

A novel role of thrombospondin-1 in cervical carcinogenesis: inhibit stroma reaction by inhibiting activated fibroblasts from invading cancer

Ming-Ping Wu^{1,2,3}, Ming-Jer Young^{1,2}, Ching-Cherng Tzeng⁴, Chii-Ruey Tzeng³, Kuo-Feng Huang¹, Li-Wha Wu^{2,5} and Cheng-Yang Chou^{2,6,*}

¹Department of Obstetrics and Gynecology, Chi Mei Foundation Hospital, Tainan 710, Taiwan, ²Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, Tainan 704, Taiwan, ³Department of Obstetrics and Gynecology, College of Medicine, Taipei Medical University, Taipei 110, Taiwan, ⁴Department of Pathology, Chi Mei Foundation Hospital, Tainan 710, Taiwan, ⁵Institute of Molecular Medicine and ⁶Department of Obstetrics and Gynecology, College of Medicine, National Cheng Kung University, Tainan 704, Taiwan

*To whom correspondence should be addressed. Tel: +886 6 2353535 ext. 5608; Fax: +886 6 2766185; Email: chouyng@mail.ncku.edu.tw
Correspondence may also be addressed to Li-Wha Wu. Tel: 886 6 2353535 ext. 3618; Fax: 886 6 2095845; Email: liwhawu@mail.ncku.edu.tw

Thrombospondin (TSP)-1, a potent angiogenesis inhibitor, has been shown to exert different biological functions on various cell types. Here, we investigate the role of TSP-1 in tumor–stroma reaction, which is mainly characterized by fibroblast activation to create a permissive microenvironment for tumor progression. Immunohistochemistry examinations in the human surgical specimens have shown that a downregulation of TSP-1 during the progression of cervical carcinogenesis was accompanied by an emergence in the upregulation of stroma markers, α -smooth muscle actin (α -SMA) and desmin. Transfection of SiHa cervical cancer cells with a plasmid expressing the TSP-1 protein exhibited antiangiogenic activity *in vitro* and resulted in reduced tumor growth in severe combined immunodeficiency (SCID) mice, which was accompanied by a decrease in tumor vascularization and lower expressions of α -SMA and desmin than those in the vector controls. Transfection with TSP-1 and purified TSP-1 added to NIH3T3 cells did not alter the protein levels of α -SMA and desmin but significantly inhibited matrix metalloproteinase-2 activity. Transforming growth factor- β (TGF- β), a major factor in the activation of fibroblasts, increased α -SMA and desmin expression and the ability of cell migration and invasion in NIH3T3 cells. The increased migration ability and the invasive ability into tumor cluster of TGF- β -treated NIH3T3 cells were dose dependently inhibited by TSP-1. In contrast, ectopic TSP-1 expression in SiHa cells has little effect on the invasive ability of the NIH3T3 cells. Together, our findings demonstrate a novel role of TSP-1 to inhibit tumor–stroma reaction that could be attributed to the blockage of activated fibroblasts from invading cancer cells.

Introduction

Our understanding of cancer has largely come from the analysis of aberrations within the tumor cell population. Recent evidence has begun to emerge regarding the important role of tumor microenvironment in tumorigenesis (1). Stroma reaction, also known as stromagenesis, is a host response of mesenchymal alteration induced in cancer that produces a progressive and permissive mesenchymal microenvironment, thereby supporting tumor progression (2). Stroma reaction is characterized by the activation of quiescent fibroblasts,

Abbreviations: CIS, carcinoma *in situ*; CM, conditioned medium; HUVEC, human umbilical vein endothelial cell; MMP, matrix metalloproteinase; SCC, squamous cell carcinoma; SCID, severe combined immunodeficiency; α -SMA, α -smooth muscle actin; SPARC, secreted protein, acidic and rich in cysteine; TGF- β , transforming growth factor- β ; TSP-1, thrombospondin.

the predominant cell type within a normal stroma, into activated fibroblast. Activated fibroblasts are defined by the expression of α -smooth muscle actin (α -SMA), desmin, vimentin, etc in the fibroblasts (3). Activated fibroblasts can produce non-cellular scaffolds in response to extracellular stimuli and create an environment promoting tumor progression (4). In addition, activated fibroblasts within tumor stroma have a propensity to migrate and invade like cancer cells (5). The proliferative activity of activated fibroblasts in cancer-induced stroma is closely linked to tumor progression, lymph node and distant organ metastasis of breast cancer (6).

Thrombospondins (TSPs) consist of a family of five extracellular proteins that participate in cell-to-cell and cell-to-matrix communications (7). Among them, TSP-1 is a 450 kDa homotrimeric matricellular glycoprotein with potent antiangiogenic effects. In many tumor types, TSP-1 can block *in vivo* neovascularization and decrease malignant tumor growth (8,9); whereas in others, it promotes cancer cell adhesion, migration and invasion (10). The differential effects of TSP-1 on tumorigenesis indicate that TSP-1 exerts different biological functions in different cell types.

We demonstrated previously that the downregulation of TSP-1 in cervical epithelium temporally and spatially coincided with the emergence of angiogenic switch during cervical carcinogenesis (11). However, the exact biological roles of TSP-1 in tumor–stroma reaction and progression require further exploration. This study was initiated to test the hypothesis that TSP-1 may inhibit stroma reaction, which supports tumor progression by the inhibition of activated fibroblasts from invading cancer cells. By studies in cell lines, SCID mice xenograft and clinical human specimens, we demonstrated a novel role of TSP-1 by which it inhibited tumor progression, at least in part, through blocking the migration and invasion of activated fibroblasts and leading to stroma normalization.

Materials and methods

Sample selections

Surgical specimen collections, obtained from cervical conization or hysterectomy, were retrieved from the Department of Pathology, Chi Mei Foundation Hospital, Tainan, Taiwan. Patients who had pathological diagnoses of carcinoma *in situ* (CIS) and International Federation of Gynecology and Obstetrics stage Ib squamous cell carcinoma (SCC), in which tumor is confined to cervix but beyond microscopic lesion ($n = 15$ from each group), were recruited in this study. They received conization for CIS or radical hysterectomy for SCC. Two representative blocks from each specimen were analyzed. In addition, two tissue sections containing normal cervical epithelium from 15 patients with benign uterine disease who underwent hysterectomy for uterine leiomyoma or adenomyosis were used as normal controls.

Immunohistochemical staining and scoring system

Formalin-fixed, paraffin-embedded tissue blocks were cut into serial sections with a thickness of 4 μ m. One section from each sample was stained with hematoxylin–eosin for confirmation of histological diagnoses by one of the authors (C.-C.T.). Adjacent sections were stained for TSP-1, α -SMA and desmin in human surgical specimens and TSP-1, α -SMA, desmin and CD34 in mice xenograft by the use of standard immunoperoxidase staining methods. The paraffin was removed in xylene, and the tissue sections were rehydrated in descending dilutions of ethanol. After sections were boiled for 13 min in a microwave oven in 10 mM citrate (pH 6.0) buffer, they were treated with a 3% hydrogen peroxide solution to block endogenous peroxidase activity. Specimens were then incubated with mouse anti-human TSP-1 (Calbiochem Co., La Jolla, CA; 1:250), α -SMA 1A4 (Dako, Glostrup, Denmark; 1:50), desmin D33 (Dako; 1:250) or CD34 monoclonal antibodies (Serotec, Oxford, UK; 1:250). Slides were then incubated with a supersensitive immunodetection system of BioGenex (San Ramon, CA). The 3,3'-diaminobenzidine tetrahydrochloride was used as a chromogen. Human placenta was used as the positive control for TSP-1, whereas smooth muscle cells were used as the positive control for α -SMA and desmin. A negative control, for which the primary

antibody was substituted with the same concentration of the appropriate IgG, was used in each staining run. Sections were counterstained with hematoxylin. All stained slides were examined by the authors (C.-C.T. and M.-P.W.) who were blinded to the patients' clinical information. The expression of α -SMA and desmin was categorized into four grades. They were arbitrarily scored as 0, no to little staining; 1, weak staining; 2, moderate staining and 3, strong staining. Microvessel density was defined as the number of blood vessels characterized as CD34-positive tube-like structures and scored as 0, no to scanty; 1, scanty; 2, moderate and 3, abundant.

