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RESEARCH ARTICLE

Thrombomodulin mediates the migratory ability of hormone-independent prostate cancer cells through the regulation of epithelial-to-mesenchymal transition biomarkers

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Abstract Thrombomodulin (TM) is highly expressed in endothelial cells and plays the key role in maintaining physical homeostasis. In addition, many pieces of evidence also show that TM contains the diagnostic value for malignant diseases. TM has been found to correlate with metastatic status in multiple cancers, but its role in prostate cancer progression remains unclear. TM expression was determined in prostate cancer cells (DU-145 and PC-3 cells) using real-time PCR and Western blotting. TM expression was manipulated in prostate cancer cells using TM-specific shRNA and an overexpression system. The proliferation, adhesion, and migratory ability of prostate cancer cells expressing various TM levels were determined using the x'Celligence biosensor system and a transwell migration assay. Higher levels of TM transcription and translation were found in DU-145 cells and were negatively correlated with the low migratory ability of DU-145 cells. After silencing TM expression in DU-145 cells, cell growth decreased, but cell adhesion and migration dramatically increased. TM overexpression in PC-3 cells reduced their metastatic ability. We investigated the possible mechanisms of this phenomenon and determined that the enhanced cell migration was mediated through the expression of E-cadherin and vimentin. TM may be a modulator of hormone-independent prostate cancer (HIPC) metastasis. The downregulation of TM expression enhanced the migratory ability of these cells via an increase in vimentin expression and a decrease in E-cadherin expression.

Keywords Thrombomodulin (TM) · Prostate cancer · Migration · Epithelial-to-mesenchymal transition (EMT)

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Introduction

Thrombomodulin (TM), a transmembrane glycoprotein, plays a crucial role in embryonic development and maintaining normal endothelial cell function in adults [1]. Several studies have reported that TM expression levels in cancer specimens may correlate with patient status [2–4]. Decreases in TM levels are correlated with tumor invasiveness in various cancers [2–4]. In addition, TM expression levels modulate cell migratory ability through the epithelial-to-mesenchymal transition (EMT) pathway [5, 6]. The soluble form of TM substantially stimulates mouse mammary tumor invasion in a dose-dependent manner [7]. Recent reports have suggested that TM regulates cancer migration through EMT biomarkers (E-cadherin, snail, and ZEB-1) [5, 6]. Immunostaining results from urological cancers have shown that TM levels may correlate with cancer malignancy [8–10].

Prostate cancer is the second most frequent cause of male cancer-related death in the USA [11, 12]. Hormone ablation therapy is an effective therapeutic strategy among early-stage prostate cancer patients [13]. However, recurrent prostate cancer may develop into a hormone-independent prostate cancer (HIPC) after hormone ablation therapy [13, 14]. At present, the response of HIPC to chemotherapy is short-lived, and those with progressive disease exhibit limited survival [13, 14]. One third of the patients who exhibit localized disease at presentation progress to metastasis [11], and prostate cancer metastasis is an obstacle to clinical therapy. However, the role of TM in prostate cancer progression and metastasis remains unclear. In this study, we found that a decrease in the TM expression enhanced the migratory ability of these cells via increasing vimentin expression and decreasing E-cadherin expression. TM may be a modulator of HIPC metastasis.

Materials and methods

Chemicals, antibodies, and cell cultures

Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Antibodies (anti-TM, anti-E-cadherin, anti-vimentin, and anti-β-actin) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The prostate cancer cell lines (PC-3 and DU-145 cell) were purchased from American Type Culture Collection (ATCC) and grown in RPMI Media 1640 (Life Technologies, Grand Island, NY) supplemented with 10 % (*v*/*v*) fetal calf serum in a humidified incubator at 37 °C and 5 % CO_2.

RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen Life Technologies) [15]. Two micrograms of total RNA was transcribed to complementary DNA (cDNA) in a 20-μL reaction vessel, using Moloney murine leukemia virus (M-MLV) reverse transcriptase at 37 °C for 90 min. The primer sequences used in quantitative PCR were as previously described [15, 16]. The quantitative reverse transcriptase (RT)-PCR reaction was conducted using a commercial SYBR Green reaction mix and performed using an ABI StepOne™ system (Applied Biosystems) under the previously described conditions [15, 17].

Western blot analysis

The control and treated cell lysates were separated using 10 % SDS-PAGE under reducing conditions and electrotransferred onto PVDF membranes (GE HealthCare, Piscataway, NJ). These membranes were subsequently blotted using anti-TM, anti-E-cadherin, anti-vimentin, and anti-actin antibodies, followed by horseradish-peroxidase-conjugated secondary antibodies (1:5,000). They were then visualized using an enhanced chemiluminescence reagent (GE Healthcare, Piscataway, NJ) and detected using a VersaDoc 5000 (Bio-Rad Laboratories, Hercules, CA) [18].

Silencing of TM in DU-145 cells

The expression of TM in DU-145 cells was ablated using MISSION short hairpin (shRNA) clones from Sigma Chemical Co. (St. Louis, MO). MISSION shRNA clones are sequence-verified shRNA lentiviral plasmids used for gene silencing in mammalian cells. The parental vector (pLKO.1-puro) allows for transient transfection or stable selection through puromycin resistance. The target sequence for the human TM mRNA (NM_000361) gene was 5′-CTTGCTCA TAGGCATCTCCATC-3′. The MISSION nontarget shRNA control vector (SHC002) was used as a scrambled control, and the sequence of the scrambled shRNA was 5′-CAACAAGA TGAAGAGCACCAA-3′. The transfection protocol has been previously described [19, 20]. Briefly, 1×10^5 cells were washed two times with phosphate-buffered saline (PBS) and mixed with 0.5 μg of plasmid. One pulse was applied for 20 ms at a fixed voltage of 1.4 kV on a Neon pipette-type microporator (Invitrogen Life Technologies, Grand Island, NY) [16]. The stably transfected cells were selected using antibiotics.

Overexpression of TM in PC-3 cells

The TM cDNA was amplified using PCR and a TM-specific primer and then ligated into the pcDNA3TOPO expression vector (Invitrogen Life Technologies, Grand Island, NY). Full-length TM sequences were confirmed based on autosequencing. The pcDNA3TOPO-TM plasmid was transfected into PC-3 cells and selected using neomycin to

obtain stably transfected cells. The TM expression levels were confirmed using real-time quantitative PCR and Western blotting, as previously described [6, 16, 21].

Cell proliferation assay using the x'Celligence biosensor system

Experiments from a previous study were modified [18] and executed using a real-time cell analyzer (RTCA) dual plate (DP) instrument (Roche Diagnostics GmbH, Germany). Growth curves were constructed using 16-well plates (E-plate 16, Roche Diagnostics GmbH). Cells were seeded in an E-plate 16 at 5,000 cells/well in fetal calf serum (FCS)-containing medium; the plate was monitored once every 30 s for 4 h and then once every half hour. The data analysis was conducted using RTCA software, version 1.2.

Adhesion assay

Adhesion assays were performed by modifying the method of Deryugina et al. [22]. A 300-μL aliquot of fibronectin (5 μg/mL) was added to each well of 24-well plates. The plates were allowed to stand overnight at 4 °C and were then washed with PBS, blocked with 1 % BSA in PBS for 1 h at 37 °C, and washed for the final time in PBS. After seeding with cell suspensions and incubating for 60 min at 37 °C, the nonadherent cells were removed by washing with PBS, and the adherent cells were fixed and stained with 0.2 % crystal violet in 10 % ethanol for 10 min. The cells were counted under a microscope (Olympus IX71, Japan).

Cell migratory ability determination

The in vitro cell migration assay was performed using an 8-μm BD Falcon™ Cell Culture Insert (BD Biosciences) [18]. Aliquots of 1×10^5 cells were seeded into the upper compartments of each chamber; the lower compartments were filled with RPMI with 10 % FCS. After 24 h of incubation, the nonmigrating cells were removed from the upper surface of the membrane. The migrated cells were stained and counted under a microscope (Olympus IX71, Japan).

