioneering study suggested that the presence of TM is critical for preventing EMT in normal human cells.

Fibronectin and vimentin are two important mesenchymal markers [39, 40]. Several studies have demonstrated that fibronectin is expressed by multiple types of cells and plays important roles in cell adhesion, migration, growth, and differentiation [41]. A previous study found that malignant ascites from ovarian cancer patients have higher expression levels of fibronectin compared with those in benign ascites [42]. High expression of fibronectin is also an indicator of poor prognosis in ovarian cancer patients who have higher

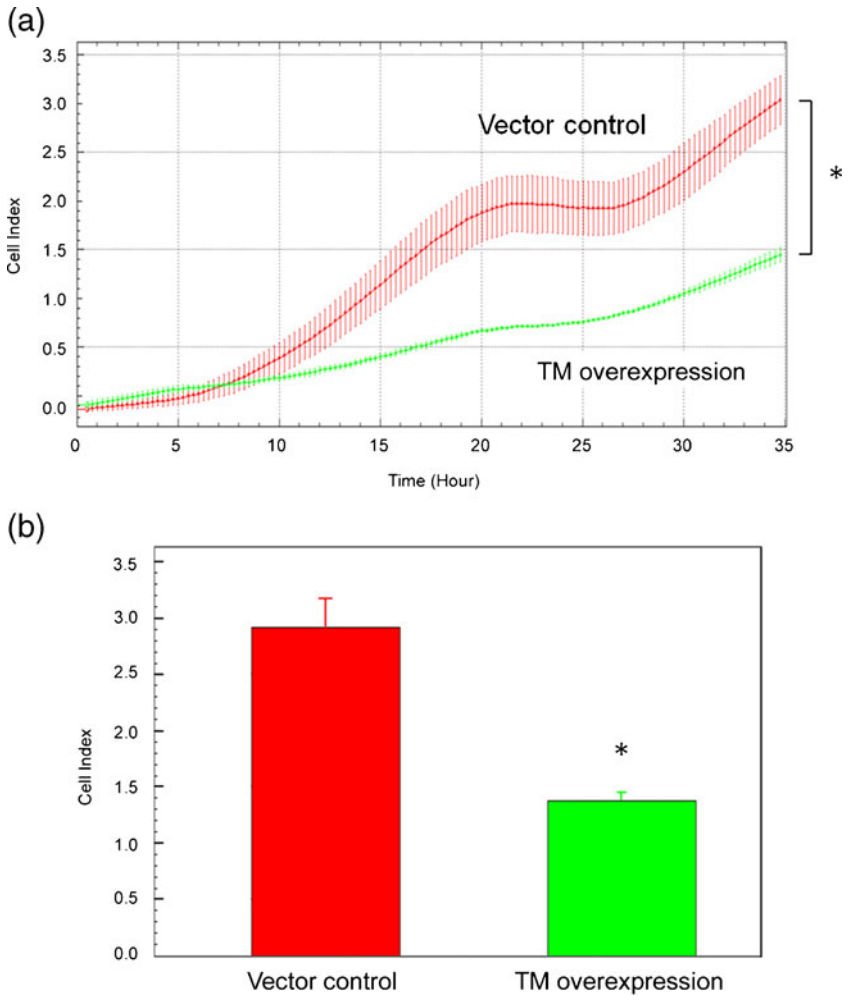

Fig. 4 Overexpression of thrombomodulin suppresses cancer cell migration. The xCELLigence biosensor system was used to measure the migration of skov-3 cells transfected with pCDNA3-topo or pCDNA3-topo-TM. **a** The biosensor system demonstrated that TM overexpression reduced the number of migrating skov-3 cells. **b** Cell migration was quantified using the RTCA software. The *y*-axis represents the cell index (*$p<0.05$)

chances of tumor recurrence[43]. A proteomic study has demonstrated that vimentin is a potential biomarker that can identify subgroups of women with polycystic ovarian syndrome who have an increased risk of ovarian cancer [44]. Vimentin plays a significant role in cisplatin resistance in ovarian cancer [38]. Additionally, paclitaxel-resistant epithelial ovarian cancer cells

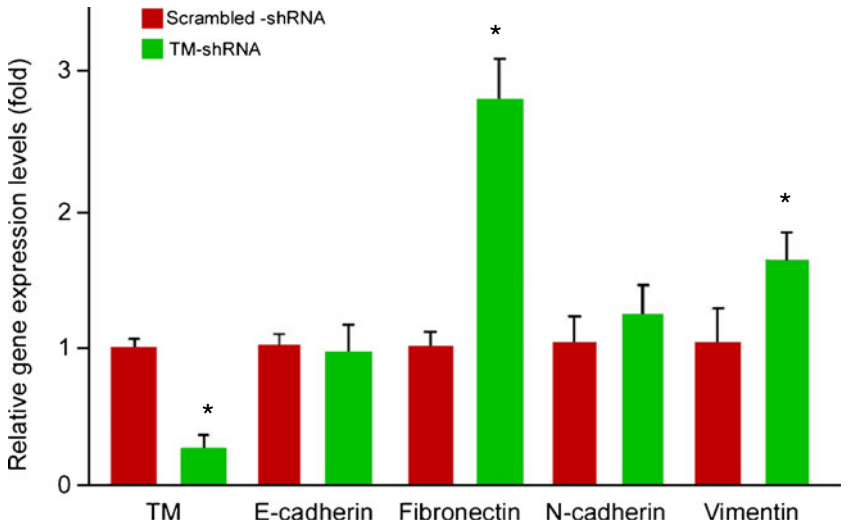

Fig. 5 Thrombomodulin modulates the expression of EMT biomarkers. The expression levels of several EMT biomarkers (E-cadherin, N-cadherin, fibronectin, and vimentin) were determined in TM-shRNA and scrambled control skov-3 cells by quantitative PCR. The knockdown of TM altered fibronectin and vimentin expression levels (*$p<0.05$)

have higher migratory abilities and display higher expression of fibronectin and vimentin [45].

Despite the difficulty of early diagnosis, the main challenge in treating advanced ovarian cancer cases is that the majority of cases remain incurable due to recurrence and chemoresistance [46, 47]. This critical problem has resulted in high mortality rates among patients. Because ovarian carcinogenesis involves several different pathways, including the BRAF/KRAS and TP53 pathways [48], biomarkers capable of differentiating between specific ovarian cancer cells will not only help to select suitable chemotherapeutic regimens with higher response rates but will also improve the sensitivity and specificity of the diagnosis of early ovarian cancer. Moreover, biomarker-based identification of chemoresistant ovarian cancer cells—particularly those with an EMT phenotype—and a clearer understanding of the molecular mechanisms of chemoresistance will improve the development of targeted molecular therapeutic regimens for eliminating resistant cancer cells.

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Conflicts of interest None

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