Cell cultures and transfection

The cervical cancer SiHa cell line and mouse fibroblast NIH3T3 cells were used to study TSP-1 function. Epithelial cells and fibroblasts derived from normal uterine cervix were used as representatives of normal, quiescent cells. Normal cervical epithelial cells were prepared as described previously (12). Normal cervical fibroblast cells were derived in a similar manner except that Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (Biological Industries Co., Haemek, Israel) was used instead of serum-free keratinocyte medium for epithelial cells.

Full-length human TSP-1 complementary DNA construct, a kind gift from Prof. Frazier, W.A. at Washington University, St Louis, MO (13), was subcloned into the eukaryotic expression vector pCDNA3.1 (Invitrogen, Rockville, MD) and transfected into SiHa or NIH3T3 cells by using Lipofectamine 2000 (Invitrogen). G418 at a final concentration of 1 mg/ml was added 24 h after transfection. The G418-resistant colonies were expanded and examined for the expression of TSP-1. To assess proliferation, cells were plated at the density of 10^5 per dish on 60 mm dishes and the medium was changed every 2 days. Viable cells were counted with a hemocytometer using trypan blue (0.4%) exclusion.

Immunoblotting

Equal amounts of protein lysates were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 80–100 V for 2.5–3 h. The proteins were transferred to polyvinylidene difluoride membrane (Amersham Corp., Arlington Heights, IL). The blots were incubated with anti-human TSP-1 antibody (Neomarker, Fremont, CA; 1:1000), anti-human α -SMA 1A4 antibody, anti-desmin antibody (Abcam, Cambridge, MA) or anti-matrix metalloproteinases (MMP)-2 and -9 (Calbiochem, Darmstadt, Germany; 1:500). Blots were then processed by chemiluminescent substrate detection system (Amersham Corp.).

Matrigel angiogenesis assay

Matrigel (BD Biosciences, Bedford, MA) was thawed overnight at 4°C and then was added (80 μ l) to each well of a 96-well plate at 37°C and allowed to polymerize (14). Before angiogenesis assay, human umbilical vein endothelial cells (HUVECs) were cultured at low serum condition (M199 with 2% fetal bovine serum) overnight. A suspension of 20 000 HUVECs in M199 medium was seeded into each well coated with Matrigel. The cells were treated with 40 μ g/ml bovine serum albumin, indicated culture medium from different cell lines, or purified TSP-1 (Sigma, St Louis, MO) in various concentrations. Cells were incubated for 6–8 h at 37°C and viewed using an Olympus Optical (Tokyo, Japan) CK30 microscope. The formation of tubular network structures represents angiogenesis ability. All assays were performed in triplicate.

Animal models

Subcutaneous tumors were generated by inoculating 10^7 SiHa-vector or SiHa-TSP-1 cells into six aged 9-week-old SCID mice in each group. All manipulations were conducted under aseptic conditions in a laminar flow hood. The smallest and largest tumor diameters were measured weekly, using a digital caliper, and the tumor volumes were calculated using the following formula: volume = $4/3\pi \times (1/2 \text{ smaller diameter})^2 \times 1/2 \text{ larger diameter}$. The mice were killed for histological examination 12 weeks after the tumor cell inoculation. The animal experiments were performed according to the ethical guidelines and approved by the institutional ethical committee.

Fibroblast activation

Transforming growth factor- β (TGF- β) (Sigma) at 2–20 ng/ml was used to activate NIH3T3 fibroblasts for the indicated time (15). TGF- β at 10 ng/ml was later used to activate fibroblasts for cell migration assay and 20 ng/ml for Matrigel multicellular coculture invasion assays.

Cell migration assay and MMPs zymography

Cell migration was assayed in the 48-well Boyden chamber (Neuro Probe, Gaithersburg, MD) as described (16). A 50 μ l aliquot containing 5000 cells of NIH3T3 or NIH3T3-TSP-1 was placed on the upper compartment, and fibronectin was used as the chemoattractant in the lower compartment of the chamber. The assays were run for 6 h in Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin at 37°C. After incubation, cells were

fixed with methanol, stained with Giemsa solution (Merck, Darmstadt, Germany) and counted immediately after staining. Conditioned medium (CM) from the culture was cleared of cells and debris by centrifugation at 3000g for 10 min and concentrated by 10-fold using Amicon Microcon (Ym-10). MMP-2 and MMP-9 activities in the CM were measured by gelatin zymography as described previously (17).

Matrigel multicellular coculture invasion assay of activated fibroblasts

To detect the invasive ability of fibroblasts into tumor cells cluster, we applied the Matrigel multicellular coculture system as described by Walter-Yohrling *et al.* (18) with a slight modification. A layer of Matrigel (80 μ l) was added to each well of a 96-well plate and allowed to polymerize. In the central area of each well, a Matrigel plug of ~ 2 μ l was removed and then filled with 10^5 cancer cells (SiHa or SiHa-TSP-1) in 5 μ l of Matrigel. The Matrigel was allowed to polymerize for 30 min to form a tumor cell cluster. A total of 10^5 fluorescent dye PKH26-labeled (red) or PKH67-labeled (green) (Sigma) fibroblasts (normal fibroblasts, NIH3T3 or NIH3T3-TSP-1) in 150 μ l aliquot were dispersed diffusely in the periphery of each well. Following 16–24 h incubation, red fluorescence for PKH26 dye or green fluorescence for PKH67 and bright field images were captured with an inverted fluorescent light microscope (Olympus IX70). Positive invasion of activated fibroblasts was defined as the presence of fibroblasts accumulation in the central tumor cluster following activated fibroblasts migration and invasion toward tumor cluster. In contrast, absence of invasion is defined as diffuse distribution of fibroblasts in the periphery of tumor clusters.

Statistical analysis

All values were reported as mean \pm SD. Kruskal–Wallis test with Dunn *post hoc* comparison test was used for statistical analysis in α -SMA and desmin expression in normal cervix, CIS and SCC among the non-parametric measurement multigroups comparison. Mann–Whitney *U*-test was used for two-group comparison in SiHa-induced or SiHa-TSP-1-induced tumors. Repeated analysis of variance measurement was used for tumor growth curves. Student's *t*-test was used for NIH3T3 cell migration assay in which TSP-1 was added in lower, upper or both or comparing NIH3T3 with or without TGF- β treatment.

Results

The downregulation of TSP-1 coincides with the upregulation of stroma markers during cervical carcinogenesis

A representative immunohistochemistry staining of TSP-1, α -SMA and desmin in various human cervical epithelial lesions was shown in Figure 1. In the group of normal cervical epithelium, TSP-1 expression was localized mainly in basal epithelial cell layer, but became invisible in CIS and SCC epithelia (upper panel, Figure 1). In contrast, the stains for α -SMA (middle panel, Figure 1) and desmin (lower panel, Figure 1) were hardly visible in the stromal area of normal cervix. The staining intensities of α -SMA and desmin increased overtly in stromal area of CIS and SCC. The mean α -SMA and desmin scores in the three groups are summarized in Table I. Statistical analyses with Kruskal–Wallis test revealed that α -SMA and desmin scores were significantly different among normal, CIS and SCC groups. In addition, α -SMA scores were significantly different when compared normal with CIS, CIS with SCC and normal with SCC with *P* values of 0.002, 0.006 and <0.001 , respectively. Similarly, desmin scores were also significantly different among pair-wise comparisons with *P* values of 0.019, <0.001 and <0.001 , respectively (Dunn *post hoc* comparison test).

