Original Article
TEM1 knock-down inhibits the proliferation and metastasis of MES-SA uterine sarcoma cells

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Abstract: Aims: Uterine sarcomas are associated with a poor prognosis because of their high recurrence rate and metastatic potential. Tumor endothelial marker (TEM1) is associated with tumor metastasis and invasiveness. It is over-expressed in uterine sarcomas but has not been investigated fully. Our study aimed to elucidate the function of the TEM1 protein in uterine sarcoma cells MES-SA. Methods: A TEM1 knock-down MES-SA uterine sarcoma cell line was established using a small interfering RNA (siRNA) targeting TEM1. Cell proliferation, invasion and metastasis were assessed using Cell Counting Kit (CCK)-8, scratch, transwell and colony formation assays. Protein markers of the epithelial-mesenchymal transition (EMT) were investigated to evaluate the metastatic potential of TEM1 in uterine sarcoma cells. Results: TEM1 downregulation inhibits the migration, metastasis and proliferation of MES-SA cells. The ability of these cells to form colonies was also impaired by TEM1 knock-down. The EMT was significantly inhibited in MES-SA cells after TEM1 silencing. Conclusion: TEM1 promotes the invasiveness and EMT of uterine sarcoma cells, and a TEM1-targeted therapy may be a potential option for patients with uterine sarcoma.

Keywords: TEM1, MES-SA, uterine sarcoma, metastasis, proliferation, EMT

Introduction

Uterine sarcomas are rare malignant tumors that account for approximately 3% of all uterine malignancies [1]. These tumors are associated with a poor prognosis because of their high recurrence rate and metastatic potential [1-3]. The most common treatments for uterine sarcoma are complete surgical excision, with or without adjuvant or neoadjuvant therapies [4]. However, the overall 5 year survival rate for patients with uterine sarcoma remains only 15-25% [1, 5]. The development of efficient therapies has been hindered by the rarity of these tumors and the diversity of their histopathological results [1, 4]. Limited data concerning uterine sarcoma have been reported to date, and the mechanisms underlying uterine sarcoma proliferation and metastasis are not fully understood.

Tumor endothelial marker 1 (TEM1), also known as endosialin or CD248, is located at 11q13.2 and is a bulk transmembrane glycoprotein that was first identified as an antigen associated with the tumor endothelium [6, 7]. Its expression is increased in tumors, particularly in stro- mal cells and pericytes surrounding blood vessels. Moreover, TEM1 is expressed at low levels in healthy blood vessels and other tissues from adult individuals [8, 9]. Hypoxia-inducible factor-2 (HIF-2) is involved in regulating TEM1 expression under hypoxic conditions in cooperation with other transcription factors [10]. TEM1 promotes tumor growth and metastasis by interacting with extracellular matrix (ECM) proteins in the microenvironment [11]. TEM1 suppression was recently shown to be associated with decreased cellular proliferation *in vitro* and the inhibition of tumor growth, invasion and metastasis in orthotopic models; in addition, the platelet-derived growth factor receptor-β (PDGFR-β) pathway was shown be involved in these functions in a subset of cells [11-13].

TEM1 was recently shown to be a biological marker for tumors with a mesenchymal origin, including sarcoma, and it promotes tumor proliferation and metastasis [9, 14]. Rouleau et al. [14] screened human clinical sarcoma specimens and sarcoma cell lines and detected TEM1 in advanced diseases and 22 out of 42 cell lines, claiming for the first time that TEM1 might be a suitable target for sarcoma therapy

and prognosis. As shown in the study by Sun et al. [15], primary human osteosarcoma samples containing cancer stem-like side population (SP) cells displayed increased expression of TEM1, contributing to their increased self-renewal and deregulated cell proliferation properties and subsequently leading to drug resistance and high invasive potential. Because of the promising therapeutic value of TEM1, a TEM1-targeted monoclonal antibody (MORAb-004) has recently been investigated in the first-in-Human Phase I Study as a treatment for solid tumors [16].

TEM1 is also overexpressed in uterine sarcomas [9, 14] but has not been investigated fully. Our study aimed to evaluate the function of TEM1 in uterine sarcoma using a previously validated TEM1-over-expressing MES-SA cell line [14], which was established from a 56-year-old female patient with uterine sarcoma. After knocking down TEM1 expression, we explored the function of TEM1 in the MES-SA cell line.

Materials and methods

Drugs and reagents

Uterine sarcoma MES-SA, Ewing's sarcoma A673 and leiomyosaroma SKUT-1 cell lines were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). Anti-TEM1, E-cadherin, Vimentin, and Neural-cadherin (N-cadherin) antibodies were synthesized by Abcam (Cambridge, MA, USA). The High Capacity cDNA Reverse Transcription Kit and TaqMan Universal Master Mix II were obtained from Abcam (Cambridge, MA, USA). Primers and small interfering RNAs (siRNAs) were designed by GenePharma (Shanghai, China). McCoy's 5a Medium, fetal bovine serum (FBS), TRIzol reagent and Lipofectamine 2000 were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). The RNeasy Mini Kit and RNase-Free DNase Set were purchased from QIAGEN Shenzhen Company Limited (Shenzhen, China). The penicillin streptomycin solution was obtained from Corning Cellgro (New York, NY, USA).

