

# Endogenous thrombospondin-1 is not necessary for proliferation but is permissive for vascular smooth muscle cell responses to platelet-derived growth factor

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## Abstract

We have reexamined the role of endogenous thrombospondin-1 (TSP1) in growth and motility of vascular smooth muscle cells (SMCs). Based on the ability of aortic-derived SMCs isolated from TSP1 null mice and grown in the absence of exogenous TSP1 to grow at comparable rates and to a slightly higher density than equivalent cells from wild-type mice, TSP1 is not necessary for their growth. Low concentrations of exogenous TSP1 stimulate growth of TSP1 null SMCs, but higher doses of TSP1 or its C-terminal domain are inhibitory. However, SMCs from TSP1 null mice are selectively deficient in chemotactic and proliferative responses to platelet-derived growth factor and in outgrowth in three-dimensional cultures. Recombinant portions of the N- and C-terminal domains of TSP1 stimulate SMC chemotaxis through different integrin receptors. Based on these data, the relative deficiency in SMC outgrowth during an ex vivo angiogenic response of muscle tissue from TSP1 null mice is probably due to restriction of platelet-derived growth factor dependent SMC migration and/or proliferation.

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## 1. Introduction

Regulation of SMC proliferation and motility are critical for normal embryonic vascular development. In adult animals, dysregulation of these SMC responses play important roles in the pathogenesis of atherosclerosis and in the recurrence of disease following vascular interventions (Chen et al., 1999). Furthermore, evidence has accumulated

that SMCs also play a critical role in pathological angiogenesis associated with tumor growth (Wesseling et al., 1995; Bergers and Benjamin, 2003). Therefore, pharmacological inhibitors of SMC proliferation or motility could have applications for treatment of both cardiovascular disease and cancer.

To identify new targets for developing such drugs, we must understand the molecular mechanisms that regulate these SMC responses. Extracellular matrix plays important roles in regulating cell growth and motility (Ingber, 2002; Kaverina et al., 2002). The extracellular matrix protein thrombospondin-1 (TSP1) was shown to stimulate SMC proliferation (Majack et al., 1986). Subsequently, the ability of TSP1 antibodies to block proliferation was interpreted to

*Abbreviations:* IGF1; insulin-like growth factor-1; PDGF; platelet-derived growth factor- $\beta\beta$ ; TSP1; thrombospondin-1.

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indicate an essential role for TSP1 in SMC proliferation (Majack et al., 1988). Further studies have supported a positive role of TSP1 in SMC proliferation and have defined roles for specific TSP1 receptors, including  $\alpha 3\beta 1$  integrin (Lynn et al., 2002),  $\alpha v\beta 3$  integrin (Lele et al., 2001), and CD47 (Maile and Clemmons, 2003). Other studies have identified some of the molecules involved in the signaling pathway and found that TSP1-mediated proliferation involves activation of Erk (Lynn et al., 2002) and S6 kinase (Scott Burden et al., 1988).

TSP1 has been reported to act separately from the well-known SMC mitogen platelet-derived growth factor (PDGF) (Patel et al., 1997), although others have shown that TSP1 binds directly to PDGF (Hogg et al., 1997), and under some conditions, PDGF and TSP1 clearly have synergistic effects on SMC (Yabkowitz et al., 1993). Furthermore, PDGF induces synthesis of TSP1 by SMCs (Majack et al., 1986; Majack et al., 1988). Therefore, apart from their known direct interaction, TSP1 expressed by SMCs likely contributes to or modulates some responses of SMCs to PDGF.

TSP1 also stimulates chemotaxis of SMCs (Yabkowitz et al., 1993).  $\alpha v\beta 3$  integrin (Lynn et al., 2002),  $\alpha 2\beta 1$  and CD47 (Wang and Frazier, 1998) have been identified as receptors that mediate motility responses of SMCs to TSP1. TSP1 induced chemotaxis is sensitive to inhibitors of PI 3 kinase and focal adhesion kinase but not to a MAP kinase inhibitor (Lynn et al., 1999; Lynn et al., 2002). The chemotactic response to TSP1 also differs from the mitogenic response in its sensitivity to pertussis toxin, implicating G protein signaling (Wang et al., 1999; Lynn et al., 2002). Therefore, growth and motility are mediated by different TSP1 receptors and involve distinct signaling pathways in SMCs.

The derivation of TSP1 null mice and their relatively normal circulatory systems, however, raises questions about the hypothesis that TSP1 is essential for SMC growth (Lawler et al., 1998). In an effort to better define the role of TSP1 in modulating VSMC responses, we have used both isolated SMCs from these mice and muscle explant cultures that permit assessment of coordinated outgrowth of both endothelial and perivascular cells in a three-dimensional matrix culture (Calzada et al., 2004). We also compared the proliferative and migratory activity of aortic-derived VSMCs from wild-type and TSP1 null mice. We present herein evidence that TSP1 null VSMC proliferation is comparable to that of TSP1 expressing cells. In contrast, both migratory and proliferative responses of SMC and endothelial cells to PDGF are deficient in the absence of endogenous TSP1.

## 2. Results

### 2.1. TSP1 null endothelial and vascular smooth muscle cells are sustainable in vitro in TSP1-deficient medium

Aortic-derived VSMC cultures from wild-type and TSP1 null mice were stained with  $\alpha$ -smooth muscle actin and

CD31 antibodies. At least  $96 \pm 2\%$  of total cells counted from wild-type or TSP1 null cell cultures stained positive for smooth muscle actin and were negative for CD31. Lung-derived endothelial cells and VSMCs from TSP1 null and wild-type mice proliferated comparably in medium containing serum (data not shown). Because serum provides an exogenous source of bovine TSP1 that could support SMC growth (Patel et al., 1997), SMC isolated from TSP1 null and wild-type mice were also grown directly in medium lacking any TSP1 detectable by immunoassay. SMCs from null and wild-type mice demonstrated similar growth and morphologies in medium deficient in TSP1 (Fig. 1A and B). VSMCs from the wild-type mice achieved surface saturation somewhat earlier than those from TSP1 null mice, but the surface saturation density was reproducibly greater in SMC cultures from TSP1 null mice (Fig. 1C). The wild-type and null SMCs also grew at similar rates in a proprietary TSP1-free medium without serum, although the interval to confluence was longer than for cells grown using TSP1-deficient medium containing heparin-stripped serum (results not shown).

