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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Knockdown of Thrombomodulin Enhances HCC Cell Migration through Increase of ZEB1 and Decrease of E-cadherin Gene Expression

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ABSTRACT

Background. Thrombomodulin (TM) is a key molecule mediating circulation homeostasis through its binding to thrombin. The TM–thrombin complex can activate protein C and thrombin-activatable fibrinolysis inhibitor to form a tight clot. In many cancer tissues, decrease of TM expression may correlate with cancer metastasis. However, the role of TM in hepatocellular carcinoma (HCC) progression is still unclear.

Methods. We characterized TM expression in HCC cells (HepJ5 and skHep-1 cells) using real-time polymerase chain reaction (PCR) and Western blotting. We then manipulated TM expression using both TM-specific short hairpin RNA (shRNA) and overexpressing it in HCC cells. Transwell migration assay was performed to monitor the migratory ability of HCC cells under different levels of TM expression.

Results. We found that TM was ectopically highly expressed in skHep-1 at both transcriptional and translational levels. After silencing TM expression in skHep-1 cells, we found that metastatic capability was dramatically increased. Conversely, overexpression of TM in HepJ5 cells decreased metastatic ability. We investigated the possible mechanism and found that decreased TM-mediated enhancement of cell migration was dependent on upregulation of ZEB1, a repressor of E-cadherin.

Conclusions. TM may be a modulator of cancer metastasis in HCC. Downregulation of TM expression may increase ZEB1 and decrease E-cadherin levels.

Hepatocellular carcinoma (HCC) is a cancer that is prevalent worldwide, particularly in East Asia.[1–3] Although there have been some advancements in diagnosis and treatment of HCC, the mortality rate remains high.[2,3] Prognosis of HCC is heavily dependent on tumor stage at time of diagnosis. In early-stage cancer, curative hepatic resection and partial ablation therapy are possible.[4,5] However, disease recurrence and metastasis after treatment remain major obstacles and contribute to poor prognosis. Therefore, better understanding of the components that modulate tumor metastasis is crucial to improving HCC therapy.[6] Metastasis is a complex process that involves many factors.[7] Several studies have indicated that epithelial–mesenchymal transition (EMT) is found in the most aggressive metastatic cancer cells[8–13] A key feature in initiation and execution of EMT is downregulation of E-cadherin expression, for which several mechanisms have been reported.[8–10,14–16]

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Thrombomodulin (TM) is highly expressed in endothelial cells and acts as a natural anticoagulation factor in maintaining circulation homeostasis.[17,18] Previously, it was thought that the function of TM was limited solely to modulating coagulation.[18] However, recently TM has been shown to be an interesting molecule with many other functions, including in inflammation, thrombosis, and carcinogenesis.[19,20] TM can be detected on the surface of many tumor cells. It is also thought to be involved in the hemostatic shift that occurs locally around malignant tumors, although the mechanism of this process is still under investigation.[21–25] Further studies have shown that there is a role for TM as a diagnostic, therapeutic, and prognostic factor in tumors. An immunohistochemical and clinicopathological study of TM in 141 cases of resected HCC indicated that TM may inhibit adhesion of tumor cells and prevent the spread of intrahepatic metastasis.[21] The anticancer effects of soluble-form TM has been identified in many different cancers, and soluble-form TM significantly stimulates mouse mammary tumor invasion in a dose-dependent manner.[26–30] These studies indicate that soluble- and membrane-form TM may act differently in cancer progression.

Previous studies focused on elucidating thrombin and thrombin-receptor-dependent TM function; however, the role of TM independent of thrombin and its receptor is still unclear. We believe that understanding the role of TM in HCC will allow the development of future therapeutic targets.

MATERIALS AND METHODS

Chemicals, Antibodies, and Cell Culture

Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Antibodies [anti-TM, and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH)] were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The HCC cell lines (Hep J5 and skHep-1) were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY, USA) supplemented by 10% (v/v) fetal calf serum (FCS) in 5% CO_2 humidified incubator at 37°C.