Cell culture

MES-SA, A673 and SKUT-1 cells were cultured in McCoy's 5a or DMEM supplemented with 10% FBS and 100 U/ml penicillin in a humidified atmosphere containing 5% CO_2 at 37°C.

Cells were propagated according to the protocol provided by the American Type Culture Collection (ATCC).

siRNA design and transfection

FAM-labeled siRNAs 5'-GGCUUCGAGUGUUAU-UGUATT-3' (siRNA1) and 5'-CAGCCAACUAUCC-AGAUCUTT-3' (siRNA2) against TEM1 and a scrambled control (5'-UUCUCCGAACGUGUCA-CGUTT-3') were designed and synthesized by GenePharma (Shanghai, China). Cells were grown to 50% confluence in serum-free medium and transfected with 60 nM TEM1 siRNAs or the scrambled control using Lipofectamine 2000, according to the manufacturer's instructions. The culture medium was replaced with complete medium containing 10% FBS 6 h after transfection.

Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted using TRIzol reagent and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit, according to the manufacturer's instructions. The resulting cDNA samples were subjected to qPCR analysis using TaqMan Universal Master Mix II. The cDNAs were quantified using an ABI ViiA 7 System (Applied Biosystems) with inventoried TEM1 primers (18S probe as an endogenous control) using a two-step reaction with the following conditions: predenaturation (95°C for 15 min), denaturation (95°C for 10 s), annealing (for 30 s), and elongation (for 30 s); the annealing and elongation steps were performed 40 times. Samples were normalized to leiomyosaroma SKUT-1 cells. The qPCR reactions were performed in triplicate and the comparative CT method (2-ΔΔCT method) was used to calculate the relative gene expression levels.

Flow cytometry

The expression of the TEM1 protein was assessed by flow cytometry. Cells were trypsinized and $1×10^4$ cells per sample were washed with PBS and resuspended in 100 μl of fluorescence-activated cell sorting (FACS) buffer (PBS+0.5% FBS). One microliter of 78Fc (1 mg/ml) was incubated with cells for 1 h on ice and then washed three times with FACS buffer. Cells were then incubated with 1 μl of goat anti-human IgG-APC (Jackson Immoresearch, USA) for 30 min on ice. Cells were washed three times with PBS and analyzed on a FACSCanto
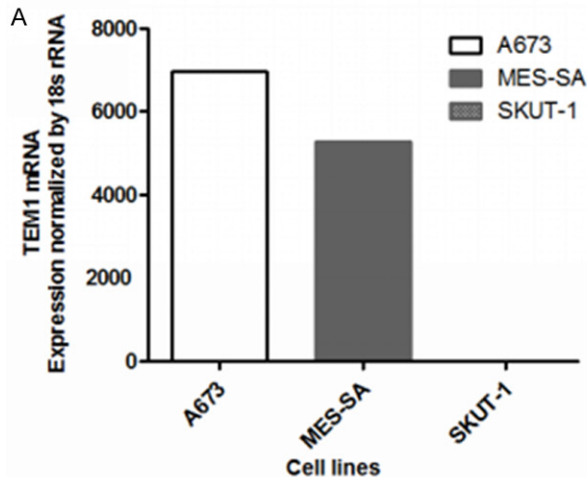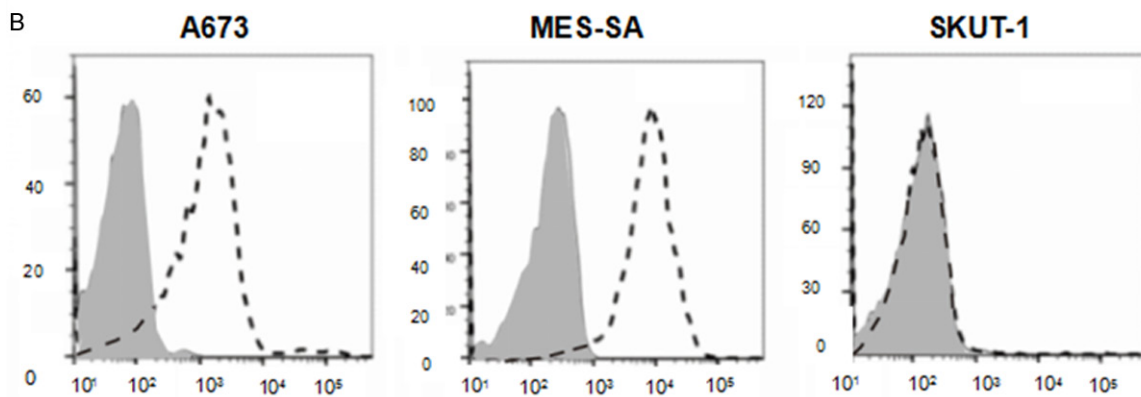
Figure 1. TEM1 is expressed at high levels in MES-SA cells. TEM1 expression in A673, MES-SA and SKUT-1 cells was detected by qPCR (A) and flow cytometry (B). A673 and MES-SA cells expressed high levels of the TEM1 transcript and protein. SKUT-1 cells were used as a negative control for TEM1 expression.

instrument (BD Biosciences, USA). Data were analyzed using FlowJo software.