### 2.2. TSP1 has biphasic effects on vascular smooth muscle cell proliferation

Consistent with previous reports, addition of exogenous TSP1 increased wild-type VSMC proliferation at low doses ( $\leq 1 \mu\text{g/ml}$ , Fig. 2A). SMCs from TSP1 null mice were somewhat more responsive to low doses of TSP1 up to  $5 \mu\text{g/ml}$ . In contrast, high doses of exogenous TSP1 ( $\geq 10 \mu\text{g/ml}$ ) inhibited proliferation of both null and wild-type cells (Fig. 2A). Exogenous TSP1 demonstrated a similar biphasic effect upon proliferation of lung-derived endothelial cells from null and wild-type mice, although stimulation of proliferation by low doses of TSP1 was less than for SMCs (Fig. 2B).

### 2.3. Human smooth muscle cell proliferation is promoted by the collagen binding fragment and inhibited by recombinant C-terminal domain and 4N1K peptides derived from TSP1

Human VSMCs had a qualitatively similar biphasic response to exogenous TSP1, although somewhat higher concentrations were required (Fig. 3A). A growth-promoting activity was localized to the core region of TSP1, in that the 50/70 kDa chymotryptic fragment of TSP1 significantly promoted cell proliferation (Fig. 3B). This fragment contains a collagen-binding site and is consistent with reports that monomeric collagen directly promotes SMC proliferation (Koyama et al., 1996). In contrast, recombinant C-terminal domain of TSP1 and the 4N1K peptide derived from this domain both inhibited SMC proliferation (Fig. 3C). This is in agreement with reports that 4N1K peptide decreases DNA synthesis in mitogen stimulated SMCs (Maile and Clemmons, 2003). The 4N1K peptide also inhibits angiogenesis, as quantified by tube formation (Kanda et al., 1999), but not cell proliferation in brain endothelial cells.

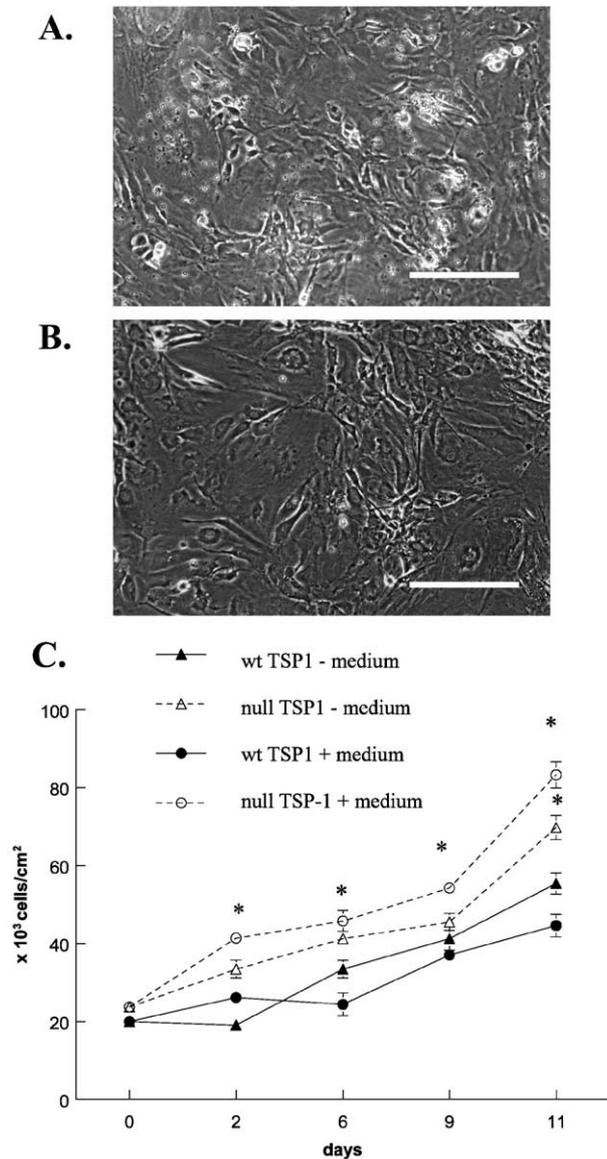


Fig. 1. TSP-1 null vascular smooth muscle cell growth does not require TSP1. Aortic-derived murine vascular smooth muscle cells from a TSP1 null and wild-type animals were cultured for 14 days in DMEM supplemented with 5% heparin-stripped fetal calf serum. Microscopy demonstrates comparable cell morphology between wild-type (A) and TSP1 null (B) VSMCs. (C) Growth curve analysis of murine aortic-derived wild-type (closed symbols) and TSP1 null (open symbols) vascular smooth muscle cells. VSMCs were cultured in 12-well plates in either TSP1 containing or deficient medium. Viable cells (as determined by Trypan blue exclusion) were counted from wells in triplicate using a hemocytometer. Both wild-type and TSP1 null cells obtained surface saturation by culture day 11. Scale bars in A and B=250  $\mu$ m. (\*indicates  $p \leq 0.05$  for null vs. wild-type).

#### 2.4. Endogenous TSP1 is necessary for inhibition of cell proliferation by TSP1 antibodies

Previous observations that TSP1 antibodies inhibit proliferation of SMCs were interpreted as evidence that TSP1 is necessary for growth, i.e., the antibodies were assumed to inhibit binding of TSP1 to a cell surface receptor that mediates a growth response (Majack et al., 1988).