RNA Extraction and Quantitative Real-Time PCR Analysis

Total RNA was extracted using the Trizol reagent according to the manufacturer's instructions (Invitrogen Life Technologies) as described previously.[31] Two micrograms total RNA was transcribed to complementary DNA (cDNA) in a 20 µl reaction using Moloney murine leukemia virus (M-MLV) reverse transcriptase at 37°C for 90 min. The quantitative PCR primer sequences of thrombomodulin were: forward: 5′-ACCTTCCTCAATG CCAGTCAG-3′; reversed: 5′-GCCGTCGCCGTTCAGTA G-3′. The primers of E-cadherin (forward: 5′-CGGGAATG CAGTTGAGGATC-3′; reversed: 5′-AGGATGGTGTAA GCGATGGC-3′), ZEB1 (forward: 5′-AGCAGTGAA AGA GAAGGGAATGC-3′; reversed: 5′-GGTCCTCCTCAGG TGCCTCAG-3′), and β-actin (forward: 5′-AGCGCGGCT ACAGCTTCA-3′; reversed: 5′-GGCCATCTCTTGCTC-GAAGT-3′) were the same as previously described.[31] The quantitative RT-PCR reaction was carried out using a commercial SYBR Green reaction mix. Thermal cycling was performed using ABI StepOne^{TM} equipment (Applied Biosystems), and the quantitative PCR conditions were: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Protein Extraction and Western Blot Analysis

Cells were washed with cold phosphate-buffered saline (PBS) and lysed in culture dishes using cell lysis buffer [40 mM Tris-HCl, pH 7.4, 10% glycerol, 50 mM BGP, 5 mM ethylene glycol tetraacetic acid (EGTA), 2 mM ethylenediamine tetraacetic acid (EDTA), 0.35 mM vanadate, 10 mM NaF, and 0.3% Triton X-100] containing protease inhibitors (Complete Protease Inhibitor Tablets; Boehringer Mannheim, Indianapolis, IN). Equal amounts of protein (determined using Bio-Rad detergent compatible protein assays) from control and treated cell lysates were separated by 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) under reducing conditions, and electrotransferred onto polyvinylidene diflouride (PVDF) membranes (Bio-Rad Laboratories), which were subsequently blotted using anti-TM (1:1,000), anti-GAPDH (1:5,000) antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000), visualized with enhanced chemoluminescence reagent (Amersham, Piscataway, NJ), and detected by VersaDoc 5000 (Bio-Rad Laboratories).

Manipulating TM Expression in HCC Cells

Silencing Thrombomodulin Expression in skHep-1 Cells We ablated thrombomodulin expression in skHep-1 cells using MISSION shRNA clones purchased from Sigma Chemical Co. (St. Louis, MO). MISSION shRNA clones are sequence-verified shRNA lentiviral plasmids for gene silencing in mammalian cells. The parental vector (pLKO.1 ← puro) allows for transient transfection or stable selection via puromycin resistance. The target sequences for the human thrombomodulin mRNA (NM_000361) gene was 5′-CTTGCTCATAGGCATCTCC ATC-3′. The MISSION non-target shRNA control vector (SHC002) was used as a scrambled control, and the

sequence of scrambled-shRNA was 5′-CAACAAGA TGAAGAGCACCAA-3′. The transfection protocol has been described previously.[32,33] Briefly, 1.5×10^5 cells were washed twice with 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 pipette-type microporator MP-100 (Digital Bio, Seoul, Korea).

Overexpression of TM in HepJ5 Cells cDNA of TM was conducted by TM-specific primer, amplified by PCR, and subcloned into pCDNA3 expression vector (Invitrogen Life Technologies). The full-length sequences of TM were confirmed by autosequencing. The pCDNA3-TM plasmid was transfected into HepJ5 cells and selected by neomycin to obtain stably transfected cells. TM expression levels were confirmed by real-time PCR and Western blotting.

Transwell Migration Assay

In vitro cell migration was investigated using the 8-μm BD Falcon™ cell culture insert (BD Biosciences). Cells (1×10^5) were suspended in 500 μl serum-free DMEM then seeded into the upper compartments of each chamber, and the lower compartments were filled with 1 mL DMEM with 10% FCS. After 24 h incubation at 37°C in 5% CO_2, nonmigrating cells were removed by scrubbing from the upper surface of the membrane. Cells on the reverse side were stained with 0.1% crystal violet, and migrating cells were counted under microscope at 100× magnification (Olympus IX71).