Western blot

Proteins were extracted from cells and the protein concentration was determined using a bicinchoninic acid (BCA) kit. Equal amounts of protein (20 µg) from cell lines were separated on 10% SDS-PAGE gels and then transferred onto polyvinylidene fluoride membranes (Millipore). After blocking, membranes were incubated with milk containing the TEM1 (1:5,000 dilution), E-cadherin (1:5,000), Vimentin (1:2,500), or N-cadherin (1:2,500) antibody and then incubated overnight at 4°C. Subsequently, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (dilution 1:5,000) was added to the membranes and incubated for 1 h before detection. Endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. The immunoreactive bands were detected with an enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA).

Cell counting kit (CCK)-8 assays

A commercial CCK-8 assay (Sigma-Aldrich, St. Louis, MO, USA) was used to evaluate cell proliferation, according to the manufacturer's instruction. MES-SA cells were seeded onto 96-well plates at a density of 1×10^3 cells per well and cultured at 37°C in air with 5% CO_2. CCK-8 reagents were added to each well at 24, 48 and 72 h. The absorbance was measured after an additional 2 h of incubation. A microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to detect the absorbance at a wavelength of 450 nm.

Cell invasion assay

A cell invasion assay was performed using 6.5 mm transwells with 8.0 µm pore polycarbonate membrane inserts coated with a 0.5 mg/ml Matrigel matrix placed in a 24-well plate. MES-SA cells were suspended in 100 µl of serum-free medium and seeded in each upper chamber at a density of 1×10^5 cells/ml. Five hundred microliters of medium containing 10% FBS
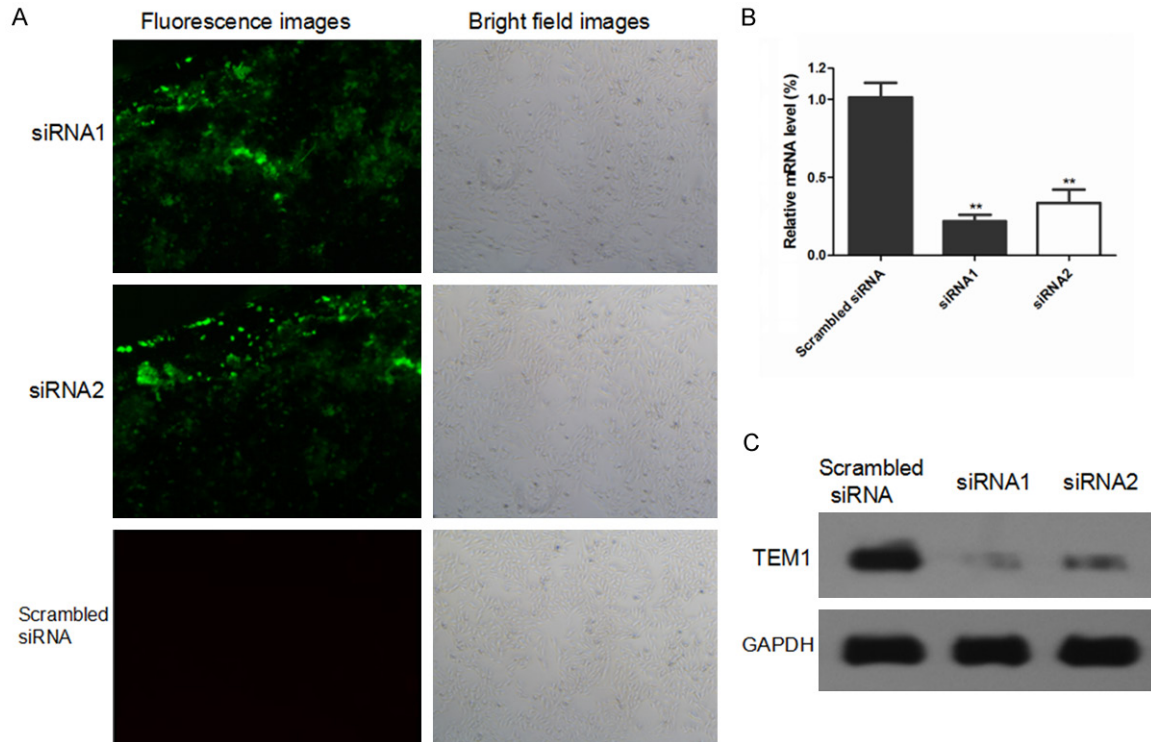
Figure 2. TEM1-targeted siRNAs down-regulate TEM1 expression in MES-SA cells. MES-SA cells were transfected with FAM-conjugated TEM1 siRNA1, siRNA2 and a scrambled siRNA (A). The expression of the TEM1 mRNA was inhibited by siRNA1 and siRNA2, with siRNA2 showing a more obvious inhibitory effect (B). Transfected cells were lysed and subjected to western blotting (C). The data are shown as the means ± SEM (n=3, *P<0.05, **P<0.01 compared with the scrambled siRNA group).
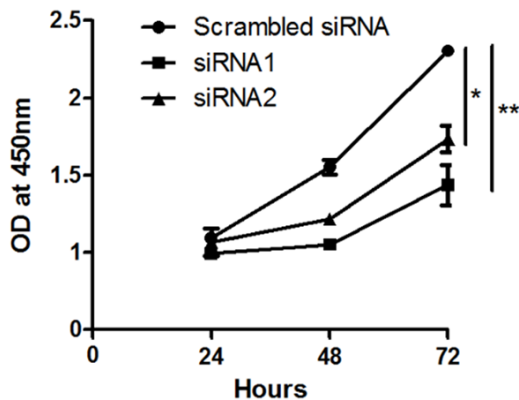