TSP-1 inhibits angiogenesis, tumor development and SCC-induced stromal reaction in vivo

To test whether manipulation of TSP-1 activity would alter tumor formation *in vivo* and whether TSP-1 is involved in tumor–stroma reaction, we developed SiHa-TSP-1 cell lines that were stably transfected with the full-length TSP-1 complementary DNA. Immunoblots confirmed the overexpression of TSP-1 in C44 and C57 clones as compared with parental cells (SiHa) or vector control (C5, C9 and C12) (Figure 2A). The C44 clone (SiHa-TSP-1) was used for further study. As shown in Figure 2B, increased expression of TSP-1 did not alter the growth rate of cancer cells. We next examined the angiogenic activity of TSP-1 by Matrigel angiogenesis assay. HUVECs seeded on Matrigel rapidly formed tubule networks within 6 h (left

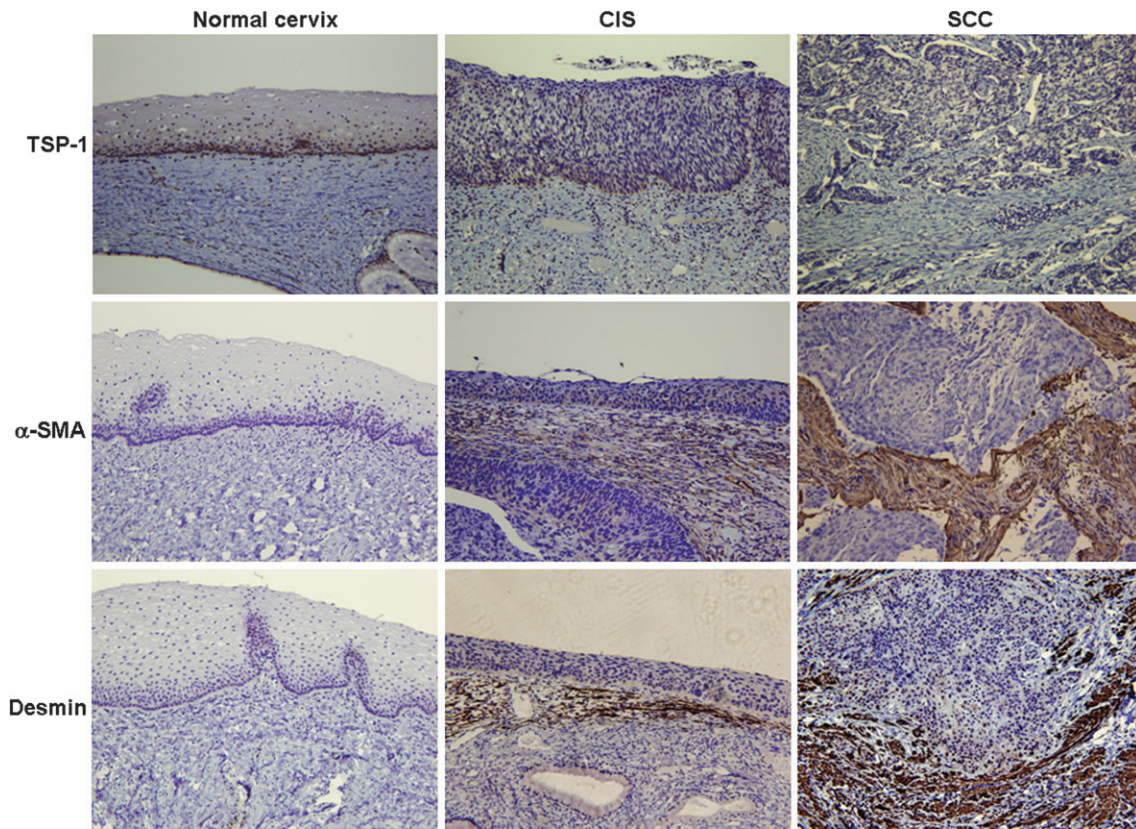


Fig. 1. Immunohistochemistry ($\times 200$) of representative subjects with normal cervical epithelium, CIS and SCC revealed that TSP-1 was localized in basal epithelial layer of normal cervix and was downregulated in CIS and SCC. In direct contrast, the staining intensities of α -SMA and desmin were significantly upregulated in the stroma of CIS and SCC lesions compared with those in normal cervix.

Table I. Distribution of stroma markers, α -SMA and desmin scores, in the three groups of normal cervical epithelium, CIS or SCC

α -SMA				Desmin			
Cervical lesions	<i>n</i>	Mean \pm SD	<i>P</i> value	Cervical lesions	<i>n</i>	Mean \pm SD	<i>P</i> value
Normal	15	0.67 \pm 0.42	<0.001	Normal	15	0.21 \pm 0.18	<0.001
CIS	15	2.13 \pm 0.61		CIS	15	0.57 \pm 0.31	
SCC	15	2.78 \pm 0.21		SCC	15	1.78 \pm 0.44	

Statistical analyses with Kruskal–Wallis test.

upper panel, Figure 2C), which was attenuated by either the purified TSP-1 at 10 μ g/ml or by the CM from normal cervical epithelium (upper panel, Figure 2C). In the same line of observations, only TSP-1 containing CM from SiHa-TSP-1 (SiHa-TSP-1 CM) but neither parental SiHa (SiHa CM) nor vector control cells (SiHa-vector CM) had potent inhibitory effect on the tube-forming ability of HUVECs (lower panel, Figure 2C).

The SiHa-TSP-1 and its vector control were inoculated into SCID mice subcutaneously. Rapid tumor growth was evident in SCID mice inoculated with vector control cells. In contrast, SiHa-TSP-1 cells exhibited significantly reduced rate of tumor growth and tumor size ($P = 0.013$, analysis of variance test; Figure 3A and B). Moreover, the SiHa-TSP-1-inoculated tumor was less vascularized as compared with SiHa-vector control (CD34, Figure 3C) (0.58 ± 0.18 versus 1.67 ± 0.19 , $n = 6$, $P = 0.002$). In SiHa-vector-inoculated tumor, the expression level of TSP-1 was negligible, whereas TSP-1 was much highly expressed in tumor site and stroma area of SiHa-TSP-1-inoculated tumor. In contrast, the expression levels of α -SMA and desmin were high in SiHa-vector-inoculated tumor, but significantly reduced in SiHa-TSP-1-inoculated tumor (Figure 3C; 0.95 ± 0.42

versus 2.17 ± 0.43 for α -SMA, $P = 0.002$; 0.48 ± 0.46 versus 1.52 ± 0.63 for desmin, $P = 0.015$, Mann–Whitney *U*-test).

TSP-1 does not downregulate α -SMA and desmin expression but inhibits MMP-2 activity

Phenotypic switching of quiescent fibroblasts into activated fibroblasts is the most prominent stromal reaction during carcinogenesis. Activated fibroblasts are often characterized by acquisition of high α -SMA expression. Due to difficulties in long-term culturing primary cells, NIH3T3 fibroblasts were used instead. Western blot analysis showed that NIH3T3 cells had higher level of α -SMA expression than those in normal primary cervical fibroblasts and HUVECs (Figure 4A). To examine if TSP-1 could directly modulate the α -SMA expression, we established clones stably expressing TSP-1 protein in NIH3T3 cells (NIH3T3-TSP-1). Western blot confirmed the expression of TSP-1 protein in NIH3T3-TSP-1 but not NIH3T3 or vector control cells (Figure 4A and B). TSP-1 overexpression by transfection or by exogenous addition of TSP-1 (1–20 μ g/ml) did not have any detectable effect on the expression of the α -SMA and desmin in these cells