Based on our data showing that TSP1 is not necessary for proliferation, we considered an alternative hypothesis that TSP1 antibodies may inhibit proliferation by cross-linking cell surface receptors occupied by TSP1. If so, the TSP1 antibodies should only inhibit proliferation of wild-type cells. Aortic-derived VSMCs from wild-type and TSP1 null mice were incubated with either TSP1 (1  $\mu$ g/ml), TSP1 monoclonal antibody (clone A6.1, 20  $\mu$ g/ml) or both. As previously reported for human SMCs (Majack et al., 1988), TSP1 antibody A6.1 inhibited proliferation of wild-type murine VSMCs (Fig. 4A). However, proliferation of TSP1 null SMCs was not inhibited by this antibody. Proliferation of wild-type SMCs was restored by the addition of exogenous TSP1 at a concentration that did not directly significantly alter proliferation (Fig. 4A).

Similar responses were seen using murine lung-derived endothelial cells (Fig. 4B). Addition of A6.1 selectively

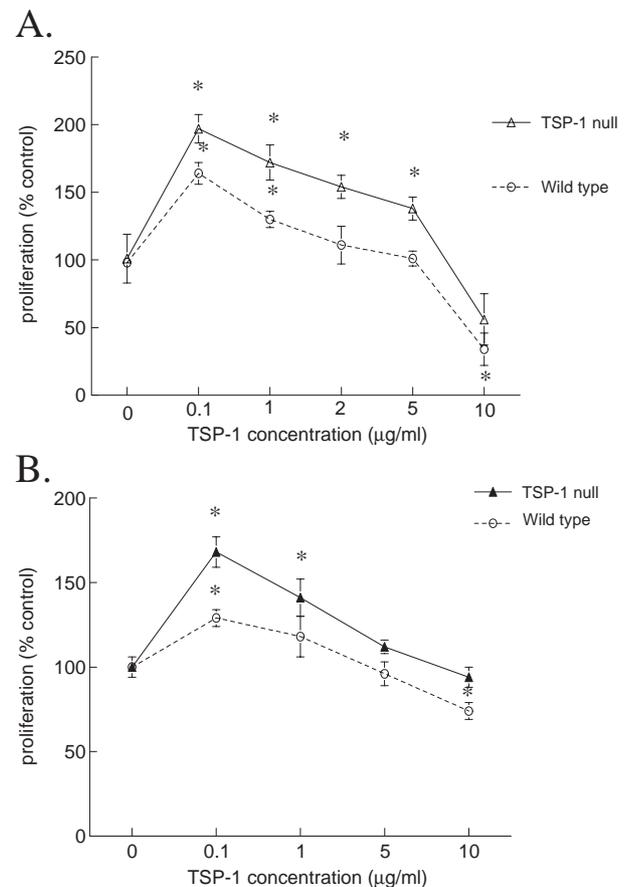


Fig. 2. Biphasic effects of exogenous TSP-1 on proliferation of TSP1 null and wild-type vascular smooth muscle cells. (A) Cultures of VSMCs obtained from fresh murine aortas were grown in DMEM supplemented with 5% heparin-stripped FCS. (B) Lung-derived endothelial cells were grown in EGM with 5% heparin-stripped FCS. When cells had obtained roughly 80% confluence, they were harvested and plated at ~5000 cells/well in 96-well culture plates and incubated at 37 °C and 5% CO<sub>2</sub> for 72 h in the presence of the indicated concentrations of platelet TSP1. Proliferation was quantified using an MTT assay and is presented normalized to initial cell numbers (0%) and proliferation at 72 h in the absence of TSP1 (100%). Results represent the mean  $\pm$  S.D. of at least three separate experiments.

inhibited proliferation of wild-type but not null endothelial cells. Addition of exogenous TSP1 (10 µg/ml) also reversed inhibition of endothelial cell proliferation by the antibody. A6.1 and two other TSP1 antibodies also inhibited proliferation of bovine corneal endothelial cells (Fig. 4C). A6.1, D4.6 and A2.5 inhibited proliferation, but a fourth TSP1 antibody, A4.1, did not. Because TSP1 is clearly not required for endothelial cell proliferation and typically inhibits proliferation of these cells (Taraboletti et al., 1990), inhibition by TSP1 antibodies of both smooth muscle and endothelial cell proliferation probably occurs

through a mechanism other than blocking a growth-promoting activity of TSP1.

2.5. Vascular smooth muscle cell proliferation is moderately enhanced by antibody ligation of CD47 and integrin  $\alpha3\beta1$

One potential mechanism by which TSP1 antibodies could modulate proliferation is through inducing clustering of cell surface receptors occupied by TSP1. We therefore asked whether direct binding of antibodies specific for known TSP1 receptors could induce a similar response (Fig. 5). Antibodies recognizing CD47, CD36 and  $\alpha3\beta1$  integrin led to moderate yet statistically significant increases in human aortic VSMC proliferation (Fig. 5A and B). Immunohistochemical analysis of the human aortic VSMCs verified cell surface expression of CD36 but not of the endothelial marker CD31 (data not shown).

2.6. Increased neovascularization of TSP1 null muscle explants in 3D collagen gels

To further characterize the role of endogenous TSP1 during neovessel formation, an ex vivo model of neovessel formation was developed wherein muscle tissue explants from wild-type or TSP1 null mice were embedded in 3D collagen gels (Fig. 6). During the first 3–5 days of culture, single cells migrated into the gel and gradually organized into capillary-like structures. After an initial lag phase, cell migration from TSP1 null muscle explants was much faster than that from wild-type explants (Fig. 6A, D). Capillary-like tubes began to form on day 7 and peaked by day 14, with a consistently higher density of tubules in TSP1 null explants than in wild-type muscle explants (Fig. 6B and E). Digital image analysis of cultures at day 14 also showed a significant increase in the formation of tube-like structures from TSP1 null explants (Fig. 6C and F). The total area of outgrowth for the typical wild-type and TSP1 null explants shown was  $14,970 \pm 220$  pixels<sup>2</sup>/field,  $31,800 \pm 760$  pixels<sup>2</sup>/field, respectively ( $P \leq 0.01$ ). A mean tubule path length for the wild-type and TSP1 null explants was  $4790 \pm 50$  pixels/