Cell migration assayed with the x'Celligence biosensor system

Experiments were conducted using an RTCA DP instrument (Roche Diagnostics GmbH, Germany) that was placed in a 5 % CO_2 humidified incubator maintained at 37 °C, as

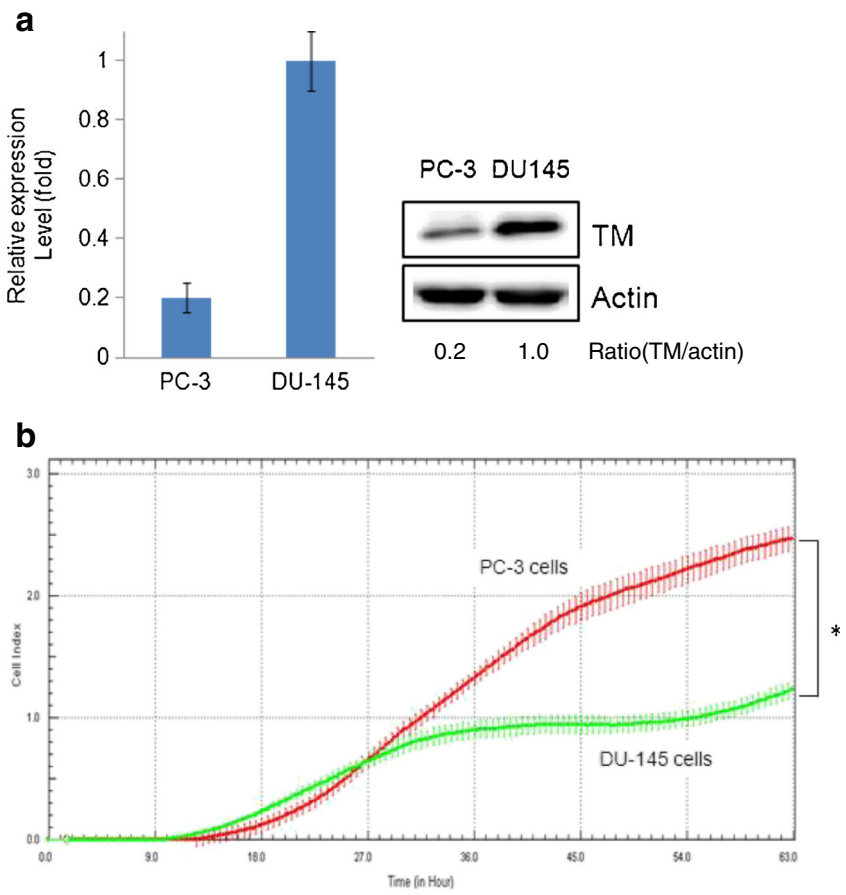
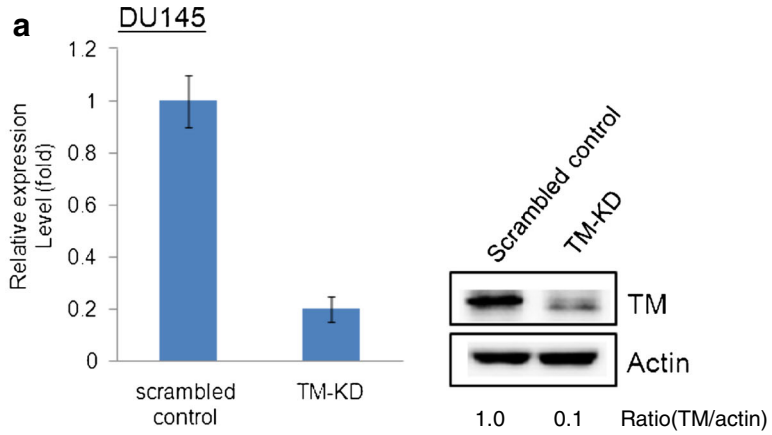
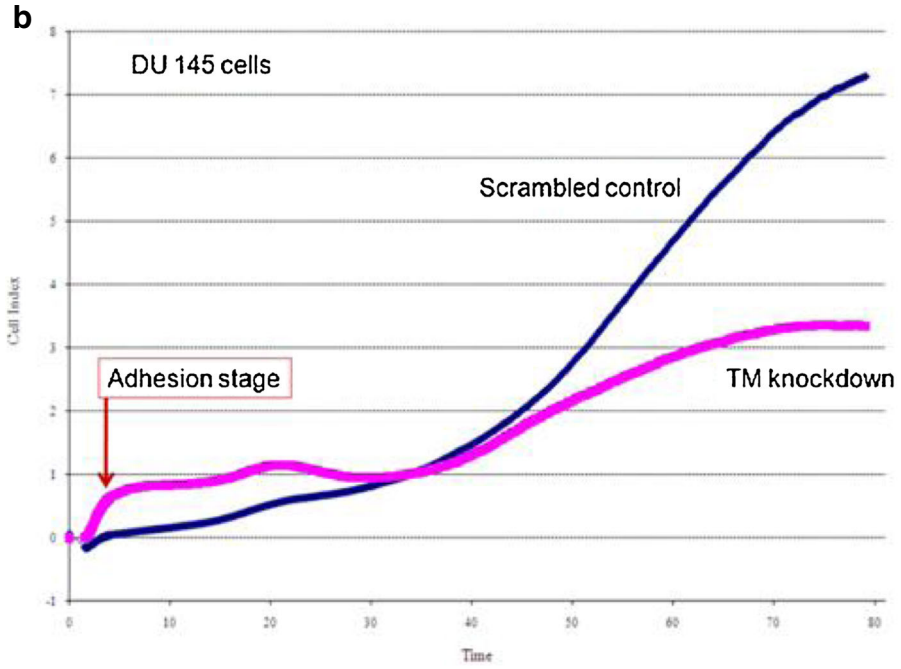


