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RESEARCH ARTICLE

Thrombomodulin mediates the migration of cervical cancer cells through the regulation of epithelial–mesenchymal transition biomarkers

Cheng-Jeng Tai · Chao-Wen Cheng · Hou-Yu Su · Wei-Yu Chen · Chun-Te Wu · Feng-Yen Lin · Chien-Kai Wang · Chen-Jei Tai · Po-Li Wei

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Abstract Thrombomodulin (TM) has been shown to regulate many physiological and pathological processes, including inflammation, thrombosis, and tumor progression. TM is also a natural anticoagulant that maintains circulatory homeostasis in endothelial cells. However, little is known regarding the role of TM in the progression and metastasis of cervical cancer. TM-specific RNA interference and a cDNA expression vector were used to manipulate TM expression in cervical cancer cells. Cell growth and cell migration were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, transwell migration assays, and a biosensor system. TM silencing did not affect the growth rate of the cells. However, cell migration was dramatically enhanced after silencing of TM in HeLa cells. The overexpression of TM in cervical cancer cells only slightly influenced their proliferative capacity. After overexpression of TM in HeLa cells, their migratory

Cheng-Jeng Tai and Chao-Wen Cheng these authors contributed equally.

C.-J. Tai · C.-K. Wang
Division of Hematology and Oncology, Department of Internal Medicine, Taipei Medical University Hospital, Taipei, Taiwan, Republic of China

C.-J. Tai · F.-Y. Lin · C.-K. Wang
Department of Internal Medicine, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan, Republic of China

C.-W. Cheng
Graduate Institute of Clinical Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan, Republic of China

H.-Y. Su
Department of Surgery, Saint Mary's Hospital Luodong, Yilan County, Taiwan, Republic of China

W.-Y. Chen
Department of Pathology, Wan-Fang Hospital and Taipei Medical University, Taipei, Taiwan, Republic of China

C.-T. Wu
Chang Gung University College of Medicine, Taipei, Taiwan, Republic of China

C.-T. Wu
Department of Urology, Chang Gung Memorial Hospital at Keelung, Keelung, Taiwan, Republic of China

F.-Y. Lin
Division of Cardiology, Taipei Medical University Hospital, Taipei, Taiwan, Republic of China

C.-K. Wang · C.-J. Tai
Department of Chinese Medicine, Taipei Medical University Hospital, Taipei, Taiwan, Republic of China

C.-K. Wang · C.-J. Tai
Department of Obstetrics and Gynecology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan, Republic of China

P.-L. Wei
Department of Surgery, College of Medicine, Taipei Medical University, Taipei, Taiwan, Republic of China

P.-L. Wei
Department of Surgery, Taipei Medical University Hospital, Taipei, Taiwan, Republic of China

P.-L. Wei (✉)
Cancer Research Center, Taipei Medical University Hospital, 250 Wu-Xin Street, Taipei, Taiwan, Republic of China
e-mail: poliwei@tmu.edu.tw

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capability was suppressed. Furthermore, we found that the decreased expression of E-cadherin and increase of zeb-1 and snail expression in TM-silenced cells which may be correlated with the results of knocking-down TM increases the migratory ability in this study. Our results demonstrate that TM may slightly regulate the growth but played the important role in the migratory ability of cervical cancer cells, suggesting that TM could potentially serve as a novel prognostic and therapeutic target in cervical cancer.

Keywords Thrombomodulin · Cervical cancer · Migration · EMT

Abbreviations

TM	Thrombomodulin
EMT	Epithelial–mesenchymal transition

Introduction

Cervical cancer is one of the most common gynecological cancers worldwide, and more than 80 % of cases occur in developing countries [1, 2]. As a result of the introduction and popularization of cervical cancer screening programs, the incidence of cervical cancer has decreased in the last four decades [2]. Regular papanicolaou smear screening is effective for the early diagnosis of premalignant cervical dysplasia [3], and surgery/chemoradiotherapy can currently cure 80 to 95 % of stage I/II patients [4, 5]. However, the 5-year survival rate of patients with invasive cervical cancer drops to 60 % [6]. Currently, the prognosis for advanced cervical cancer cases primarily depends upon the clinical staging and histology of the tumor [7]. Novel molecular prognostic markers, which have been shown to participate in specific pathways involved in tumorigenesis and tumor progression of cervical cancer, may provide useful information to determine patient prognosis, predict survival, and design therapeutic strategies.