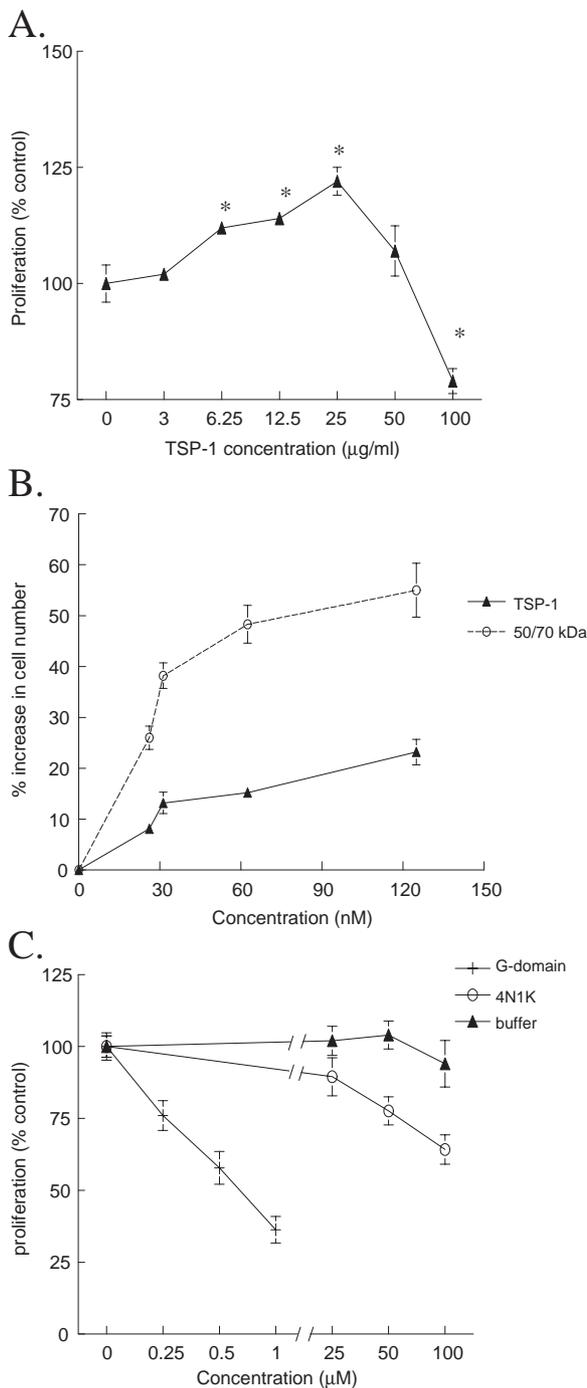


Fig. 3. The collagen-binding fragment of TSP1 stimulates SMC proliferation whereas the CBD and 4N1K peptide inhibit proliferation of SMC. (A) Human SMC were plated on 96-well plates overnight, and serum-starved for 48 h. The cells were treated with TSP1 (human platelet TSP) at the concentrations indicated for 3 days. The relative cell number was determined using the Promega (Madison, WI) Aqueous Nonradioactive Cell Proliferation Kit. Comparable data was obtained with SMC isolated from rat aorta. Human SMC were plated on 96-well plates overnight, and serum-starved for 48 h. The cells were treated with intact TSP1 or the 50/70 chymotryptic fragment of TSP1 at the concentrations indicated for 3 days. The relative cell number was determined (B). Comparable data was obtained with SMC isolated from rat aorta. Note that the 50/70-kDa fragment is threefold more active than intact TSP1 on a molar basis. In another series of experiments human SMC were treated with recombinant C-terminal (G) domain (0–1.0 µM, upper scale) or 4N1K peptide (0–100 µM, lower scale) (C). (the C-terminal domain production and purification is described in (McDonald et al., 2003))

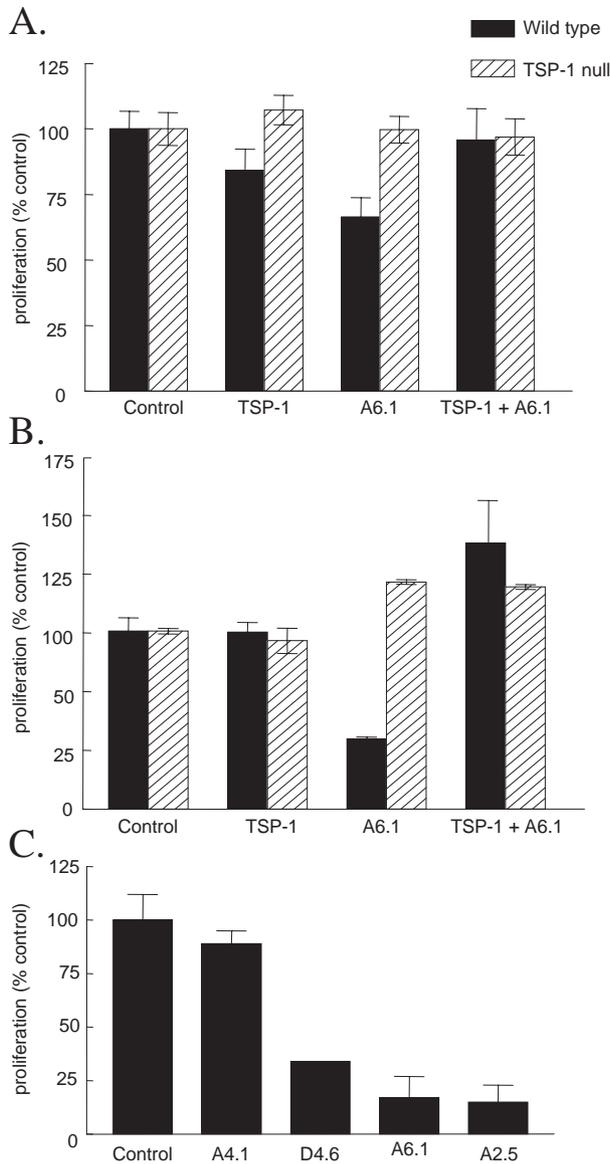


Fig. 4. Inhibition of proliferation by a monoclonal antibody to TSP-1 requires endogenous TSP1 but is not specific for vascular smooth muscle cells. A monoclonal antibody to TSP-1 (clone A6.1 10  $\mu\text{g/ml}$ ) was added to proliferating vascular smooth muscle cells (A) and plates incubated for 72 h in the absence of TSP-1. Wild-type and TSP-1 null lung-derived endothelial cells (B) were plated in 96-well culture plates and incubated in TSP-1-deficient medium. Net proliferation was determined after 72 h by subtracting the signal from time 0. (C) In a separate series of experiments, antibodies to TSP-1 were added to proliferating bovine cornea endothelial cells. Results represent the mean  $\pm$  S.D. of at least three independent experiments.