Figure 3. TEM1 silencing inhibits MES-SA cell proliferation. The CCK-8 assay was conducted to evaluate the growth rates of MES-SA cells transfected with siRNA1, siRNA2 and the scrambled siRNA from days 1 to 3. Values were normalized to the average absorbance measured on day 1 (n=3, *P<0.05 and **P<0.01 compared with the scrambled siRNA group).

were added to the lower compartments of the chamber. Following an 18 h incubation at 37°C with 5% CO_2, non-invading cells were removed from the upper compartment using moist cot-

ton swabs. The invaded cells were then fixed with paraformaldehyde, stained with 0.5% crystal violet for 30 min and the number of cells in 3 randomly selected fields per membrane was immediately counted. Experiments were repeated three times.

In vitro scratch assay

MES-SA cells (5×10^5) were added to 6-well plates with complete medium supplemented with 10% FBS and allowed to adhere for 8 h. Cells were then incubated in serum-free medium and transfected with siRNAs. A line was scratched within the monolayer of cells using a 10 µl micropipette tip 20 h after transfection (time 0). The cells were subsequently cultured in complete medium containing 10% FBS for up to 24 h. Images of migrating cells in the wounded region were captured at baseline (time 0 h) and 24 h later (time 24 h).

Colony formation

MES-SA cells were seeded in 6 cm dishes at a density of 400 cells per well and cultured at
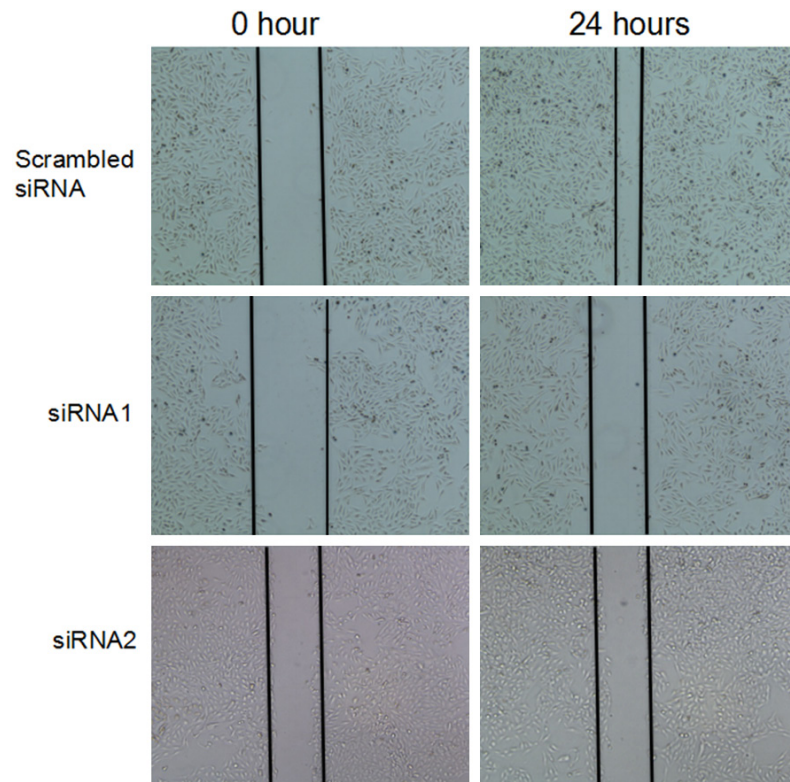
Figure 4. TEM1 silencing inhibits ME-SA cell migration. Monolayers of MES-SA cells transfected with siRNA1, siRNA2, or the scrambled siRNA were scratched and evaluated for wound closure after 24 h.

37°C in air with 5% CO_2 for 14 days to examine the survival and proliferation of MES-SA cells. Colonies were fixed with 4% paraformaldehyde and stained with Giemsa stain. Colonies were observed under a microscope and the number of colonies containing >50 cells was counted.

Statistical analysis

GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA) was used for the statistical analysis. Continuous variables were compared using Student's t-test. Comparisons between groups were analyzed using one-way analysis of variance (ANOVA). A *P* value of less than 0.05 was considered a significant difference.