Fig. 1 Thrombomodulin expression levels in prostate cancer cells. Total RNA was extracted from DU-145 and PC-3 cells and analyzed using quantitative PCR and Western blotting. Actin was used as an internal control. **a** Thrombomodulin was identified in both DU-145 and PC-3 cells. DU-145 cells expressed a higher level of thrombomodulin than what the PC-3 cells did. **b** The migratory abilities of the DU-145 and PC-3 cells were determined using an x'Celligence biosensor system. The number of cells is represented as the cell index (*y*-axis). The experiments were independently performed three times in triplicate; **P*<0.05


a
DU145
Relative expression Level (fold)
1.2
1
0.8
0.6
0.4
0.2
0
scrambled control
TM-KD
Scrambled control
TM-KD
TM
Actin
1.0
0.1
Ratio(TM/actin)


b
DU 145 cells
Scrambled control
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TM knockdown
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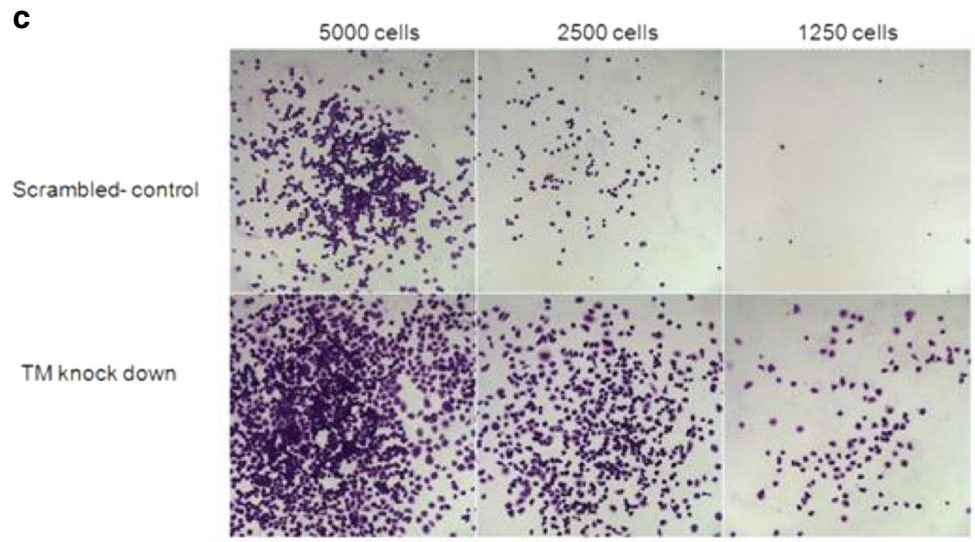
c
5000 cells
2500 cells
1250 cells
Scrambled- control
TM knock down