Statistical Analysis

All experiments were repeated a minimum of three times. All data collected from real-time RT-PCR, cell proliferation, and migration assays are expressed as mean ± standard deviation (SD). The data presented in some figures are from a representative experiment, which was quantitatively similar to the replicate experiments. Statistical significance was determined using Student's *t* test (two-tailed) comparison between two groups of data sets using Sigmaplot software.

RESULTS

Expression Pattern of TM in HCC Cells

TM plays an important role in maintaining circulation homeostasis. However, few studies have focused on the role of membrane-form TM in cancer progression. In order to study the role of TM in HCC, we examined TM expression by quantitative real-time PCR and Western blotting. As shown in Fig. 1, TM was ectopically highly expressed in skHep-1 cells compared with in HepJ5 cells.

Silencing of TM Expression in skHep-1 Cells by shRNA

In order to study TM's function in tumor progression, we introduced TM-specific shRNA to knockdown TM expression in skHep-1 cells. Then, the stably transfected cells were screened by puromycin for 2 weeks. As shown in Fig. 2, TM expression was reduced by over 85% at both transcriptional and translational levels compared with that of scrambled transfected cells.

Silencing of TM Expression Increases the Migration Ability of HCC Cells

Presently, metastatic disease is still a major problem in management of cancer. In order to determine whether TM

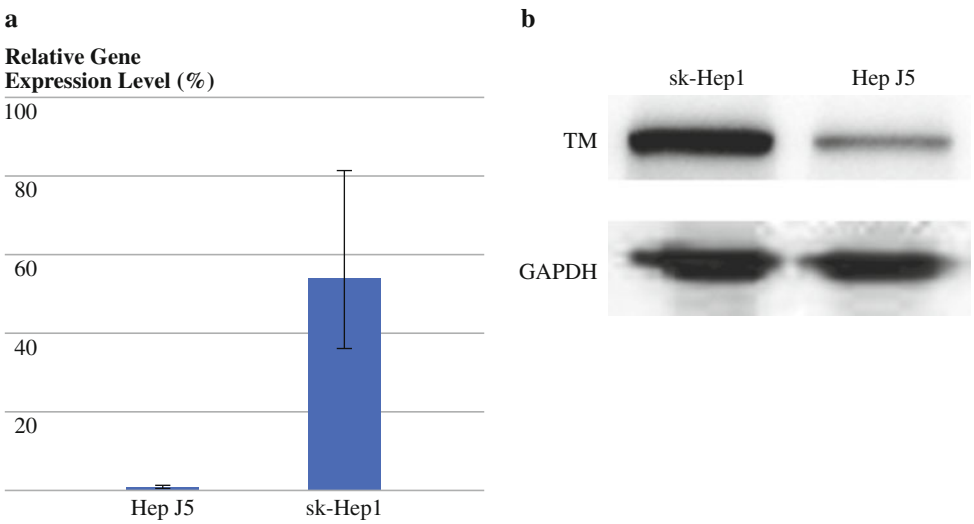

FIG. 1 Expression patterns of thrombomodulin in hepatocellular cells. Total RNA was extracted from HepJ5 and skHep-1 cells and analyzed by quantitative PCR and Western blotting. GAPDH was used as an internal control. **a** Thrombomodulin was identified in both HepJ5 and skHep-1 cells. skHep-1 cells expressed an ectopically higher level of thrombomodulin than did HepJ5 cells. **b** Western blotting analysis revealed that skHep-1 cells have a higher expression level of thrombomodulin than do HepJ5 cells