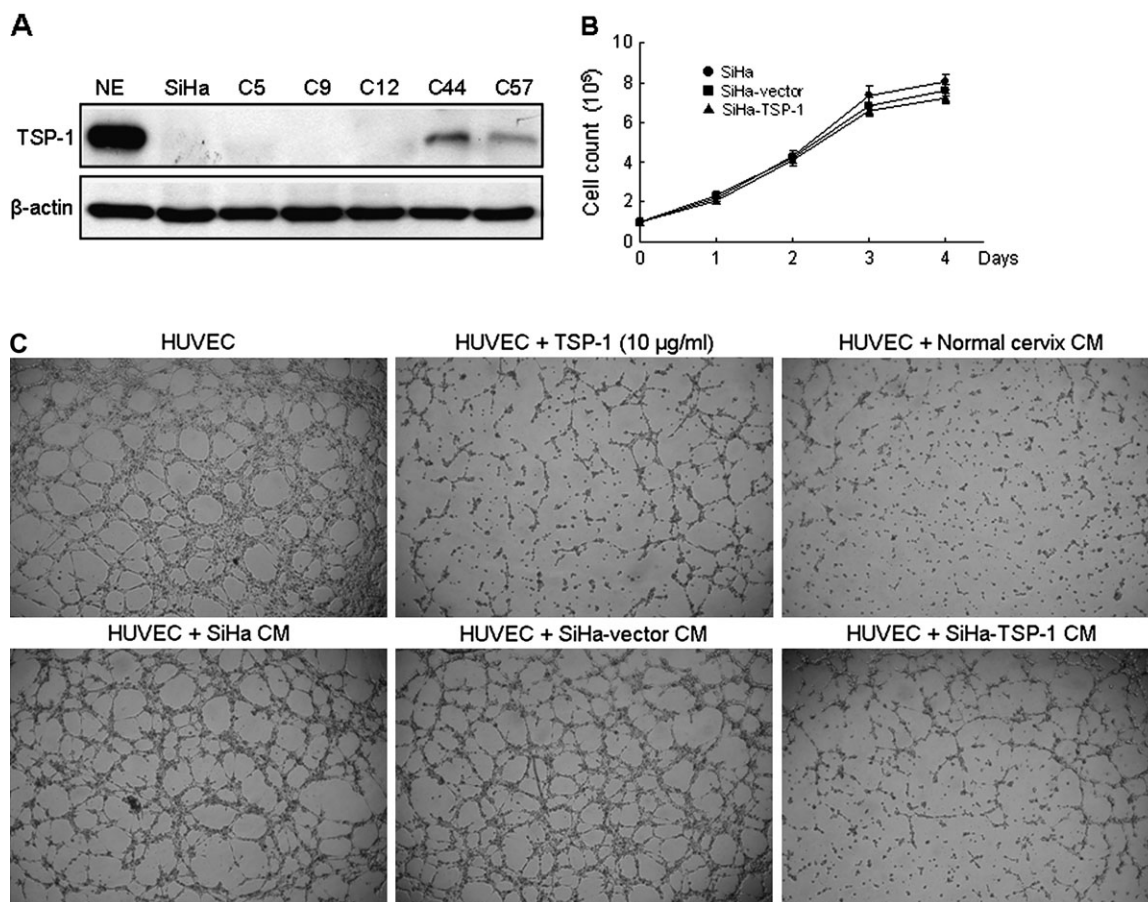


Fig. 2. Molecular characterization and functional expression of TSP-1 in SiHa cells. **(A)** TSP-1 levels measured by western blotting in parental cells (SiHa), cells transfected with empty vector (C5, C9 and C12) and cells transfected with human TSP-1 complementary DNA (C44 and C57). Positive control was total protein isolated from normal cervical epithelial cells (NE). **(B)** Cell numbers were counted at the indicated time points with a hemocytometer by using trypan blue exclusion to monitor cell viability. **(C)** The antiangiogenic ability of TSP-1. Shown are the representative results of Matrigel angiogenesis assay to determine the formation of vascular network by HUVECs under normal culture condition, treated with indicated CM from different cell lines (SiHa, SiHa-vector and SiHa-TSP-1) or purified TSP-1 at 10 μ g/ml. All assays were performed in triplicate.

(Figure 4B). We then tested the effect of TSP-1 on the MMP-2 and MMP-9 activities, which are key regulators for matrix degradation. Elevated levels of TSP-1 either by plasmid transfection or by direct addition of TSP-1 at concentrations ranging from 1 to 20 μ g/ml did not downregulate the expression of MMP-2 protein level (Figure 4B; the MMP-9 level was negligible, and therefore the data were not shown). However, MMP-2 activity was inhibited by both ectopic expression and the addition of TSP-1 in a dose-dependent manner using gelatin zymography (Figure 4C).

TSP-1 inhibits the migration of untreated and TGF- β -treated NIH3T3 cells

TGF- β , a potent fibroblast activation and transdifferentiation factor, was used to activate NIH3T3 fibroblasts (19). To elucidate the biological functions associated with TGF- β -mediated fibroblast activation, we examined the effects of TGF- β on α -SMA, desmin expression and MMP activity in NIH3T3 cells. TGF- β concentrations as low as 2 ng/ml potentially enhanced the expression of α -SMA and desmin. TSP-1 was added to TGF- β -treated NIH3T3 to see whether or not it could change α -SMA expression after TGF- β treatment. The TGF- β -increased α -SMA expression could not be blocked with TSP-1 (Figure 4D). No significant change of MMP-2 protein levels and its gelatinolytic activity were observed in TGF- β -treated cells (Figure 4D and E). We then compared the effects of TSP-1 on the migration ability of untreated and TGF- β -treated NIH3T3 cells using a cell migration assay. TGF- β at 10 ng/ml increased NIH3T3 cell migration

($P = 0.038$). The stimulatory effect of TGF- β on cell migration was inhibited by purified TSP-1, $P = 0.010$, 0.007 and 0.006. The migration ability of untreated NIH3T3 was also markedly inhibited by TSP-1 at 10 μ g/ml in the lower, upper or both chambers (with $P = 0.021$, 0.009 and 0.009, Student's *t*-test) (Figure 4F). The inhibitory effect was in a dose-dependent manner at TSP-1 concentrations of 0.1–10 μ g/ml (data not shown). Reduced cell migration was also observed in NIH3T3-TSP-1 cells compared with NIH3T3-vector cells, $P = 0.044$ (Figure 4F).

TSP-1 inhibits activated fibroblasts from invading tumor cell cluster

Activated fibroblasts, like other invasive cancer cells, have the ability to invade tumor cell clusters (5). Since TSP-1 directly reduced MMP-2 activity and TGF- β -induced migration ability, we examined the effect of TSP-1 on the invasive ability of activated fibroblasts into tumor cell cluster. NIH3T3 cells, but not normal human fibroblasts, expressed α -SMA and had the ability to invade SiHa tumor cluster, regardless of the expression status of TSP-1 in tumor cells (Figure 5A). Exogenous TSP-1 (10, 20 or 40 μ g/ml) in tumor cluster also failed to inhibit NIH3T3 from invading SiHa tumor cluster (Figure 5B). In contrast, the invasive ability of NIH3T3-TSP-1 cells to SiHa (or SiHa-TSP-1) tumor cluster was significantly reduced when compared with NIH3T3, indicating a direct inhibitory effect of TSP-1 on the invasive ability of NIH3T3 (Figure 5A). Next, we examined the effect of exogenous TSP-1 on the invasive ability of NIH3T3 cells. Consistent with stable expression of TSP-1 in NIH3T3 cells, we found