In tumor cells of epithelial origin, the epithelial–mesenchymal transition (EMT) is considered a key step in tumor metastasis [8, 9]. The EMT is characterized by a morphological and functional shift from epithelial cells to fibroblast-like cells, which results in the loosening of intercellular junctions and increased cellular mobility [8, 9]. The loss of E-cadherin, which is the major cell adhesion molecule that forms intracellular adhesion junctions in epithelial cells, has been suggested to be the first stage of cancer cell metastasis [10]. In a recent cervical cancer study, decreased expression of E-cadherin, which is indicative of the EMT, was detected in lymph node metastases [8, 10]. This result indicates that E-cadherin and other key modulators of related molecular pathways may be useful prognostic markers in advanced cervical cancer.

Thrombomodulin (TM) is predominantly expressed in endothelial cells and functions as a natural anticoagulant, playing a critical role in the homeostasis of circulation [11, 12]. In recent years, TM has been found to regulate many other physiological and pathological processes including inflammation, thrombosis, and tumor progression [13, 14]. In several cancers, including lung, breast, colorectal, and esophageal cancer, the loss of TM in cancer cells has been associated with poor prognosis [15–18]. Furthermore, a clinical study indicated that TM expression was correlated with hepatocellular carcinoma (HCC) metastasis [19]. Another study demonstrated a decrease in E-cadherin expression after the knockdown of TM in the HCC cell lines Hep J5 and skHep-1, which ultimately resulted in enhanced cell migration [20]. However, to date, there is no conclusive evidence demonstrating the relationship between cervical cancer metastasis and TM expression. Nevertheless, previous clinical and in vitro findings suggest that TM may be a potential prognostic marker and therapeutic target for patients diagnosed with cancers in which there exists a known correlation between TM expression and cancer progression. Based on this perspective, the aim of the present study was to investigate the role of TM in modulating E-cadherin expression and related pathways that are associated with the metastasis of cervical cancer cells. The results of this study will help determine the value of measuring TM expression to predict cervical cancer prognosis and select appropriate therapies.

Materials and methods

Chemicals, reagents, and cell culture

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

Manipulation of TM expression

Generation TM knockdown cervical cells The expression of TM was knocked down in cervical cells using MISSION shRNA clones from Sigma Chemical Co. (St. Louis, MO). The parental vector (pLKO.1<-puro) enabled transient transfection or stable selection via puromycin resistance. The

targeting sequence for the human TM mRNA (NM_000361) was 5′-CTTGCTCATAGGCATCTCCATC-3′. The MISSION non-targeting shRNA control vector (SHC002) was used as a scrambled control, and the sequence of the scrambled shRNA was 5′-CAACAAGATGAAGAGCACCAA-3′. The transfection protocol has been described previously [21–25]. Briefly, 1×10^5 cells were washed twice with phosphate-buffered saline (PBS) and mixed with 0.5 μg of plasmid. We applied one pulse for 20 ms at a fixed voltage of 1.4 kV on the pipette-type Neon microporator (Invitrogen, Grand Island, NY) [26, 27].

Overexpression of TM in cervical cancer cells The full-length TM cDNA was amplified using polymerase chain reaction (PCR) and subcloned into the pcDNA3-TOPO vector (Invitrogen), generating the pcDNA3-TOPO-TM expression vector. The full-length sequence was verified using automated sequencing (Applied Biosystems, Grand Island, NY). The pcDNA3-TOPO-TM plasmid was transfected into HeLa cells, and stably transfected cells were selected with neomycin. The TM expression levels were confirmed using real-time quantitative PCR and Western blotting as previously described [20, 26, 27].

Protein extraction and Western blot analysis

Protein expression levels were determined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting 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). Equal amounts of proteins were separated in a 10 % SDS-polyacrylamide gel under reducing conditions and electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were subsequently blotted using anti-TM or anti-actin antibodies and horseradish peroxidase-conjugated secondary antibodies (1:5,000). The specific proteins were subsequently visualized using Enhanced Chemiluminescence Reagent (GE Healthcare, Piscataway, NJ) and detected using the VersaDoc 5000 (Bio-Rad Laboratories) [23, 24].