field,  $12,480 \pm 180/\text{pixels/field}$ , respectively ( $P \leq 0.01$ ). Vascular cells from TSP1 null explants consistently migrated farther than those from wild-type explants over a period of 12 days (Fig. 6G). This ex vivo assay, therefore, replicates the known anti-angiogenic activity of TSP1 in vivo and supports a neovascularization response that can be quantified and is much more stable than typical for Matrigel tube forming assays (Benelli and Albin, 1999).

## 2.7. Reciprocal regulation of endothelial and perivascular/smooth muscle cell outgrowth by TSP1

Outgrowth from the muscle explants may better reflect a normal angiogenic response than do pure endothelial cell cultures in that perivascular cells, mast cells and stromal fibroblasts that play important roles in physiological angiogenesis are present. To quantify the dependence of endothelial and perivascular cell outgrowth on endogenous TSP1, we recovered outgrowth cells from the 3D cultures and characterized them by immunohistochemical staining (Table 1). Endothelial cells represented a significantly larger fraction of the total outgrowth from TSP1 null explants ( $32.9 \pm 3.4\%$ ) than from wild-type explants ( $24.8 \pm 3.2\%$ ) ( $P \leq 0.05$ ). In contrast, smooth muscle actin-positive pericytes or SMCs were significantly increased in wild-type explants ( $34.1 \pm 3.2\%$ ) compared to TSP1 null explants ( $20.8 \pm 2.2\%$ ) ( $P \leq 0.05$ ) (Table 1). Therefore, opposing effects of TSP1 on endothelial cell and perivascular SMC

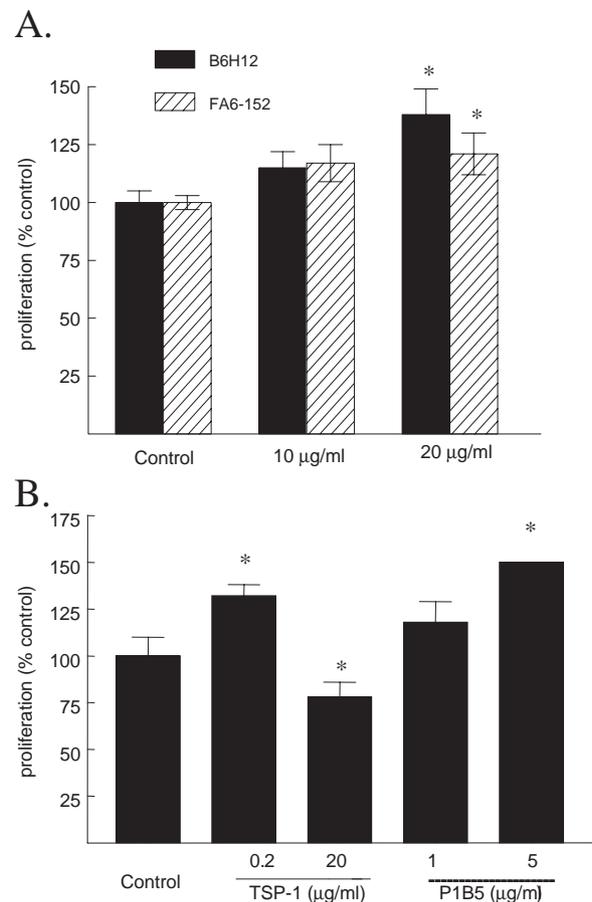


Fig. 5. Vascular smooth muscle cell proliferation is moderately enhanced by antibody ligation of CD47 and  $\alpha 3\beta 1$ . Monoclonal antibodies to CD36 (clone FA6-152, 10–20  $\mu\text{g/ml}$ ), CD47 (clone B6H12, 5–10  $\mu\text{g/ml}$ ) (A) and  $\alpha 3\beta 1$  (clone P1B5, 1–5  $\mu\text{g/ml}$ ) (B) were added to proliferating human aortic-derived vascular smooth muscle cells and plates incubated for 72 h. Net proliferation was determined after 72 h by subtracting the signal from time 0. Results represent the mean  $\pm$  S.D. of at least three independent experiments. \* $p \leq 0.05$ .