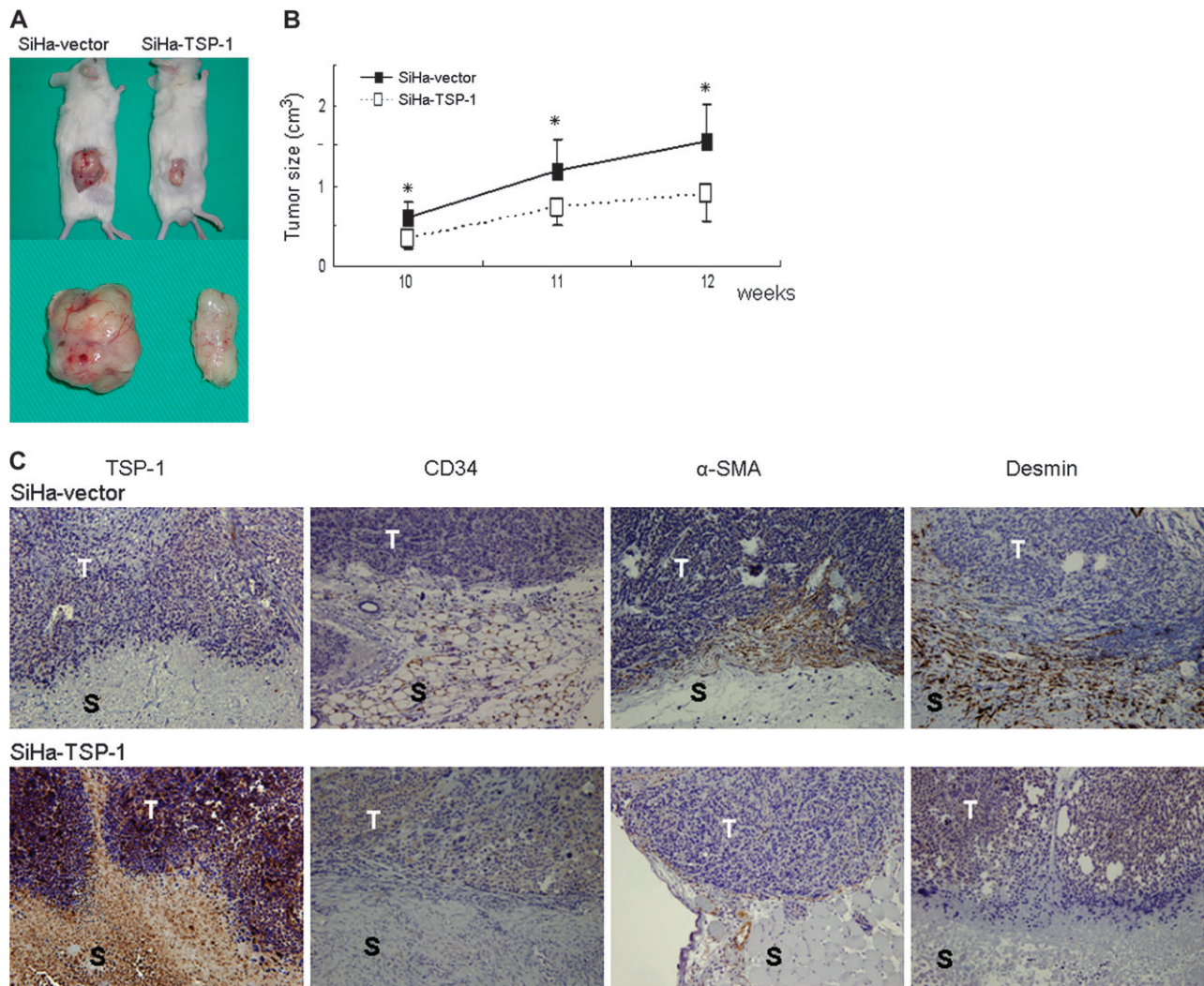


Fig. 3. TSP-1 inhibits tumor development, tumor angiogenesis and stroma marker expression in SCID mice. (A) Representative gross tumor xenografts resulting from the transplantation of vector control (SiHa-vector) or cells transfected with human TSP-1 complementary DNA (SiHa-TSP-1) at 12 weeks after tumor transplantation. (B) Tumor growth curves of SCID mice subcutaneously inoculated with SiHa-vector or SiHa-TSP-1 cell lines. Each point is mean \pm standard deviation ($n = 6$ in each group). (C) Immunohistochemistry ($\times 200$) of representative sections from SCID mice subcutaneously inoculated with SiHa-vector or SiHa-TSP-1 cell lines revealed a negligible level of TSP-1 in tumor (T) and stroma (S) area, highly vascularized as shown by positive CD34 stain and strong staining intensities for α -SMA and desmin in SiHa-vector-induced tumor stroma. In contrast, the SiHa-TSP-1-induced tumor showed less vascularization and overexpressed TSP-1 with downregulated α -SMA and desmin staining intensities compared with the SiHa-vector-induced tumor.

a dosage-dependent inhibition of NIH3T3 cell invasion. A complete inhibition was observed at 20 μ g/ml or higher (Figure 5C). We further used TGF- β to activate NIH3T3 and investigated the effect of TGF- β on TSP-1-mediated inhibition of NIH3T3 invasion. TSP-1 potentially inhibited fibroblast invasion regardless of the presence of TGF- β , although a higher dose of TSP-1 was required for a complete inhibition of TGF- β -treated NIH3T3 cells (Figure 5C and D). Taken together, our results indicate that the TSP-1-mediated inhibition of the invasive ability could only be demonstrated when the manipulation of TSP-1 expression was in fibroblasts, but not in tumor cells.

Discussion

Our current understanding on the role of TSP-1 in tumor progression and clinical prognosis has extended far beyond antiangiogenesis. The present study provides evidence that TSP-1 has the potential to inhibit stroma reaction during cervical carcinogenesis. This conclusion is supported by the following findings: (i) The concordance of the downregulation of TSP-1 and the upregulation of stroma markers in surgi-

cal specimens of cervical carcinoma suggests that TSP-1 plays an inhibitory role in stroma reaction. (ii) Transfection of SiHa cells with a plasmid expressing the TSP-1 protein resulted in reduced tumor growth in SCID mice that was accompanied by a decrease in tumor vascularization and a lower level of stroma markers, α -SMA and desmin, than the vector transfection. (iii) Ectopic expression of TSP-1 or by addition of purified TSP-1 manifested an inhibition of MMP-2 activity, TGF- β -enhanced cell migration and the invasive ability of activated fibroblasts from tumor cell clusters.

We previously pointed out that the switch of angiogenic phenotype, partly due to the downregulation of TSP-1, occurred during the transition from low-grade to high-grade squamous intraepithelial lesion (11). In this study, we demonstrated a temporal inverse correlation of TSP-1 and stromal marker expression during cervical carcinogenesis using human clinical specimens. The inhibitory effect of TSP-1 on stromal marker expression was further confirmed in SCID mouse xenografts using transfection of TSP-1 complementary DNA expression vectors. Genetic manipulation of TSP-1 expression level in the cells demonstrated that TSP-1-mediated inhibition of stroma reaction was primarily due to the inhibition of activated fibroblast migration