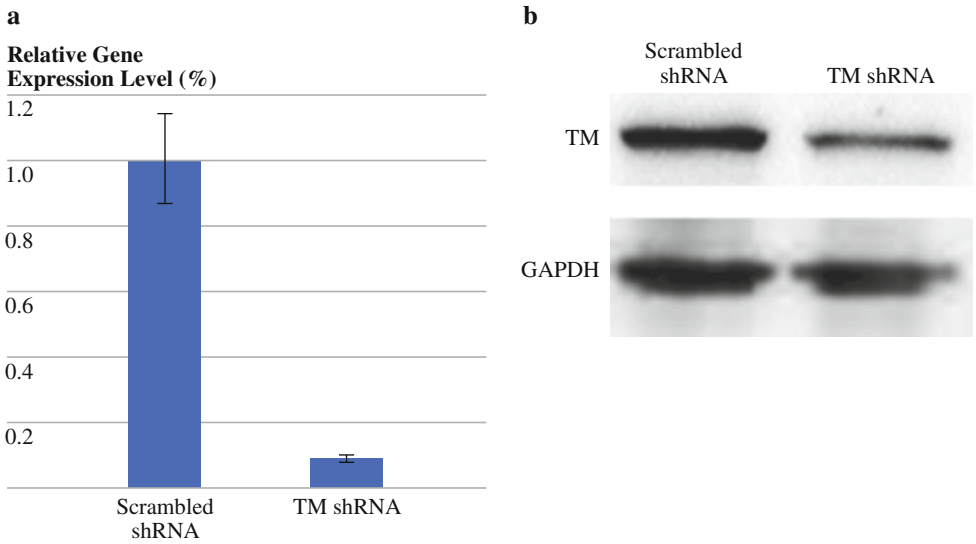

FIG. 2 Knockdown of thrombomodulin expression by shRNA. skHep-1 cells were transfected with either TM-shRNA or scrambled-shRNA construct, and stably transfected cells were selected by puromycin. Thrombomodulin expression was determined by quantitative PCR and Western blotting. TM-shRNA skHep-1 cells showed 85% reduction in thrombomodulin expression compared with scrambled-shRNA skHep-1 cells at both **a** transcriptional and **b** translational levels

influences HCC metastasis, a migration assay was performed using the Transwell migration system (BD Biosciences). TM-shRNA skHep-1 and scrambled-shRNA skHep-1 cells (1×10^5) were seeded in the upper chamber of filter inserts with serum-free medium. Migration ability was significantly increased in TM-shRNA skHep-1 cells compared with in scrambled-shRNA skHep-1 cells (Fig. 3a, b).

Overexpression of TM Suppresses Migration Ability in HepJ5 Cells

In order to confirm the role of TM in cancer migration, we cloned the TM gene into an expression vector, pCDNA3, and overexpressed TM in HepJ5 cells to determine and compare migratory ability. As shown in Fig. 4a, b, expression levels of TM were dramatically increased with transfected pCDNA3-TM plasmids. We found that the number of migrated cells was dramatically decreased after overexpression of TM in HepJ5 cells, which is consistent with our previous finding in skHep-1 cells.

TM Expression Knockdown Enhances HCC Migration via Downregulation of E-cadherin and Upregulation of ZEB1

Since EMT is a major pathologic event in cancer metastasis and the loss of E-cadherin is one of the hallmarks of EMT, we further evaluated E-cadherin expression in HCC cells after silencing of TM expression. E-cadherin expression was reduced after TM knockdown in skHep-1 cells (Fig. 5a). We studied ZEB1 and snail expression in TM-shRNA and scrambled-shRNA skHep-1 cells, because they are major transcriptional repressors of E-cadherin in cancer. We found that ZEB1 expression increased after TM knockdown in skHep-1 cells, but snail expression was unchanged (Fig. 5b). These results suggest that downregulation of TM may enhance migration ability through decreased E-cadherin expression and increased ZEB1 expression.

DISCUSSION

TM is recognized as an essential vessel wall cofactor of the antithrombotic mechanism and is also expressed by a wide range of tumor cells. Many studies have shown that coagulation and anticoagulation factors are correlated with tumor progression. Overexpression of wild-type TM in melanoma cells decreases cell proliferation in vitro and tumor growth in vivo, and deletion of the cytoplasmic or lectin domain nullifies this effect.[27] This also indicates that TM exerts a modulating effect on cell proliferation independent of thrombin and the thrombin receptor. The interaction between thrombin and its receptor plays an important role in cancer metastasis and has been well defined. Studies have shown that a cooperative proteinase-activated receptor-1 (PAR-1)/PAR-4 signaling network also contributes to thrombin-mediated tumor cell migration in HCC, but there is little information about the role of TM in HCC metastasis.[34]

The leading cause of death in HCC patients is metastatic disease. Clinical evidence has shown that highly expressing TM in cancer cells reduces the incidence of metastasis. It has also been reported that soluble TM in the blood correlates with cancer stage, indicating that TM may be a biomarker of metastatic cancer. Some studies also showed that TM's antitumor function is mediated through its influence on cell–cell adhesion interactions that depend on the lectin-like domain of TM.[23,25,35–37] Here we used skHep-1 cells that ectopically expressed TM at high levels