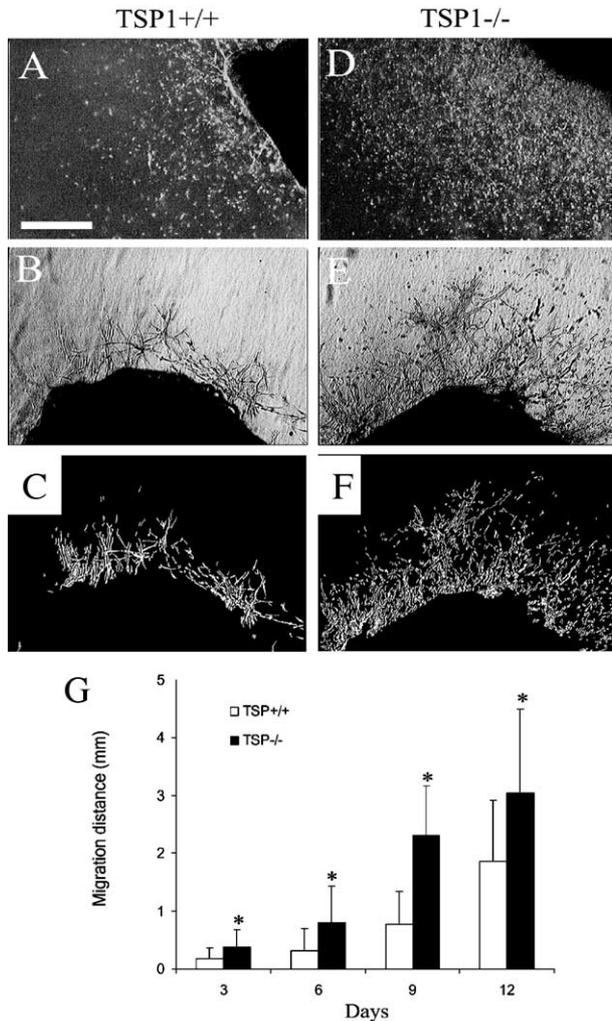


Fig. 6. Endogenous TSP1 inhibits vascular outgrowth from murine muscle explants in a 3D collagen gel. Wild-type (A–C) and TSP1<sup>-/-</sup> (D–F) mouse muscle fragments were embedded in 3D collagen matrices and examined by dark field microscopy after 10 days (A and D) or phase contrast after 14 days in culture (B and E), at which time the vascular cells formed capillary-like tube structures. C and F are digital masks for quantitative analysis of the outgrowth in B and E, respectively, produced using ImagePro software. Scale bar in panel A=500  $\mu$ m. G, Time-course for cell outgrowth in a 3D collagen matrix. Vascular outgrowths were evaluated at the indicated times by measuring the maximal cell migration distance in each of four quadrants from each explant. Data are the means of measurements from 12 explants at each time point, and the error bars represent the S.E.M. Results for TSP1 null explants that differed significantly from wild-type are indicated by \* ( $p \leq 0.01$ ). The results are representative of at least three independent experiments.

responses may each contribute to the observed differences in outgrowth between the wild-type and TSP1 null explants.

### 2.8. Exogenous TSP1 inhibits neovessel formation of TSP1 null muscle explants

Two approaches were used to test whether exogenous TSP1 could restore a wild-type phenotype to TSP1-null mouse muscle explants. MDA-MB-435 breast carcinoma cells express high levels of angiogenic growth factors and

induce strong angiogenic responses in mouse xenografts (Weinstat-Saslow et al., 1994) but do not express significant levels of endogenous TSP1 (Zabrenetzky et al., 1994). Co-cultures of MDA-MB-435 cells suspended in soft agar with TSP1 null explants in adjacent Vitrogen gels strongly induced vascular outgrowth from the explants (Fig. 7A). After 12 days, the cells formed well-organized vascular networks (Fig. 7C). In contrast, explants co-cultured with TSP1 over-expressing MDA-MB-435 cells (clone TH-26) exhibited little outgrowth, with minimal organization of the cells into networks (Fig. 7B and D). This result is consistent with the decreased angiogenic response of TH-26 tumors in mammary fat pad xenografts (Weinstat-Saslow et al., 1994).

To more directly assess the dose dependency for exogenous TSP1 to inhibit vascular outgrowth, the explants were placed in collagen gels containing growth factors and varying concentrations of human platelet TSP1 (Fig. 7E). TSP1 maximally suppressed neovessel formation at 2  $\mu$ g/ml, but the response was biphasic and reproducibly decreased at higher TSP1 concentrations. The decrease at the high TSP1 concentration may be due to the pro-angiogenic activities that have been identified in the N-terminal regions of TSP1 (Taraboletti et al., 2000; Chandrasekaran et al., 2000; Calzada et al., 2003, 2004) or to stimulation of SMC outgrowth. However, doses that inhibited outgrowth are more representative of endogenous TSP1 levels secreted by cells in tissue culture and confirms that the enhanced vascular response of the null explants can be reversed by restoring TSP1.

### 2.9. Vascular cell chemotaxis to PDGF is enhanced in the presence of TSP1

Because endogenous TSP1 is not necessary for SMC proliferation in culture, we considered that the lower percentage of SMCs in vascular outgrowths from the TSP1 null muscle explants may be due to a defect in SMC migration. PDGF plays a major role in angiogenic responses in vitro and in vivo as a stimulator of perivascular

Table 1  
Immunocytochemical analysis of vascular cells from murine muscle explants

Cell type	Wild type (%)	TSP1 <sup>-/-</sup> (%)
Endothelial cells	25 $\pm$ 3 <sup>a</sup>	33 $\pm$ 3*
Smooth muscle and pericytes	34 $\pm$ 3	21 $\pm$ 2*
Fibroblasts and other cell types	41 $\pm$ 6	46 $\pm$ 4

Wild-type and TSP1 null mouse muscle explants were cultured in 3D matrices for 14 days. Vascular outgrowths were collected after digesting the collagen gels using collagenase type 2. Slides were prepared by cytospin and stained using CD31 (PECAM-1) or  $\alpha$  smooth muscle actin antibodies. CD31-positive endothelial cells and  $\alpha$  smooth muscle actin-positive perivascular cells were counted in 10 randomly selected fields. Results are the means of at least three independent experiments.

<sup>a</sup> Values are the means  $\pm$  S.E.M.;  $n=3$ .

\*  $P \leq 0.05$ , compared with wild-type group.