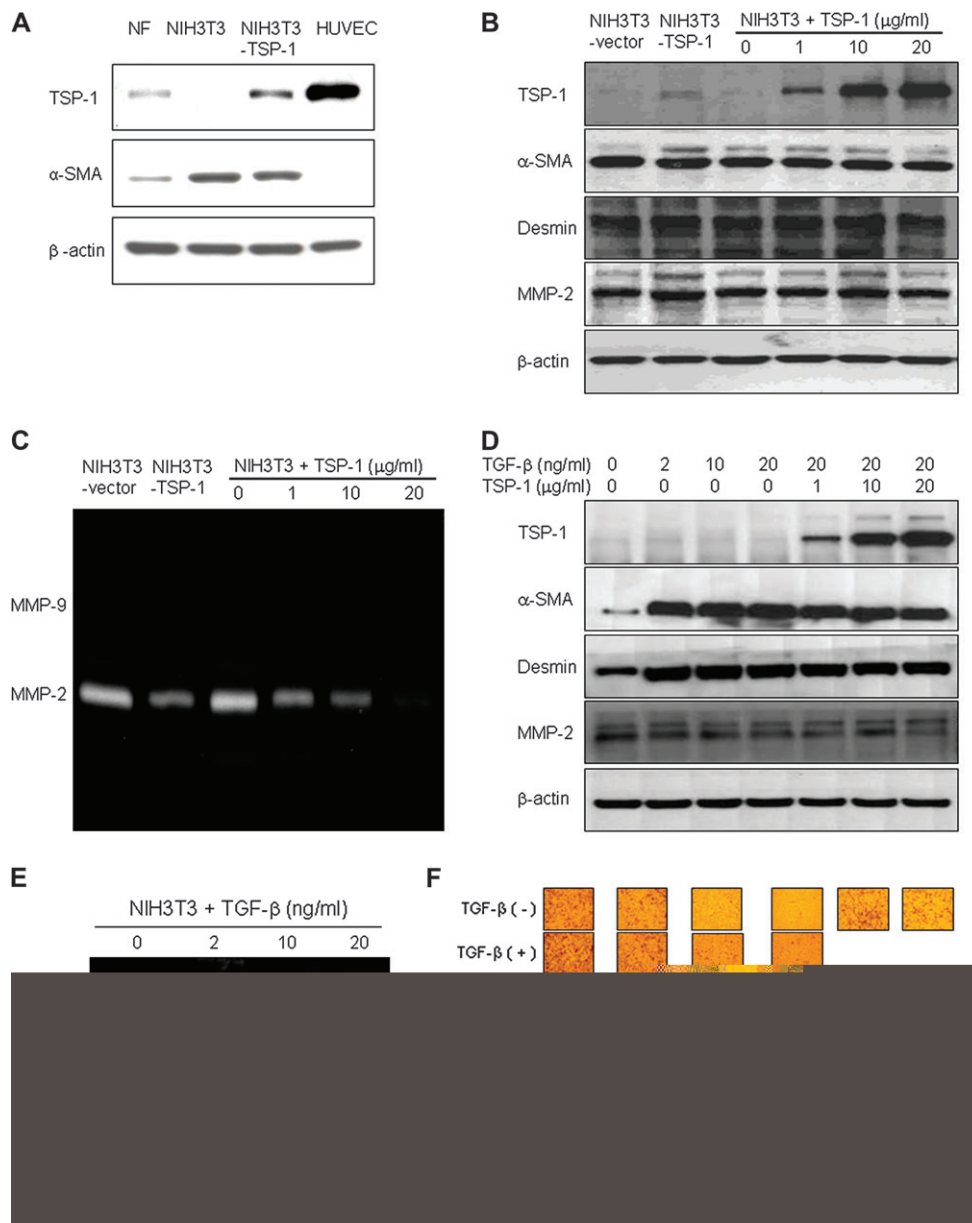


Fig. 4. Augmentation of TSP-1 activity is associated with decreased NIH3T3 cell migration and MMP-2 activity, but not with α -SMA and desmin expression. TGF- β treatment for 24 h increases NIH3T3 cell migration and α -SMA and desmin expression. The TGF- β -increased α -SMA expression cannot be blocked with TSP-1. (A) A representative western immunoblot showing that stable transfection of TSP-1 in NIH3T3 cells upregulated the protein level of TSP-1, but did not downregulate the protein level of α -SMA, as compared with normal fibroblast (NF) derived from normal uterine cervix with higher protein level of TSP-1 and lower α -SMA. (B) A representative western immunoblot showing that TSP-1 transfection (NIH3T3-TSP-1), as well as purified TSP-1 at various concentrations, did not change the protein levels of α -SMA, desmin and MMP-2. Experiments were repeated three times. (C) Decreased MMP-2 activity was noted with the transfection of TSP-1 complementary DNA or the addition of TSP-1 to NIH3T3 cells. Shown is a gelatin zymography of conditioned media (a representative of three similar experiments). (D) A representative western immunoblot showing that TGF- β -treatment (2, 10 and 20 ng/ml) upregulated α -SMA and desmin, but did not change MMP-2 expression in NIH3T3 cells. The addition of TSP-1 at 1, 10 and 20 μ g/ml did not change the TGF- β -increased α -SMA expression. Experiments were repeated three times. (E) There was no change on the MMP-2 activity after TGF- β treatment. Shown is a gelatin zymography of conditioned media (a representative of three similar experiments). (F) Cell migration assay with modified Boyden chamber of NIH3T3-vector, NIH3T3-TSP-1 or NIH3T3 cells in the absence (0/0) or presence of purified TSP-1. Each column represents mean \pm standard deviation ($n = 6$). TSP-1 was added on the lower (0/10), upper (10/0) or both (10/10) compartments of the Boyden chamber. * or † $P < 0.05$; ** or †† $P < 0.01$.

and invasion, rather than a direct effect on the stromal marker expression. These results indicate that TSP-1 participates not only in the negative regulation of angiogenesis but also stroma reaction during cervical carcinogenesis.

Cancer progression is a complex process involving transformation, invasion, angiogenesis and metastasis (20). Although TSP-1 is commonly believed to have antitumor effects due to its antiangiogenic

ability, however, results from various studies have demonstrated different correlations among the levels of TSP-1 and tumor progression and clinical prognosis in different tumor types, indicating different biological functions of TSP-1 in different cancer cell types. The lower expression levels of TSP-1 in cervical cancer than those in normal tissue are similar to some human tumors in which decreased TSP-1 expression is associated with malignancy (21,22). Among these