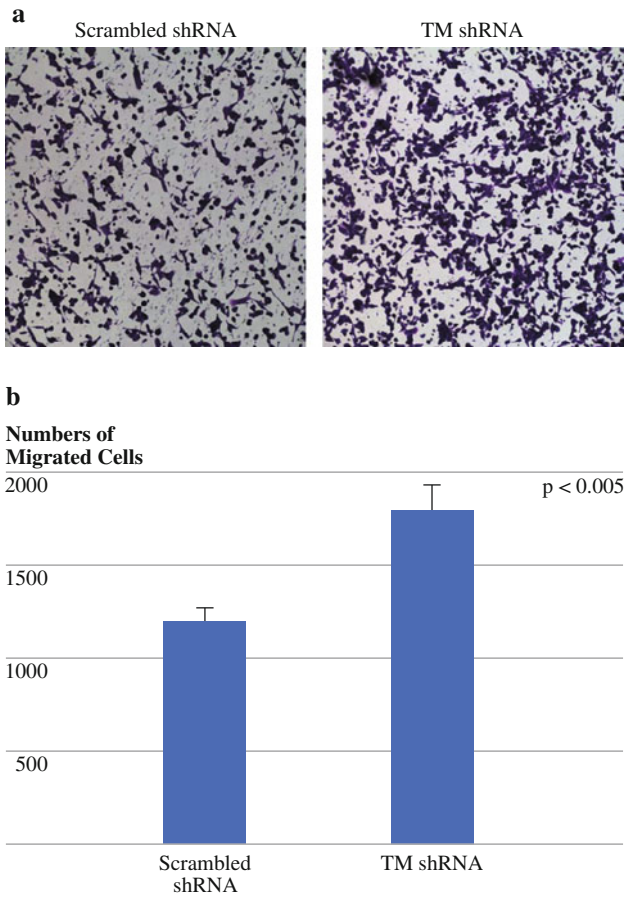

FIG. 3 Reduction of thrombomodulin expression enhances cancer migration. The Transwell migration system was used to evaluate migration ability. **a** The Transwell migration system demonstrated enhanced migration ability after knockdown of TM expression in skHep-1 cells. The pictures of migratory cells were taken by phase-contrast microscopy under 100× magnification. **b** Quantitative Transwell migration assay. *Y*-axis represents the number of migrated cells. The data are the average numbers of cells that migrated in a representative experiment, measured in triplicate and presented as mean ± SD