RT-PCR and quantitative RT-PCR analysis Total RNA was extracted from cells using the TRIZOL reagent as recommended by the manufacturer (Invitrogen). cDNA was amplified from 2 μg of total RNA in a final volume of 20 μL using M-MLV (Moloney murine leukemia virus) reverse transcriptase at 37 °C for 90 min. The primers for real-time PCR (RT-PCR) have been described in previous studies [20, 23, 24]. The quantitative RT-PCR was carried out using ABI SYBR Green Master Mix, and thermal cycling was performed using the ABI StepOne (Applied Biosystems) and the following quantitative PCR protocol: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Cell viability assay

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were plated at 2×10^4 cells per well in 24-well

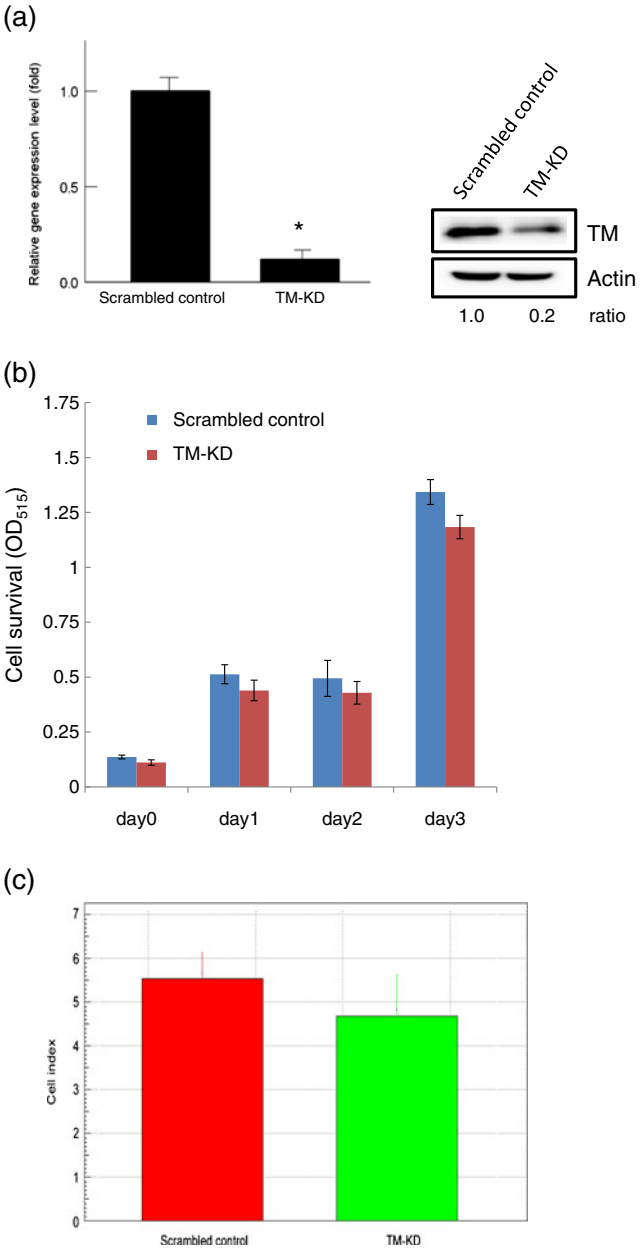

Fig. 1 Knockdown of TM expression in cervical cancer cells don't influence cell proliferative capability. The expression of TM was silenced in HeLa cells using a TM-specific shRNA (*TM-KD*) or a scrambled shRNA (*scrambled control*). **a** Western blot analysis revealed that TM expression was reduced over 80 % in TM-KD HeLa cells compared with scrambled control HeLa cells. Total RNA was extracted from cells and analyzed using quantitative PCR and Western blotting. *β*-actin was used as an internal control. **b**, **c** The proliferation of TM-KD and scrambled control HeLa cells was determined using an MTT assay and the xCELLigence biosensor system

plates and incubated overnight at 37 °C in a humidified incubator containing 5 % CO_2. At selected time intervals, the medium was aspirated. The remaining cells were further incubated with 0.25 mg/mL MTT for 1 h. The MTT was extracted with DMSO, and the extract color change was measured at 550 nm using a spectrophotometer (GE Healthcare, Piscataway, NJ).