◀ **Fig. 2** A decrease in thrombomodulin levels influences proliferation and adhesion. TM expression was silenced by TM-shRNA knockdown (TM-KD) and scrambled shRNA (scrambled control) in DU-145 cells. **a** The TM expression levels of TM-KD and scrambled control DU-145 cells were determined by real-time PCR and Western blotting. **b** The proliferation abilities of TM-KD and scrambled control DU-145 cells were determined using an x'Celligence biosensor system. The number of cells is represented as the cell index (*y*-axis). **c** The adhesive abilities of TM-KD and scrambled control DU-145 cells were examined using fibronectin-coated 96-well plates. Adhered cells were counted under a microscope. All the experiments were independently repeated at least three times

previously described [17, 18]. Cells (20,000 cells/well) were seeded into the upper chamber of a cell invasion/migration (CIM)-plate 16 in serum-free medium (4 wells/sample). The CIM-plate 16 was monitored every 10 s for 40 min and once every hour thereafter. The data were analyzed using RTCA software 1.2 (supplied with the instrument).

Statistical analysis

All the experiments were repeated in a minimum of three times. All the data collected from the real-time RT-PCR and cell proliferation and migration assays were expressed as mean±standard deviations. Statistical significance was determined using Student's *t* test (two-tailed) to compare two groups of data in Microsoft Excel.

Results

Expression pattern of TM in prostate cancer cells

TM is a membrane protein with multiple functions in the maintenance of circulation homeostasis and the inhibition of the progression of malignant diseases. Previous reports have demonstrated that TM directly regulates the metastatic ability of various cancers [5, 6]. However, there is little information regarding the role of TM in HIPC. To elucidate this role, we performed experiments using androgen receptor-negative cells (DU-145 and PC-3 cells) and analyzed the expression of TM in prostate cancer cells. As shown in Fig. 1a, TM levels were detected during transcription and translation in both DU-145 and PC-3 cells. TM was more highly expressed in DU-145 cells compared with PC-3 cells. The migratory ability of DU-145 and PC-3 cells was determined using the x'Celligence biosensor system. As shown in Fig. 1b, the migratory ability of PC-3 cells was greater than that of the DU-145 cells. These results suggest that the TM expression level is negatively correlated with the migratory ability of cells.

Silencing of TM expression in DU-145 cells by shRNA

To further examine the role of TM in cell migration, we introduced TM-specific shRNA to silence TM expression in DU-145 cells and analyzed the stably transfected cells after screening with an antibiotic for 2 weeks. As shown in Fig. 2a, both the transcriptional and translational expression levels of TM were reduced by greater than 85 % in the TM-shRNA knockdown (TM-KD) cells compared with the scrambled shRNA-transfected (scrambled control) cells.

Silencing TM expression suppressed the proliferation of DU-145 cells

We analyzed the proliferation ability of TM-KD cells and scrambled control cells using a biosensor system. The results demonstrated that silencing TM inhibited DU-145 cell proliferation (Fig. 2b). However, adhesion increased in TM-KD cells. We confirmed this result using an adhesion assay and determined that the adhesion ability was increased in TM-KD cells compared with the scrambled control cells (Fig. 2c).