Results

TEM1 expression in human sarcoma tumors and cell lines

TEM1 expression was evaluated in three human sarcoma cell lines: a positive control A673 Ewing's sarcoma cell line, the MES-SA leiomyo-

sarcoma cell line and a negative control SKUT-1 leiomyosarcoma cell line. Based on the qPCR (**Figure 1A**) and flow cytometry results (**Figure 1B**), MES-SA cells expressed high levels of both the TEM1 mRNA and protein that were comparable to the levels observed in the A673 cells and consistent with findings reported in previous studies [9, 14].

Knock-down of TEM1 protein expression in the MES-SA cell line

Three FAM-labeled siRNAs targeting the TEM1 gene were generated to investigate the effect of TEM1 expression on MES-SA cell growth. FAM fluorescence was observed in >90% of MES-SA cells 72 h after transfection (**Figure 2A**). As shown in the results of the qPCR and western blot assays, lower levels of the TEM1 mRNA and protein were observed in cells transfected with siRNA1 and siRNA2 (P<0.01; **Figure 2B** and **2C**) than in non-transfected cells. Notably, siRNA1 revealed the greatest knock-down efficiency.

Silencing of the TEM1 protein inhibits the proliferation of MES-SA cells

Cell proliferation assays were performed to determine whether TEM1 knock-down inhibited MES-SA cell proliferation *in vitro*. As shown in **Figure 3**, the cell proliferation rates were significantly reduced in the siRNA1 and siRNA2 groups at 48 and 72 h compared with the scrambled siRNA control group, with greater inhibition observed in the siRNA1 group (P< 0.01) than in the siRNA2 group (P<0.05) at 72 h.

Silencing of the TEM1 protein suppresses the migration and invasion of MES-SA cells

Scratch and transwell assays were performed to evaluate whether TEM1 down-regulation inhibited the wound-healing and invasion abilities
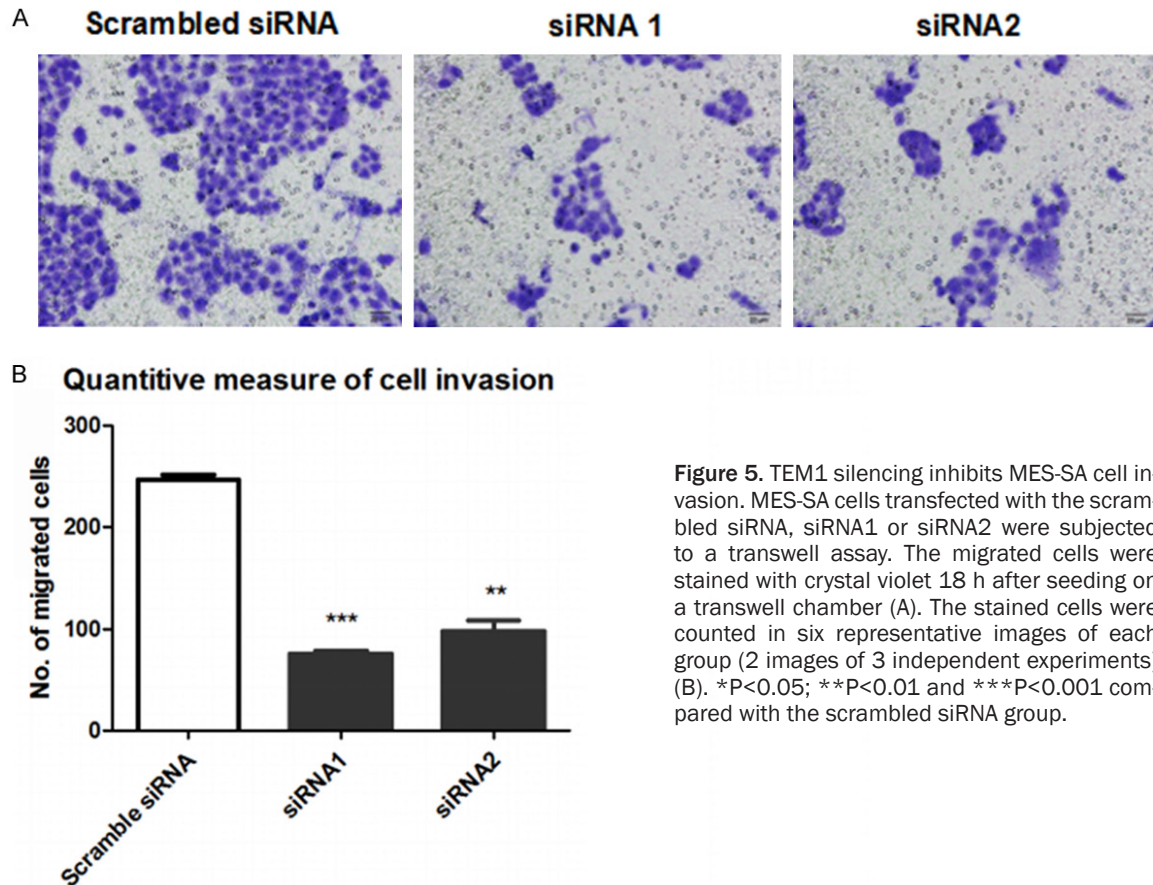
A