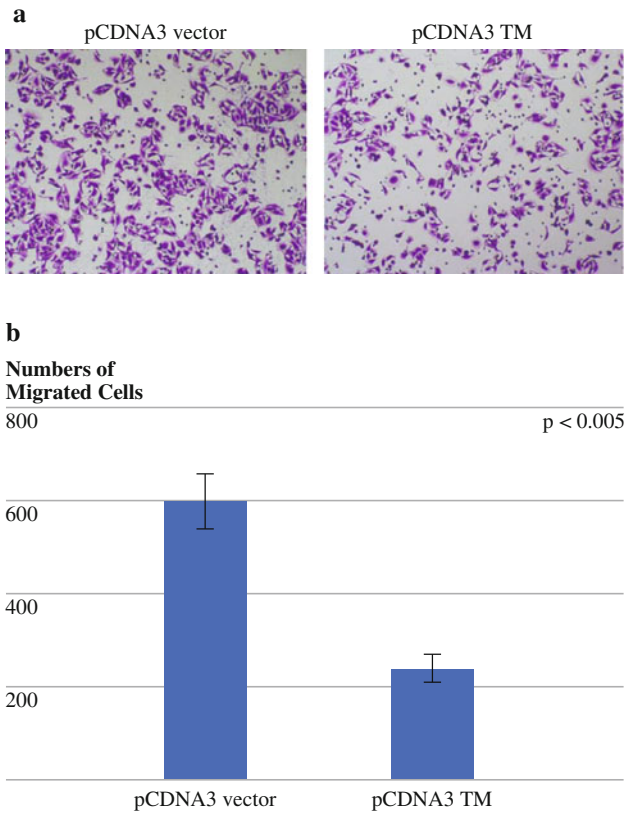

FIG. 4 Overexpression of thrombomodulin suppresses cancer migration. The Transwell migration system was used to evaluate migration ability between HepJ5 cells transfected with pCDNA3 or pCDNA3-TM. **a** The Transwell migration system demonstrated that overexpression of TM caused reduction of migrated cell numbers in HepJ5 cells. The images of migratory cells were taken by phase-contrast microscopy under 100× magnification. **b** Quantitative Transwell migration assay. *Y*-axis represents the number of migrated cells. The data are the average numbers of cells that migrated in a representative experiment, measured in triplicate and presented as mean ± SD

as a cell model. We demonstrated that silencing of TM expression dramatically enhanced cell migration. This indicates that TM also has an effect on cancer metastasis independent of thrombin and its receptor. Conversely, we overexpressed TM in Hep-J5 cells and found a reduction in migration ability. Our results suggest that TM may be a suppressor of tumor metastasis. Furthermore, we found that the enhancement of migration ability is due to increased ZEB1 and decreased E-cadherin expression. This study is the first to demonstrate that the expression level of TM may influence E-cadherin expression. Like E-cadherin, reduced expression of TM in cancer cells results in increased metastasis and poor prognosis.[38,39] However, the regulatory mechanisms of TM and E-cadherin are still unclear and require further investigation.

In this study, we used HepJ5 and skHep-1 as our experimental models. Heffelfinger et al. showed that skHep-1 is an immortal, human cell line of endothelial origin.[40] Thus, we further performed TM overexpression experiments in HepJ5 cells and demonstrated that highly expressed TM may suppress the migration ability of HCC. Our results showed that the antimetastatic role of TM may not only be due to the cell–cell interaction effects as previously reported but may also act through TM's positive modulatory effects on key molecules in the EMT.

In conclusion, we have demonstrated for the first time that TM expression may be associated with E-cadherin expression, and that knockdown of TM expression enhances cancer cell migration in HCC through decreased E-cadherin and increased ZEB1 expression. This study provides further understanding of TM's role in HCC progression.

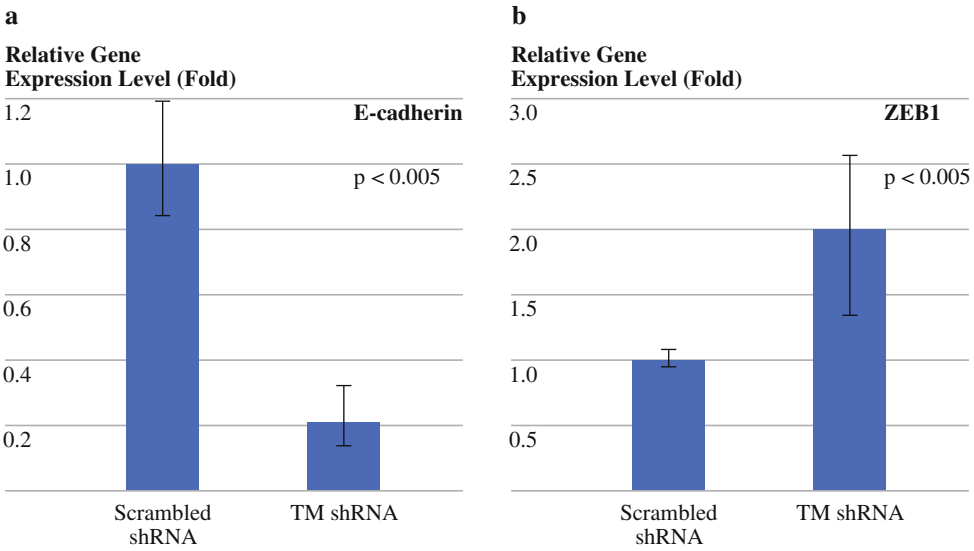

FIG. 5 Knockdown of thrombomodulin resulted in change of E-cadherin and ZEB1 expression. Quantitative PCR was used to determine expression levels of E-cadherin and ZEB1. Knockdown of thrombomodulin expression resulted in decreased E-cadherin expression (**a**) and increased ZEB1 expression (**b**). Data are the average of three independent experiments. Statistical significance was determined using unpaired two-tailed Student's *t* test

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