Cell proliferation and migration assay using the xCELLigence biosensor system

Experiments were conducted using an RTCA DP instrument (AECE Biosciences Inc. San Diego, CA), which was placed in a humidified incubator and maintained at a 5 % CO_2 and 37 °C. Growth curves were constructed using 16-well plates (E-plate 16, AECE Biosciences Inc.). For the proliferation assay, cells were seeded in an E-plate 16 at 10,000 cells/well in fetal calf serum (FCS)-containing medium. The plate was then monitored once every 30 s for 4 h and once every 0.5 h thereafter. Cell migration was assessed using specifically designed 16-well plates (CIM-plate 16, AECE Biosciences Inc.) with 8-μm pores. These plates are similar to conventional transwells and contain micro-electrodes on the underside of the membrane of the upper chamber. Medium containing 10 % FCS was added in the lower chamber, and cells (20,000 cells/well) were seeded into the upper chamber 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 RTCA software 1.2, which was supplied with the instrument.

Transwell migration assay In vitro cell migration assays were performed using a BD Falcon cell culture insert (BD Biosciences). Cells (5×10^4) were suspended in 500 μL of serum-free DMEM and seeded into the upper chamber. The lower chambers were filled with 1 mL of DMEM containing 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

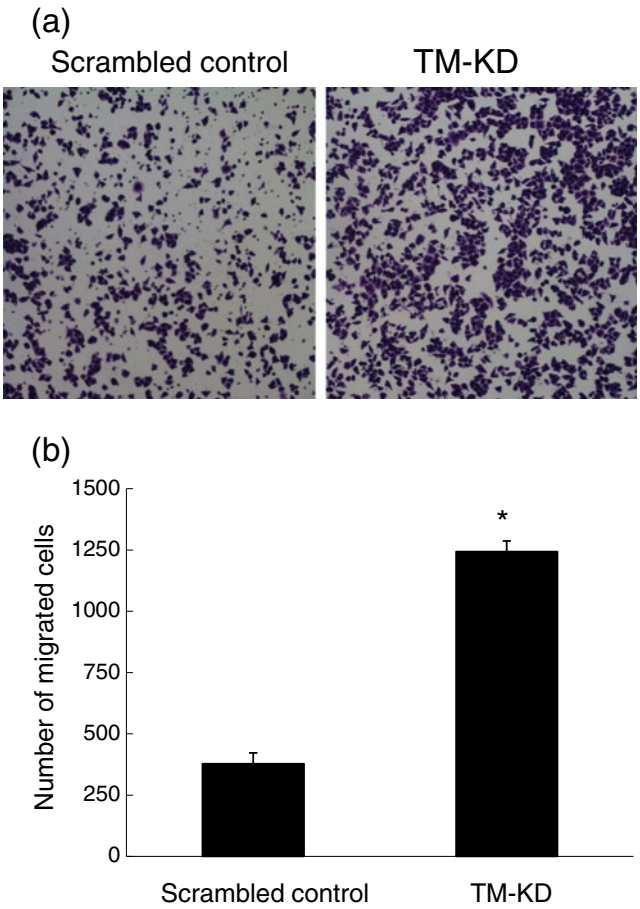

Fig. 2 Decreased TM expression enhances cancer cell migration. The transwell migration assay was used to evaluate migratory capacity. **a** Knock-down of TM expression in HeLa cells resulted in increased migration. **b** Quantitation of the transwell migration assay results are shown. The *y*-axis indicates the number of migrated cells