Scrambled siRNA   **siRNA 1**   **siRNA2**

B

Quantitive measure of cell invasion

Figure 5. TEM1 silencing inhibits MES-SA cell invasion. MES-SA cells transfected with the scrambled siRNA, siRNA1 or siRNA2 were subjected to a transwell assay. The migrated cells were stained with crystal violet 18 h after seeding on a transwell chamber (A). The stained cells were counted in six representative images of each group (2 images of 3 independent experiments) (B). *P<0.05; **P<0.01 and ***P<0.001 compared with the scrambled siRNA group.

of MES-SA cells. As shown in the results of the scratch assay, the siRNA1- or siRNA2-treated MES-SA cell showed greater distance than cells transfected with the scrambled siRNA, but no significant differences were observed between the migration distances of cells transfected with siRNA1 and siRNA2 (**Figure 4**). According to the results of the transwell assay, the average number of invaded siRNA1- and siRNA2-transfected MES-SA cells was decreased compared with cells transfected with the scrambled siRNA (siRNA1 P<0.001, siRNA2 P<0.01; **Figure 5A** and **5B**). Thus, TEM1 may contribute to the migration and invasion of MES-SA cells.

Silencing of the TEM1 protein impairs the ability of MES-SA cells to form colonies

Colony formation assays were performed using MES-SA cells transfected with or without the TEM1-siRNA. The number of viable TEM-siRNA-transfected MES-SA cell colonies was reduced compared with the cells transfected with the scrambled siRNA control (P<0.05; **Figure 6A** and **6B**), suggesting that the ability of TEM1 knock-down cells to form colonies was inhibit-

ed. Notably, siRNA1 showed a greater inhibitory effect than siRNA2 (P<0.05).

Silencing of the TEM1 protein inhibits the epithelial-mesenchymal transition (EMT) of MES-SA cells

We examined the levels of the EMT marker proteins E-cadherin, N-cadherin and Vimentin in cells with different levels of TEM1 suppression to further determine whether TEM1 affects the metastatic potential of leiomyosarcoma cells (**Figure 7A** and **7B**). According to the results of the western blot assay, TEM1 knock-down cells exhibited decreased N-cadherin (siRNA1 P< 0.05, siRNA2 P<0.01) and Vimentin (siRNA1 P<0.05, siRNA2 P<0.01) expression and increased E-cadherin expression (siRNA1 P<0.05, siRNA2 P>0.05) compared with cells transfected with the scrambled siRNA; this effect was more obvious in siRNA1-transfected cells. Based on these results, TEM1 triggers the EMT and promotes the metastasis of MES-SA cells; these effects are decreased when TEM1 expression is silenced.
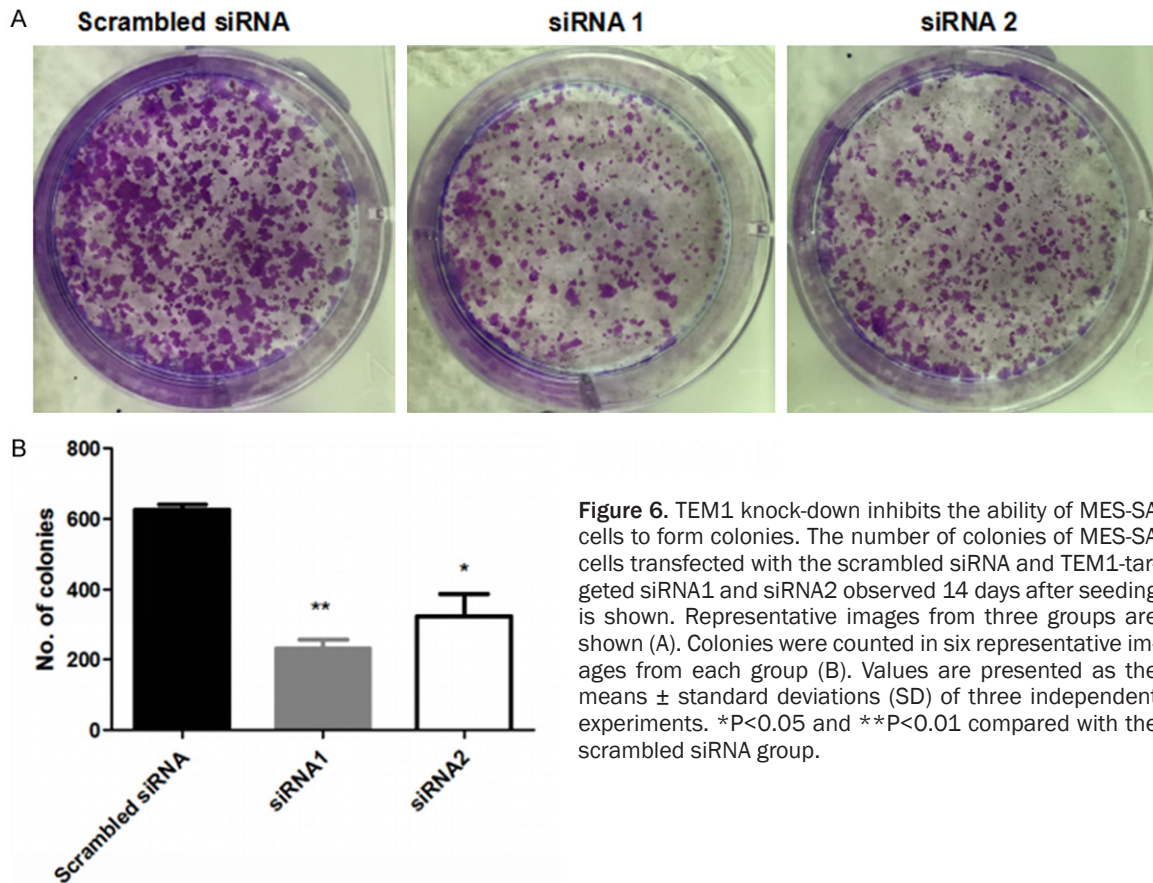
Figure 6. TEM1 knock-down inhibits the ability of MES-SA cells to form colonies. The number of colonies of MES-SA cells transfected with the scrambled siRNA and TEM1-targeted siRNA1 and siRNA2 observed 14 days after seeding is shown. Representative images from three groups are shown (A). Colonies were counted in six representative images from each group (B). Values are presented as the means ± standard deviations (SD) of three independent experiments. *P<0.05 and **P<0.01 compared with the scrambled siRNA group.