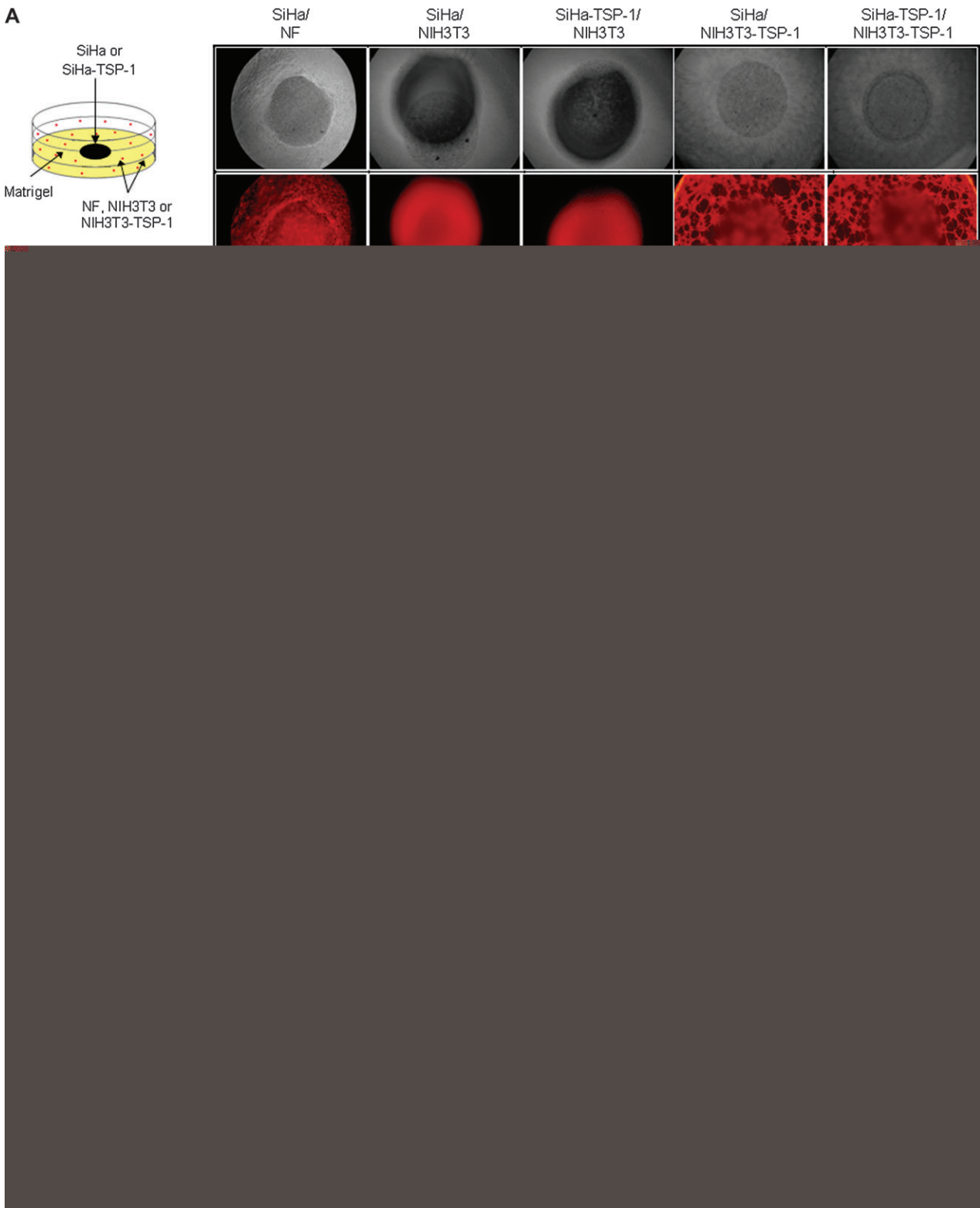


Fig. 5. TSP-1 attenuates the invasive ability of activated fibroblasts into tumor clusters. A Matrigel multicellular coculture system was used to evaluate the invasive ability of fibroblasts into tumor cell cluster. Bright field and fluorescent images were captured on an inverted phase microscope using a $\times 4$ objective for the same field. **(A)** To test the effects of gain of TSP-1 function on tumor cells and fibroblasts, tumor cells with or without TSP-1 transfection (SiHa or SiHa-TSP-1) were placed into the central area of the Matrigel to form the tumor cell cluster; fluorescence (PKH26)-labeled fibroblasts (NIH3T3 or NIH3T3-TSP-1) (red) were evenly dispersed on the peripheral area. Normal fibroblasts (NF) in the central area with SiHa tumor cell cluster in the peripheral (SiHa/NF) were used as negative control. **(B, C and D)** To test whether the addition of TSP-1 to tumor cell cluster or fibroblasts in the absence or presence of TGF- β can prevent the fibroblasts from invading into tumor cluster, SiHa cells were placed in the central area, whereas NIH3T3 cells (labeled with red fluorescence PKH26 in B or green fluorescence PKH67 in C and D) were placed in the peripheral area. Purified TSP-1 was added into SiHa (B), untreated NIH3T3 cells (C) or TGF- β (10 ng/ml)-treated NIH3T3 cells (D) with different concentrations.

cancers, TSP-1 expression is inversely correlated with tumor grade and survival rate in thyroid, colon and bladder carcinomas (21,23). The prognostic value of TSP-1 in cervical cancer requires further investigation. In contrast, TSP-1 expression was higher in tumors or tumor-associated stroma than in normal epithelial or stroma tissue in other cancer types (10). Thus, TSP-1 seems to have activatory as well as inhibitory properties in tumor progression. There are several likely explanations to account for its dualistic effects. Firstly, TSP-1 interacts with multiple extracellular macromolecules and cell surface receptors, thus exerting a wide range of responses (23,24). Secondly, the exposure to high stromal TSP-1 may induce expression of angiogenesis activators in tumor cells, which override the effects of TSP-1 (25). Thirdly, TSP-1 exerts its effects on multiple types of stromal cells such as inhibiting fibroblasts migration (26), decreasing the recruitment of inflammatory cells (27), inducing apoptosis of endothelial cells (9,28) or activating smooth muscle cells proliferation (29). Fourthly, some limitations still remain among the various models (30).

It has become increasingly clear that, from the context of tumor–stroma interactions, stroma plays an active role in tumor progression (1). Stromal cells can acquire oncogenic transformation following the exposure to carcinogen (31), manipulation of MMPs (32) and the recruitment of inflammatory cells to the stroma (27,33). Stroma reaction is often accompanied with stromal marker expression and functional changes into an invasive phenotype. Inhibition of stroma reaction by TSP-1 might be through reduced expression of stromal markers and invasiveness or inhibition of activated fibroblast migration and recruitment to tumor stroma. Using western blot analysis, migration and Matrigel coculture invasion assays, we demonstrated that TSP-1 potently exerted inhibitory effect on the migration and invasion of fibroblasts with or without TGF- β treatment; however, it has little effect on the expression level of α -SMA expression, a typical marker for activated fibroblasts. Unlike TSP-1, secreted protein, acidic and rich in cysteine (SPARC) was shown to inhibit fibroblast activation by blocking α -SMA overexpression (34). Although SPARC and TSP-1 are both matricellular proteins that inhibit angiogenesis and interfere with the organization of the extracellular matrix, however, TSP-1 inhibits stroma reaction through a mechanism distinct from SPARC.

It is worth mentioning that the stroma reaction occurred mainly at the tumor–stroma junction as shown in both clinical and animal specimens; this phenomenon was attenuated by the upregulation of TSP-1. Furthermore, this highlights the importance of the interaction between tumor and stroma and suggests a likely possibility that modulation of tumor microenvironment can potentially change the cell–matrix interactions associated with cell movement and further progression. Consistent with this notion, it has been shown that TSP-1 inhibits the activity of MMP-2 and MMP-9 via a direct interaction with these proteases in specific regions (32,35). In this study, no MMP-9 activity could be detected in NIH3T3 cells. Instead, we observed that TSP-1 inhibited MMP-2 activity, which was not altered by TGF- β treatment, followed by a decrease in the migration and invasive ability of activated fibroblasts. More studies are needed to address the exact mechanism whereby TSP-1 decreased MMP-2 activity in the context of activated fibroblasts.

Our present results show that TSP-1 inhibited TGF- β -enhanced migration and the invasive ability of NIH3T3. TSP-1 is known to function as an activator of TGF- β and this activity is mapped to the type-1 repeats of TSP-1 (36,37). Moreover, the activation of TGF- β by TSP-1 can either inhibit (38) or increase (37) malignancy depending on cell types. The complexity and duality in the functions of TSP-1 and TGF- β may be due to their ability to suppress tumor cell proliferation at early stages, but to enhance the host stroma reaction at later stages (39). Further investigation is needed to dissect the dynamic interaction between TSP-1 and TGF- β in the regulation of cervical cancer growth.

Furthermore, TSP-1 expression in both the stroma and tumor areas of SCID mouse xenografts were different from that mainly expressed in basal epithelia of normal human cervix. This discrepancy may stem from species differences, differential sensitivities of antibodies to

murine and human samples, host compensatory response or different study model (heterotopic versus orthotopic animal model). In addition, clinical observations and genetic manipulations in our study have pointed to TSP-1 as a central mediator to inhibit the invasive ability of activated fibroblasts and their subsequent recruitment to stroma. However, we cannot completely rule out the possibility of other factors, e.g. the interaction with TGF- β , involved in stroma reaction (40).

To summarize, our current study demonstrates that TSP-1 plays a broader role in tumor cell biology including the inhibition of activated fibroblast migration and invasion, in addition to its well-known anti-angiogenic effects. In viewing the fact that stromal therapy has recently emerged as a strategy for cancer treatment (41), this study carries significant implications with regard to the application of targeted interventions on the TSP-1-mediated stroma reaction that may represent a potentially new strategy for inhibiting the progression of cancer.

Funding

Chi Mei Foundation Hospital (93CM-TMU-02 and 95CM-TMU-03 to M.-P.W. and C.-R.T.); National Science Council (NSC95-2314-B-384-019 to M.-P.W.) and Center of Excellence for Clinical Trial and Research in Oncology, Ministry of Health, Taiwan (DOC-TD-B-111-004 to C.-Y.C.)

Acknowledgements

We thank Prof. Tien Kuo, Department of Molecular Pathology, MD Anderson Cancer Center for critical reading of this manuscript and Ms Chin-Li Lu, Chi Mei Foundation Hospital, for statistic analysis assistance.

Conflict of Interest Statement: None declared.