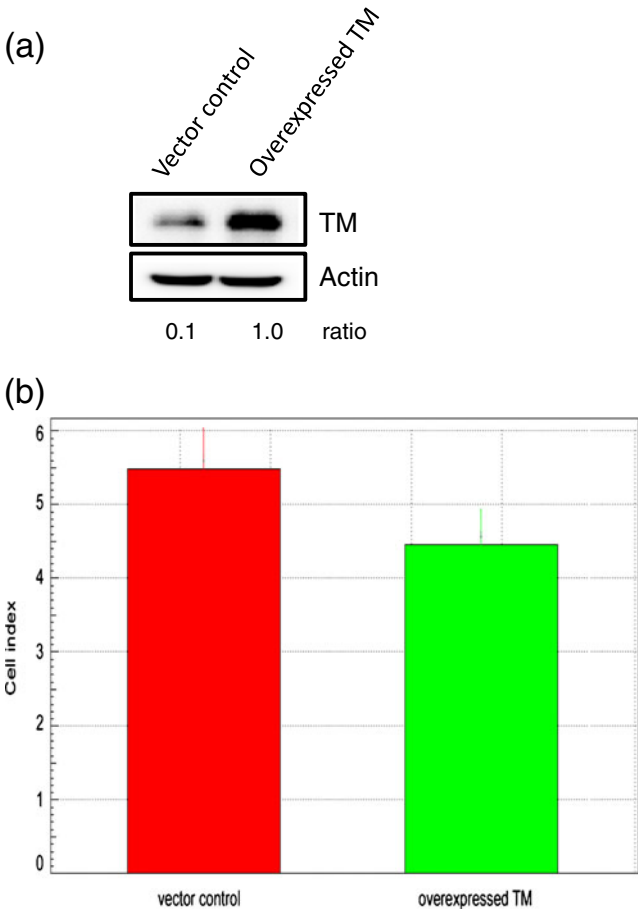

Fig. 3 Effect of TM overexpression on HeLa cell growth. HeLa cells were transfected with pcDNA3-TOPO or pcDNA3-TOPO-TM and selected using neomycin. **a** The expression of TM was determined using Western blotting. **b** The proliferation of HeLa cells overexpressing TM and HeLa cells transfected with the empty vector was determined using the xCELLigence biosensor system

the membrane by scrubbing. Cells on the reverse side were stained with 0.1 % crystal violet, and migrating cells were counted using a microscope (magnification, ×100).

Statistical analysis

All experiments were repeated a minimum of three times. All the collected data are expressed as the mean±SD. The data presented in some figures are the results of a representative experiment that was quantitatively similar in the replicate experiments. Statistical significance was determined using two-tailed Student's *t* tests.

Results

Silencing TM expression in HeLa cells using shRNA

We transfected a TM-specific shRNA into HeLa cells to silence TM expression and selected stably transfected cells after culture with puromycin for two weeks. As shown in Fig. 1a, TM expression was reduced by over 80 % at both the transcriptional level and the translational level in TM shRNA-knockdown (TM-KD) cells compared with the expression in scrambled shRNA-transfected (scrambled control) cells.