Discussion

Uterine leiomyosarcoma, which is associated with a high probability of metastasis and poor prognosis, is one of the most aggressive forms of malignancy. Recent studies have attempted to identify a reliable biomarker for uterine leiomyosarcoma treatment and prognosis. DNA topoisomerase II alpha (TOP2A) [17] is expressed in leiomyosarcoma but not in benign smooth muscle tumors. CD146 expression is correlated with lymph node metastasis and poor overall survival [18]. High p16 and phospho-histone H3 pHH3 expression and low progesterone receptor (PR) expression help distinguish leiomyosarcoma from leiomyoma [19].

TEM1 is detected in high-grade sarcomas and sites of dissemination [14], and its expression levels are positively correlated with the disease grade. Thus, TEM1 has emerged as a suitable therapeutic and prognostic target for advanced sarcoma. The TEM1 protein was reported to promote the proliferation and invasiveness of bone sarcoma [15] and brain tumors [20].

TEM1 is expressed at high levels in the uterine sarcoma MES-SA cell line and an in vivo xenograft using these cells [14]. However, the value of its expression remains unknown.

The present study confirmed that the TEM1 protein is expressed in MES-SA cells using qPCR and flow cytometry, corroborating the results of previous studies. According to the results of wound-healing, transwell and CCK-8 assays, TEM1 silencing inhibits the migration, metastasis and proliferation of MES-SA cells. The ability of these cells to form colonies was also impaired by TEM1 knock-down.