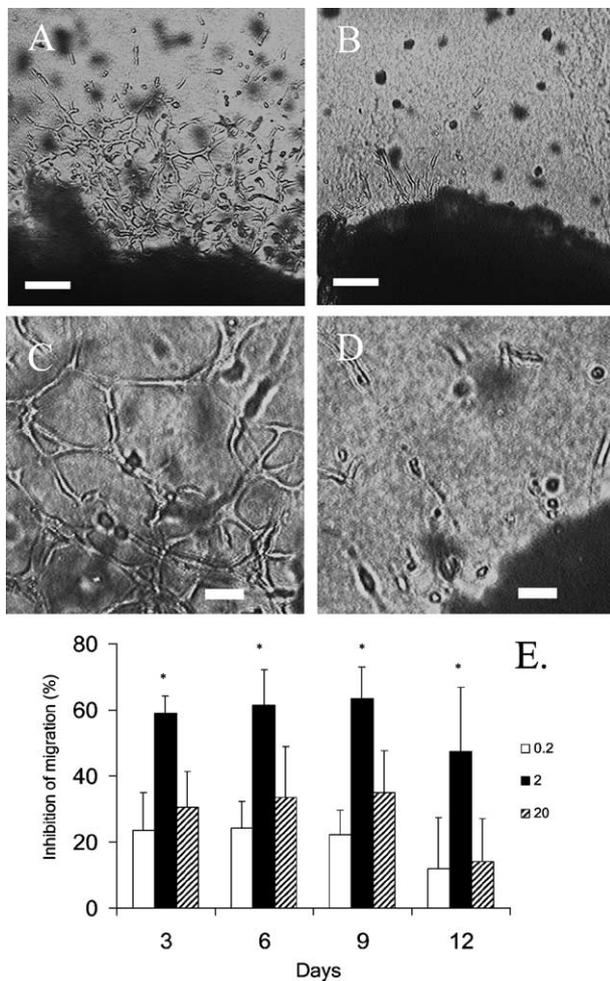


Fig. 7. Exogenous TSP1 inhibits neovascular responses of TSP1-null muscle explants in 3D collagen gels. MDA-MB-435 (A and C) or TSP1-expressing MDA-MB-435 cells (clone TH-26, B and D) were co-cultured with TSP1-null mouse muscle explants in EBM medium free of angiogenic factors. Vascular cell outgrowth and capillary-like tube formation were assessed at day 12. Scale bar in A and B=200 µm; scale bar in C and D=50 µm. (E) TSP1-null muscle explants were cultured in Vitrogen matrix in the absence or presence of 0.2, 2.0, or 20 µg/ml TSP1. Vascular outgrowths were evaluated by measuring the maximum cell migration distance in four quadrants from each explant. Inhibition of migration is presented as the percent decrease relative to untreated controls at the same time points. Data are the mean of six explants at each time point with error bars representing the S.E.M. Results that differed significantly from control are indicated: \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ . The results are representative of at least three independent experiments.

cells (Nicosia et al., 1994; Bergers and Benjamin, 2003) Exogenous TSP1 is also known to enhance SMC chemotaxis to PDGF (Yabkowitz et al., 1993).

To determine whether endogenous TSP1 similarly regulates motility responses to PDGF, we assessed PDGF-induced chemotaxis of murine VSMCs derived from wild-type and TSP1 null animals (Fig. 8A). At all PDGF doses used, chemotaxis of wild-type cells to PDGF was greater than that of TSP1 null cells. The dependence of chemotaxis on endogenous TSP1 is specific for PDGF, because migration induced by insulin-like growth factor-1 (IGF1)

was significantly greater in VSMCs from the TSP1 null mice.

The effect of TSP1 on PDGF-stimulated chemotaxis is not through binding to its own chemotactic receptors, because TSP1 null SMCs were not defective in chemotaxis to TSP1 in

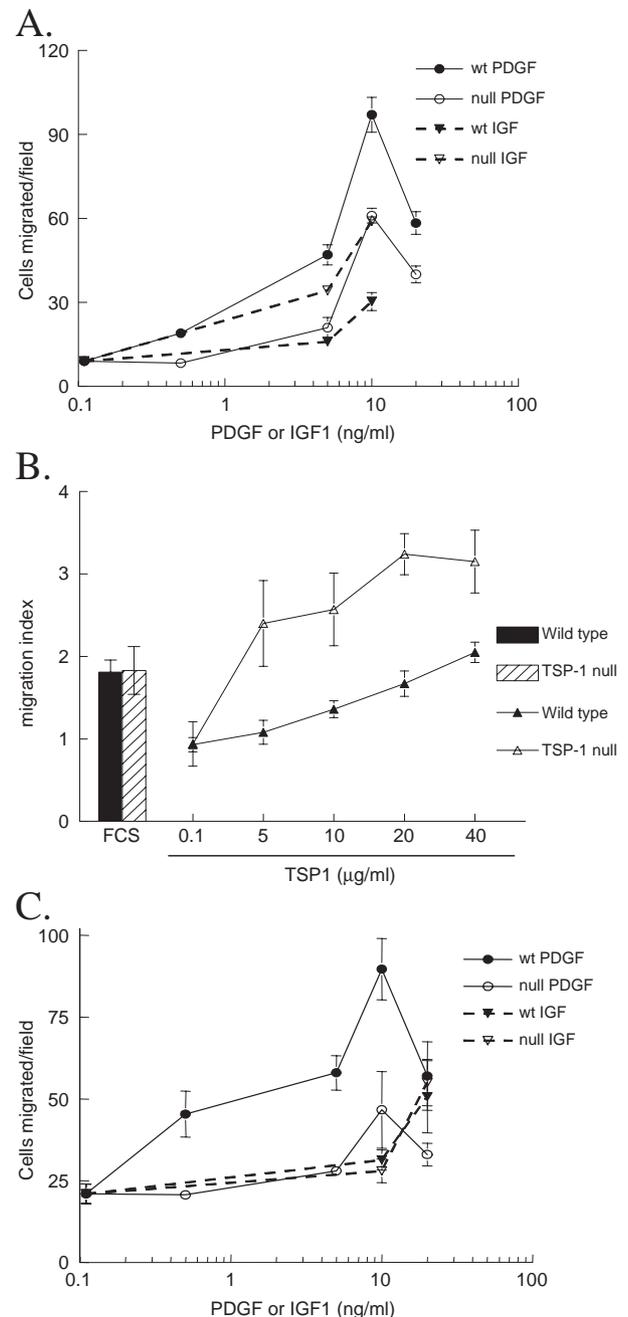


Fig. 8. TSP1 and PDGF differentially modulate chemotactic migration of TSP1 null and wild-type vascular cells. Chemotaxis was assessed in modified Boyden chambers. Primary vascular smooth muscle cells (A and B) or lung derived endothelial cells (C) were induced by TSP1 (B) and PDGF or IGF (A and C) at the indicated concentrations in the lower chamber. Cells ( $0.3\text{--}0.5 \times 10^5$ /well) added to the upper chamber were allowed to migrate for 5.5 h at 37 °C in 5% CO<sub>2</sub>. Migrated cells were counted microscopically after fixation. Results are presented as number of cells migrated/field  $\pm$  S.D.

the lower chamber (Fig. 8B). Rather, null SMC chemotaxis to TSP1 was significantly greater than for wild-type SMC. This difference was also specific in that chemotaxis to 20% serum was comparable for null and wild-type SMC (Fig. 8B).