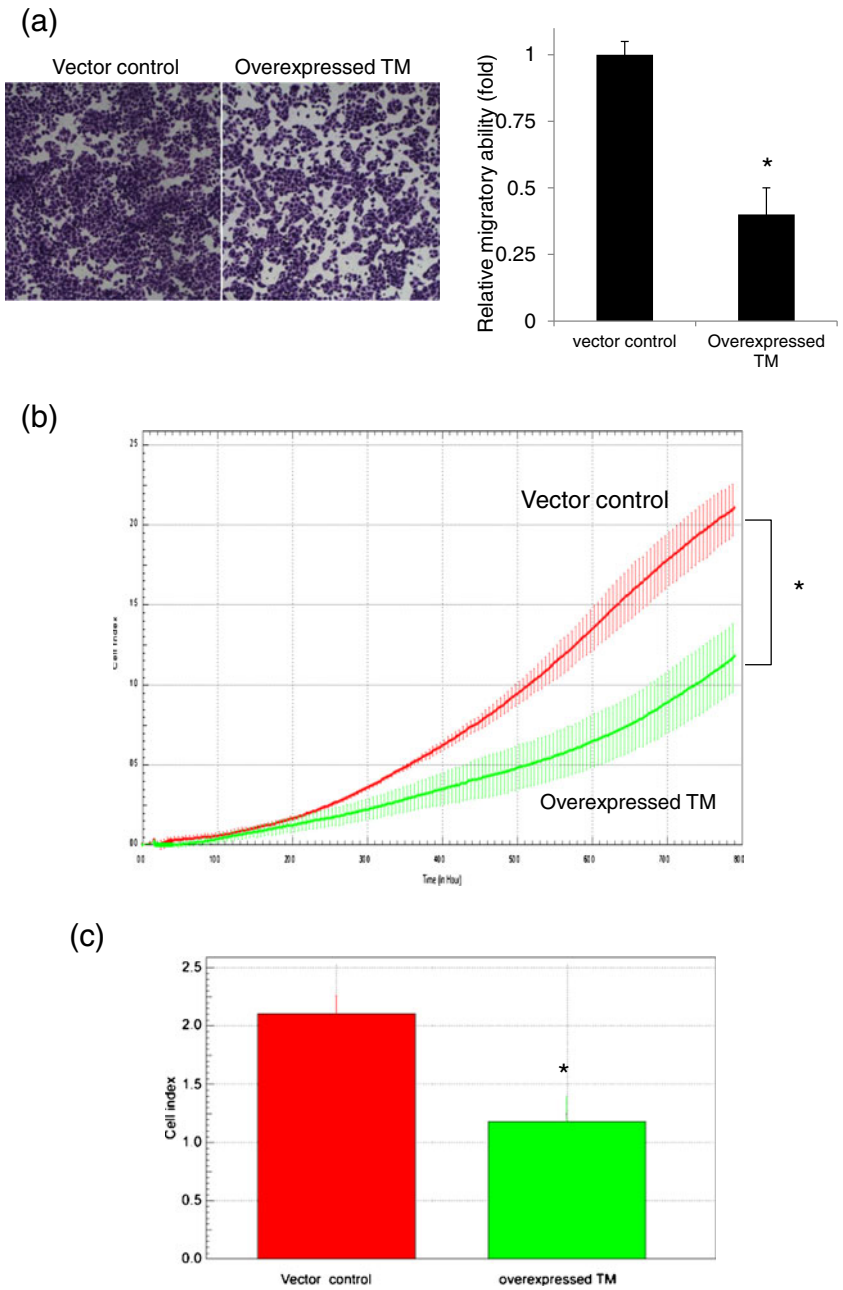

Fig. 4 Overexpression of TM suppresses cancer cell migration. The migratory capacity of HeLa cells overexpressing TM and HeLa cells transfected with the empty vector was determined using the transwell migration assay (**a**) and the xCELLigence biosensor system (**b**, **c**). The *y*-axis indicates the cell index. **c** Quantification of the migration results in an xCELLigence biosensor system

Effect of silencing TM expression on the proliferation of HeLa cells

We analyzed the proliferative ability of TM-KD cells and scrambled control cells using the MTT assay and xCELLigence biosensor system. TM silencing did not affect the proliferation of HeLa cells (Fig. 1b, c), indicating that TM most likely does not participate in the regulation of cervical cancer cell proliferation.

Silencing TM expression increases the migratory capacity of HeLa cells

Cancer metastasis remains a major problem for the successful management of cancer therapy. The impact of TM expression on the metastasis of cervical cancer was further examined using the Transwell migration assay. TM-KD and scrambled control cells were seeded into the upper chamber of the filter inserts in serum-free medium. Cell migration was significantly increased in HeLa cells following TM silencing by the TM-specific shRNA (Fig. 2). These results indicate that TM may function as a modulator of metastasis in cervical cancer.

Overexpression of TM suppresses migration of HeLa cells

To confirm the role of TM in cervical cancer progression, we cloned the TM gene into the expression vector pcDNA3-TOPO, transfected pcDNA3-TOPO-TM into HeLa cells using electroporation, and selected cells that stably overexpressed TM using antibiotics. The expression of TM was determined using real-time PCR and Western blotting. As shown in Fig. 3a, the expression of TM increased dramatically following transfection of pcDNA3-TOPO-TM into HeLa cells. As detected using the biosensor system, the growth of cells over-expressing TM was reduced by 10 % compared with control cells transfected with the empty vector (Fig. 3b). Using a transwell migration assay and biosensor system, we determined that the migratory capacity of HeLa cells overexpressing TM was dramatically decreased compared with HeLa cells transfected with the empty vector (Fig. 4). This result indicates that TM may function as a suppressor of cervical cancer cell migration.

TM affects migration via modulation of EMT biomarkers

The EMT is a major pathologic event in cancer metastasis, and changes in the expression of EMT biomarkers have been correlated with migration. Therefore, the expression of EMT biomarkers in scrambled control cells and TM-KD HeLa cells was determined. Figure 5 shows a decrease in E-cadherin expression and an increase in zeb-1 and snail expression in TM-KD cells. However, the expression of fibronectin, N-cadherin, and vimentin was similar in TM-KD cells and scrambled control cells. These results suggest that TM expression may modulate migration via the modulation of EMT biomarker expression.