References

- Mueller, M.M. *et al.* (2004) Friends or foes—bipolar effects of the tumour stroma in cancer. *Nat. Rev. Cancer*, **4**, 839–849.
- Amatangelo, M.D. *et al.* (2005) Stroma-derived three-dimensional matrices are necessary and sufficient to promote desmoplastic differentiation of normal fibroblasts. *Am. J. Pathol.*, **167**, 475–488.
- Lazard, D. *et al.* (1993) Expression of smooth muscle-specific proteins in myoepithelium and stromal myofibroblasts of normal and malignant human breast tissue. *Proc. Natl Acad. Sci. USA*, **90**, 999–1003.
- Barcellos-Hoff, M.H. *et al.* (2000) Irradiated mammary gland stroma promotes the expression of tumorigenic potential by unirradiated epithelial cells. *Cancer Res.*, **60**, 1254–1260.
- Ishii, G. *et al.* (2005) *In vivo* and *in vitro* characterization of human fibroblasts recruited selectively into human cancer stroma. *Int. J. Cancer*, **117**, 212–220.
- Hasebe, T. *et al.* (2000) Proliferative activity of intratumoral fibroblasts is closely correlated with lymph node and distant organ metastases of invasive ductal carcinoma of the breast. *Am. J. Pathol.*, **156**, 1701–1710.
- Lawler, J. (2000) The functions of thrombospondin-1 and -2. *Curr. Opin. Cell Biol.*, **12**, 634–640.
- Kodama, J. *et al.* (2001) Thrombospondin-1 and -2 messenger RNA expression in invasive cervical cancer: correlation with angiogenesis and prognosis. *Clin. Cancer Res.*, **7**, 2826–2831.
- Streit, M. *et al.* (1999) Overexpression of thrombospondin-1 decreases angiogenesis and inhibits the growth of human cutaneous squamous cell carcinomas. *Am. J. Pathol.*, **155**, 441–452.
- Bertin, N. *et al.* (1997) Thrombospondin-1 and -2 messenger RNA expression in normal, benign, and neoplastic human breast tissues: correlation with prognostic factors, tumor angiogenesis, and fibroblastic desmoplasia. *Cancer Res.*, **57**, 396–399.
- Wu, M.P. *et al.* (2004) Thrombospondin-1 acts as a fence to inhibit angiogenesis that occurs during cervical carcinogenesis. *Cancer J.*, **10**, 27–32.
- Chou, C.Y. *et al.* (1995) Volume-sensitive chloride channels associated with human cervical carcinogenesis. *Cancer Res.*, **55**, 6077–6083.
- Sheibani, N. *et al.* (1995) Thrombospondin 1 expression in transformed endothelial cells restores a normal phenotype and suppresses their tumorigenesis. *Proc. Natl Acad. Sci. USA*, **92**, 6788–6792.

14. Colorado, P.C. *et al.* (2000) Anti-angiogenic cues from vascular basement membrane collagen. *Cancer Res.*, **60**, 2520–2526.
15. Meyer-Ter-Vehn, T. *et al.* (2006) p38 inhibitors prevent TGF-beta-induced myofibroblast transdifferentiation in human tenon fibroblasts. *Invest. Ophthalmol. Vis. Sci.*, **47**, 1500–1509.
16. Albini, A. *et al.* (1987) A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Res.*, **47**, 3239–3245.
17. Shen, M.R. *et al.* (2003) KCl cotransport is an important modulator of human cervical cancer growth and invasion. *J. Biol. Chem.*, **278**, 39941–39950.
18. Walter-Yohrling, J. *et al.* (2003) Myofibroblasts enable invasion of endothelial cells into three-dimensional tumor cell clusters: a novel *in vitro* tumor model. *Cancer Chemother. Pharmacol.*, **52**, 263–269.
19. Bierie, B. *et al.* (2006) TGF-beta and cancer. *Cytokine Growth Factor Rev.*, **17**, 29–40.
20. Hanahan, D. *et al.* (2000) The hallmarks of cancer. *Cell*, **100**, 57–70.
21. Bunone, G. *et al.* (1999) Expression of angiogenesis stimulators and inhibitors in human thyroid tumors and correlation with clinical pathological features. *Am. J. Pathol.*, **155**, 1967–1976.
22. de Fraipont, F. *et al.* (2000) Expression of the angiogenesis markers vascular endothelial growth factor-A, thrombospondin-1, and platelet-derived endothelial cell growth factor in human sporadic adrenocortical tumors: correlation with genotypic alterations. *J. Clin. Endocrinol. Metab.*, **85**, 4734–4741.
23. de Fraipont, F. *et al.* (2001) Thrombospondins and tumor angiogenesis. *Trends Mol. Med.*, **7**, 401–407.
24. Brown, E.J. *et al.* (2001) Integrin-associated protein (CD47) and its ligands. *Trends Cell Biol.*, **11**, 130–135.
25. Fontana, A. *et al.* (2005) Human breast tumors override the antiangiogenic effect of stromal thrombospondin-1 *in vivo*. *Int. J. Cancer*, **116**, 686–691.
26. Streit, M. *et al.* (2000) Thrombospondin-1 suppresses wound healing and granulation tissue formation in the skin of transgenic mice. *EMBO J.*, **19**, 3272–3282.
27. Doyen, V. *et al.* (2003) Thrombospondin 1 is an autocrine negative regulator of human dendritic cell activation. *J. Exp. Med.*, **198**, 1277–1283.
28. Reiher, F.K. *et al.* (2002) Inhibition of tumor growth by systemic treatment with thrombospondin-1 peptide mimetics. *Int. J. Cancer*, **98**, 682–689.
29. Isenberg, J.S. *et al.* (2005) Endogenous thrombospondin-1 is not necessary for proliferation but is permissive for vascular smooth muscle cell responses to platelet-derived growth factor. *Matrix Biol.*, **24**, 110–123.
30. Hlatky, L. *et al.* (2002) Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. *J. Natl Cancer Inst.*, **94**, 883–893.
31. Kalas, W. *et al.* (2005) Oncogenes and angiogenesis: down-regulation of thrombospondin-1 in normal fibroblasts exposed to factors from cancer cells harboring mutant ras. *Cancer Res.*, **65**, 8878–8886.
32. Rodriguez-Manzanique, J.C. *et al.* (2001) Thrombospondin-1 suppresses spontaneous tumor growth and inhibits activation of matrix metalloproteinase-9 and mobilization of vascular endothelial growth factor. *Proc. Natl Acad. Sci. USA*, **98**, 12485–12490.
33. Vallejo, A.N. *et al.* (2000) Central role of thrombospondin-1 in the activation and clonal expansion of inflammatory T cells. *J. Immunol.*, **164**, 2947–2954.
34. Chlenski, A. *et al.* (2007) SPARC enhances tumor stroma formation and prevents fibroblast activation. *Oncogene*, **26** (31), 4513–4522.
35. Bein, K. *et al.* (2000) Thrombospondin type 1 repeats interact with matrix metalloproteinase 2. Regulation of metalloproteinase activity. *J. Biol. Chem.*, **275**, 32167–32173.
36. Crawford, S.E. *et al.* (1998) Thrombospondin-1 is a major activator of TGF-beta1 *in vivo*. *Cell*, **93**, 1159–1170.
37. Kawataki, T. *et al.* (2000) Correlation of thrombospondin-1 and transforming growth factor-beta expression with malignancy of glioma. *Neuropathology*, **20**, 161–169.
38. Miyanaga, K. *et al.* (2002) Expression and role of thrombospondin-1 in colorectal cancer. *Anticancer Res.*, **22**, 3941–3948.
39. Radisky, D.C. *et al.* (2004) Cancer. Respect thy neighbor!. *Science*, **303**, 775–777.
40. Hugo, C. (2003) The thrombospondin 1-TGF-beta axis in fibrotic renal disease. *Nephrol. Dial. Transplant.*, **18**, 1241–1245.
41. Meyerhardt, J.A. *et al.* (2005) Systemic therapy for colorectal cancer. *N. Engl. J. Med.*, **352**, 476–487.

Received October 2, 2007; revised March 11, 2008; accepted March 15, 2008