Silencing TM expression increased the migratory ability of DU-145 cells

Currently, metastasis is an important problem in the management of cancer therapy. We examined how TM expression influenced metastasis in prostate cancer using a transwell migration assay. TM-KD and scrambled control cells were seeded in the upper chamber of filter inserts with serum-free medium. We determined that the migratory ability was substantially increased after TM silencing by TM-shRNA in DU-145 cells (Fig. 3a). This finding indicates that TM expression plays a modulating role in prostate cancer metastasis.

TM overexpression suppressed the migratory ability of PC-3 cells

To confirm the role of TM in cancer migration, we cloned the TM gene into an expression vector (pcDNA3TOPO) and electroporated pcDNA3TOPO-TM into PC-3 cells to transiently overexpress TM. The TM expression levels were determined using real-time PCR and Western blotting. The migration abilities of the control and TM-overexpressing PC-3 cells were determined using a transwell migration assay. As shown in Fig. 3b, the number of migrated cells was dramatically decreased in TM-overexpressing PC-3 cells in the transwell migration assay system. This finding suggests that TM suppresses cancer migration.

TM expression mediated migration through EMT biomarkers

Because EMT is a major pathologic event in cancer metastasis and changes in EMT biomarker expression levels were correlated with migration, we determined the expression levels of