Discussion

Cervical cancer is the third most common female malignancy and second most common diagnosed malignancy of the female reproductive system [28, 29]. Given the of use of human papilloma virus vaccine and cervical cancer screening programs, metastatic cervical cancer is uncommon at initial diagnosis, and surgery is the treatment of choice for patients with localized cervical cancer. However, metastasis can develop in 15 to 60 % of patients within 2 years after surgery (SEER data for 2000–2004 http://seer.cancer.gov/). Only a few patients with limited distant metastatic disease can be successfully treated with surgical intervention; most metastatic cervical cancers are not surgically curable, and chemotherapy is the only treatment option.

In addition to being difficult to diagnose early, the main challenge in treating patients with advanced cervical cancer is the high prevalence of recurrence and chemoresistance, which render the cancer incurable in most patients, resulting in high mortality [30, 31]. The EMT has been demonstrated to play an important role in cancer cell invasion and metastasis [32], and many different biomarkers involved in the EMT have been identified including E-cadherin, N-cadherin, fibronectin, and vimentin [33, 34]. Previous results have suggested that reduced E-cadherin levels may enhance the EMT and increase the migration of cancer cells [8]. In addition, a decreased E-cadherin expression is associated with poor prognosis in cervical cancer patients [35, 36]. Increased expression of EMT-related transcription factors, such as snail, slug, twist-2, and zeb-2 has been shown to enhance chemoresistance in human cervical cancer cells [37].

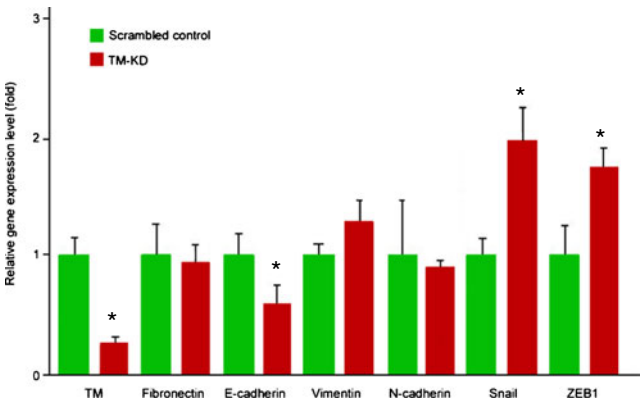

Fig. 5 TM mediates cell migration via the modulation of EMT biomarkers. The expression of EMT biomarkers (E-cadherin, N-cadherin, fibronectin, and vimentin) was determined in TM-KD and scrambled control HeLa cells using Western blotting and quantitative PCR. The suppression of TM expression resulted in decreased E-cadherin expression and increased snail and zeb-1 expression

It is critical to identify biomarkers to predict metastasis after surgery to treat cervical cancer. The loss of E-cadherin has been noted to be a prognostic factor for cervical cancer [38], but the role of the transcription repressor, snail, remains controversial [39, 40]. The suppression of TM was shown to reduce the expression level of E-cadherin and its transcription repressor, snail. zeb-1, another transcriptional repressor of E-cadherin, can also act as co-repressor of snail. zeb-1 expression was detected in tumors of the uterine origin, and high expression levels of zeb-1 positively correlated with the loss of E-cadherin expression in surgical specimens that showed increased migratory and invasive potential of endometrioid cancer cells [41, 42]. However, the relationship between zeb-1 and cervical cancer remains unclear. The present study demonstrated that the expression level of zeb-1 increased after the downregulation of TM.

Several studies have demonstrated that TM functions as a negative regulator of cell adhesion [43]. In addition, TM expression may influence the migration of HCC cells via the modulation of E-cadherin expression [20], indicating that TM plays a key role in mediating cancer metastasis. However, the role of TM in cervical cancer progression remains unclear. Here, TM expression has been shown to have a minor effect on cell proliferation but a pronounced effect on cell migration. Silencing TM expression was found to enhance cancer cell migration, whereas overexpression of TM suppresses cancer cell migration. Further investigations into the mechanism underlying this effect revealed that silencing TM expression induced a decrease in E-cadherin expression, which positively correlated with enhanced cancer cell migration. Accordingly, the overexpression of TM increased the expression of E-cadherin (data not shown). These results indicate that TM may function as a modulator of cervical cancer cell migration.

In conclusion, the present study has demonstrated that TM is a key protein that can mediate cervical cancer metastasis. These results provide a new direction to pursue in the development of new diagnostic and therapeutic strategies to treat patients with cervical cancer metastases.

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Conflict of interest None

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