EMT is considered a critical process in tumor aggressiveness and is involved in the recurrence and metastasis of sarcoma as well as the overall survival rate of patients with these tumors [21]. In the EMT process, E-cadherin expression is significantly decreased, and N-cadherin expression is elevated; subsequently, other EMT-related markers such as Vimentin are overexpressed. The EMT was reported to be triggered by Y-box-binding protein 1 (YB-1) dur-
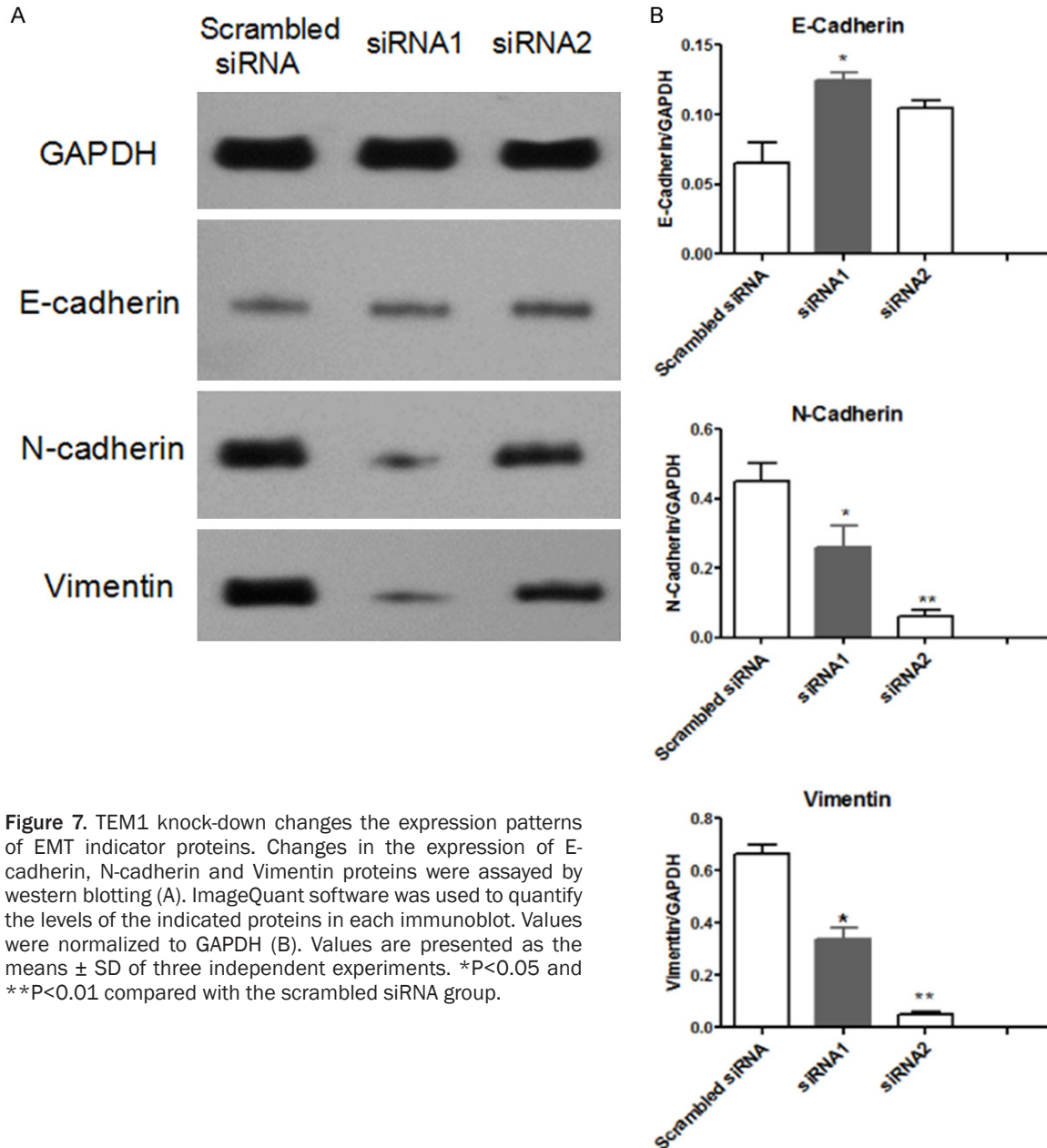
Figure 7. TEM1 knock-down changes the expression patterns of EMT indicator proteins. Changes in the expression of E-cadherin, N-cadherin and Vimentin proteins were assayed by western blotting (A). ImageQuant software was used to quantify the levels of the indicated proteins in each immunoblot. Values were normalized to GAPDH (B). Values are presented as the means ± SD of three independent experiments. *P<0.05 and **P<0.01 compared with the scrambled siRNA group.

ing sarcoma metastasis [22]. mRNA-130a promotes the metastasis and EMT of osteosarcoma [23]. Progesterone receptor membrane component 1 (PGRMC1) facilitates MES-SA cell migration and invasion by inducing the EMT [3]. According to our data, the EMT was significantly inhibited in TEM1-silenced MES-SA cells, indicating that TEM1 might contribute to the metastatic potential of uterine sarcoma cells via the EMT.

Because of the diversity of the histopathological characteristics and rarity of uterine sarco-

ma samples, studies associated with the molecular mechanisms of the disease are challenging. A recent study identified TEM1 expression in sarcoma SP cells, which are highly tumorigenic and regenerative [24]. Another study based on 945 clinical specimens revealed that TEM1 might be a marker for poor prognosis in patients with gastric cancer [25]. Clinical specimens are needed to evaluate whether altered TEM1 expression is correlated with the clinical parameters of these patients and to further elucidate the clinical value of the tumor-initiating, chemo-resistant and prognostic functions

of the TEM1 protein in uterine sarcoma development.

Based on the results of our study, TEM1 promotes the invasiveness and EMT of uterine sarcoma cells. We are performing additional in vivo studies to better elucidate the mechanism by which TEM1 expression affects sarcoma development. Based on the available published data, little to no pulmonary metastasis has been observed in mouse models of subcutaneous uterine sarcoma [2, 26]. The study by Nanda et al. [27] reported reduced tumor volumes in TEM1 knock-out orthotopic models but not in subcutaneous models, indicating that the stroma controls tumor growth in an organ-specific manner. Therefore, orthotopic uterine sarcoma models are needed to overcome the shortage of clinical uterine sarcoma specimens and to further evaluate the process of metastasis.

In conclusion, our study identified TEM1 expression in MES-SA uterine sarcoma cell line and established an in vitro TEM1-silenced model by knocking down TEM1 expression using siRNAs. TEM1 silencing inhibits the migration, metastasis, proliferation and colony formation ability of MES-SA cells, indicating that a TEM1-targeted treatment might offer new therapeutic strategies for uterine sarcoma.

Acknowledgements

Disclosure of conflict of interest

None.

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