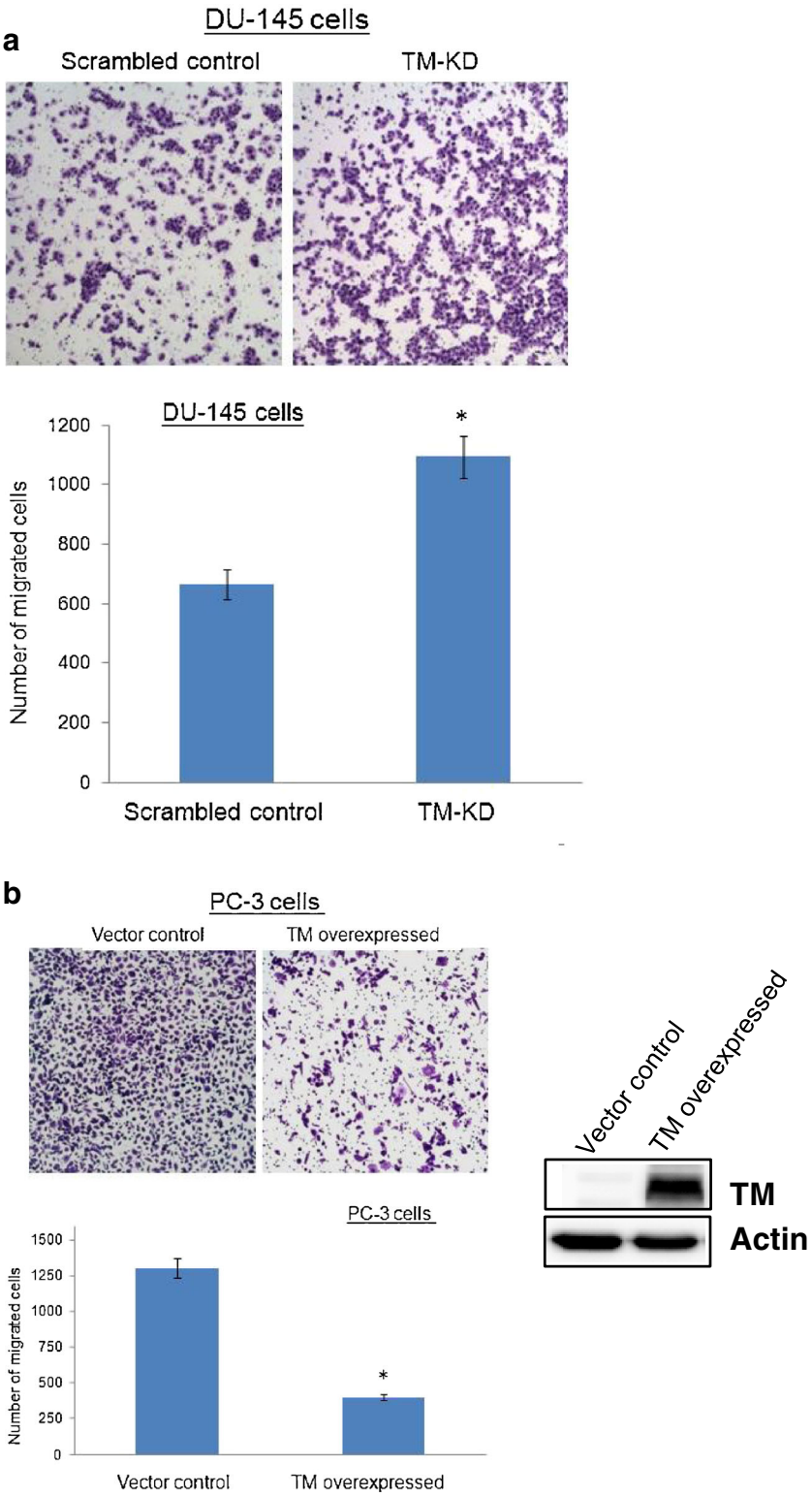

Fig. 3 Thrombomodulin expression levels influence cancer migratory ability. A transwell migration system was used to evaluate migratory ability. **a** The migratory ability of TM-KD and scrambled control DU-145 cells was determined by transwell migration assay. **b** TM was overexpressed in PC-3 cells, and the level of TM in vector control and TM overexpressed cells was determined by Western blotting. The migratory ability of vector control and TM overexpressed cells was determined by transwell migration assay. The *y*-axis represents the number of migrated cells. All the experiments were independently repeated at least three times; **P*<0.05

EMT biomarkers in scrambled control and TM-KD DU-145 cells. As shown in Fig. 4a, the TM-KD cells exhibited decreased E-cadherin expression and increased vimentin expression compared with the scrambled control DU-145 cells. By contrast, TM overexpression in PC-3 cells increased E-cadherin expression and decreased vimentin expression (Fig. 4b), reflecting a decrease in migratory ability. These results suggest that TM expression mediates migratory ability by affecting E-cadherin and vimentin expression.

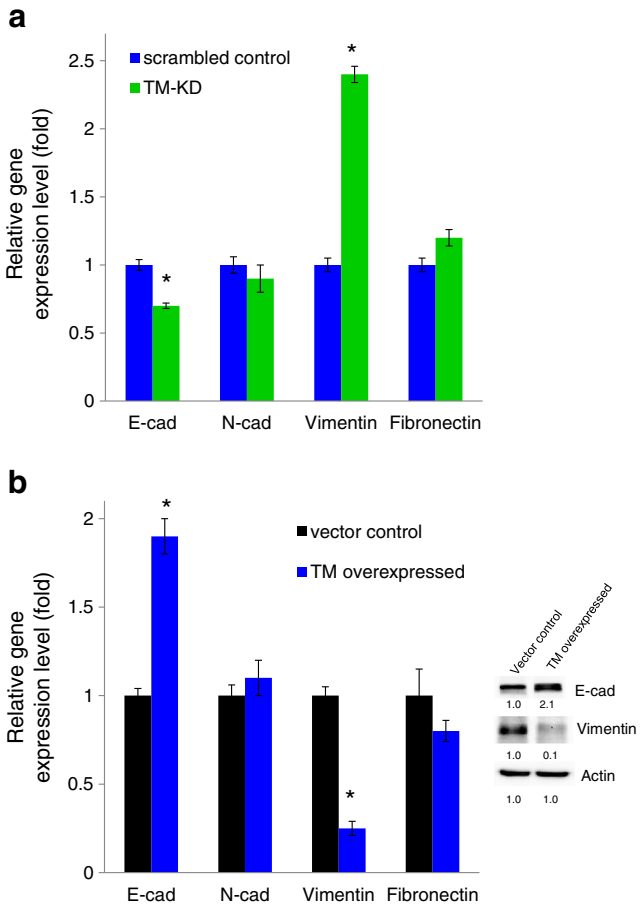

Fig. 4 Thrombomodulin mediates cell migratory ability through EMT biomarkers. The expression levels of EMT biomarkers (E-cadherin, N-cadherin, fibronectin, and vimentin) were determined using quantitative PCR and Western blotting in **a** TM-KD and scrambled control DU-145 cells and **b** TM overexpressed PC-3 cells. TM suppression altered E-cadherin and vimentin expression. *E-cad* E-cadherin, *N-cad* N-cadherin

Discussion

Prostate cancer is a critical worldwide health concern. Currently, the most crucial clinical problem in cancer therapy is metastasis. To improve therapeutic efficacy, additional information regarding cancer metastasis is required to develop prognostic and therapeutic strategies for preventing or curing metastasis. TM is an anticoagulant protein that is expressed in numerous tissues and is a valuable diagnostic and therapeutic molecule in cardiovascular diseases [23]. High TM expression in cancer specimens may also correlate with benign tumors [3, 24], suggesting that TM may be a good prognostic marker for cancers. In addition, TM may also influence the proliferation and metastasis of different cancers [6, 25, 26]. However, the role of TM in the progression of prostate cancer is unclear. Here, we demonstrated that TM expression was negatively correlated with the migratory ability of DU-45 and PC-3 cells (Fig. 1). TM silencing in DU-145 cells dramatically enhanced their migration (Fig. 3a). In addition, TM overexpression suppressed the migratory ability of PC-3 cells (Fig. 3b), suggesting that TM expression levels modulate the migratory ability of prostate cancer cells. This result is consistent with a previous report on hepatocellular carcinoma [5, 6]. To understand the relation between TM and prostate cancer migration, we analyzed the expression levels of the EMT-related biomarkers E-cadherin, N-cadherin, vimentin, and fibronectin. We determined that the TM expression level did not influence the expression levels of N-cadherin and fibronectin. However, TM silencing increased vimentin expression and decreased E-cadherin expression in prostate cancer cells (Fig. 4). Exactly how TM participates in the metastatic pathway is unclear; however, once this role is clearly defined, inducers of TM expression may yield potential therapies to control tumor behavior and impede the transendothelial spread of cancers.

Store-operated Ca(2+) entry (SOCE) is a main Ca(2+) influx pathway controlling the intracellular Ca(2+) concentration, and Ca(2+) influx is involved in cancer progression and metastasis. Stromal interacting molecule (STIM) 1 acts as a sensor for the level of Ca(2+) stored in the endoplasmic reticulum, and Orai1 protein constitutes the pore-forming subunit of the store-operated channels. STIM1 and Orai1 are critical for the proliferation, metastasis, and angiogenesis of different types of cancer, including breast cancer, liver cancer, colon cancer, and cervical cancer [27–32]. Wang et al. have also demonstrated that SOCE is involved in EGF-mediated signal activation, which induces inflammatory cascades and, eventually, CRC tumorigenesis [27]. However, the role of TM in the regulation of cancer metastasis is unclear, although it may be linked to the SOCE molecules. This hypothesis may be worth further examination. In addition, the role of TM in mediating cancer migratory ability has been reported in several cancers [25, 26]. A recent study has also demonstrated that plasminogen is a likely ligand for TM [33]. Collectively, the findings in the literature and the present study indicate that TM may be a potential target for preventing cancer metastasis.

In conclusion, our findings suggest that TM modulates the migratory ability of HIPC cells by regulating the expression of E-cadherin and vimentin. This study may provide new insight into developing a prognostic or therapeutic strategy to combat HIPC metastasis.

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Conflicts of interest None

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