Differential chemotaxis to PDGF was also seen using lung-derived endothelial cells (Fig. 8C). PDGF stimulated more migration of endothelial cells from wild-type mice at all doses. This response is also specific for PDGF in that chemotaxis induced by IGF1 was essentially the same in wild-type and null endothelial cells.

To determine whether endogenous TSP1 limits other SMC responses to PDGF, we examined proliferative responses of null and wild-type SMC in minimal medium depleted of exogenous TSP1 (Fig. 9A). Wild-type SMCs showed a greater proliferative response at 10–100 ng/ml PDGF than did TSP1 null SMCs. This dependence on endogenous TSP1 was not limited to SMC, because lung-derived endothelial cells from wild-type mice showed stronger proliferation to PDGF than those from TSP1 null mice (Fig. 9B). To confirm that this apparent endothelial proliferative response to PDGF was not due to overgrowth of contaminating SMC in the primary cell cultures, the cells were stained with Ac-LDL before and after treatment with PDGF. PDGF treatment did not change significantly the percentage of endothelial cells among total cells in wild-type and TSP1 null cultures being  $91 \pm 16\%$  and  $90 \pm 13\%$ , respectively, and the net number of positive cells increased after exposure to PDGF (results not shown).

### 2.10. N-terminal domains of both TSP1 and TSP2 induced VSMC migration

Smooth muscle cells express several TSP1 receptors (Anilkumar et al., 2002). To investigate the TSP1 receptors mediating direct chemotaxis of the VSMCs, chemotactic experiments were performed with recombinant trimeric N-terminal domains of TSP1 (NoC1) and TSP2 (NoC2) and a recombinant protein containing the EGF repeats, the type 3 repeats, and the carboxyl terminal domain of TSP1 (E123CaG1). Human aortic VSMCs demonstrated significantly increased migration to the N-terminal domains of both TSP1 and TSP2 as compared to controls, with the response to NoC2 and NoC1 being comparable (Fig. 10A). A  $\beta_1$  integrin-specific blocking antibody, mAb13, completely inhibited both NoC1- and NoC2-stimulated cell migration and partially inhibited chemotaxis stimulated by intact TSP1.

In contrast, the selective  $\alpha_v$  integrin antagonist SB223245 partially inhibited chemotaxis stimulated by TSP1 but somewhat stimulated chemotaxis to NoC1 and NoC2. As previously shown using recombinant fusion proteins (Lee et al., 2003) and implied from RGD peptide blocking studies (Lynn et al., 1999), a recombinant C-terminal region of TSP1 containing its RGD sequence stimulated migration of VSMCs. Addition of the selective  $\alpha_v$  integrin antagonist SB223245 completely inhibited cell migration to the carboxyl terminal region of TSP1. Thus,

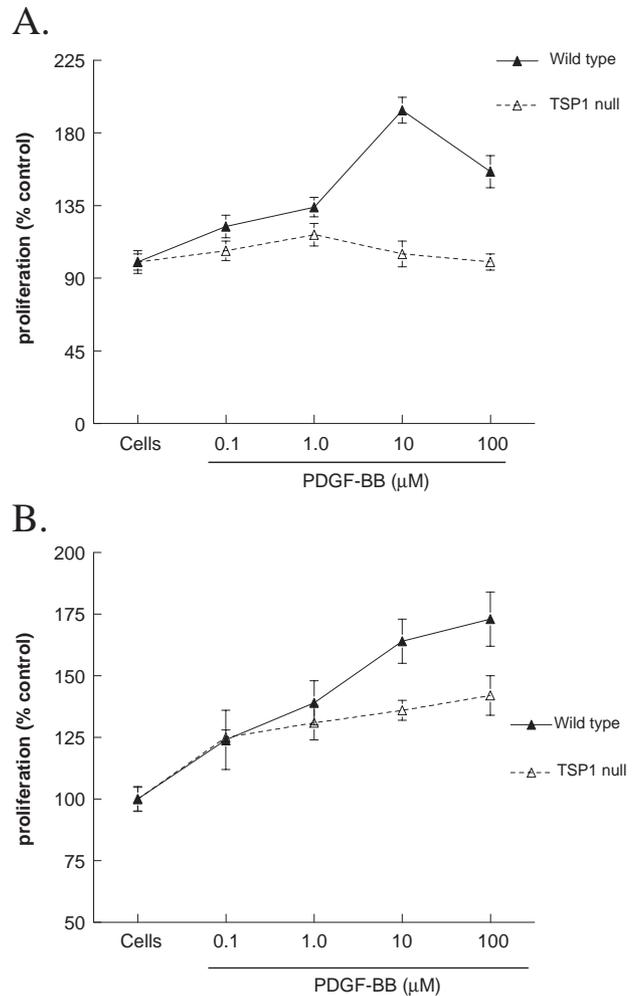


Fig. 9. PDGF differentially induces proliferation of TSP1 null and wild-type vascular cells. Cultures of VSMCs obtained from fresh murine aortas were grown in DMEM supplemented with 5% heparin-stripped FCS (A). Lung-derived endothelial cells were grown in EGM with 5% heparin-stripped FCS (B). When cells had obtained roughly 80% confluence, they were harvested and plated at  $\sim 5000$  cells/well in 96-well culture plates and incubated for 72 h in the presence of the indicated concentrations of PDGF. Proliferation was quantified using an MTT assay and is presented normalized to initial cell numbers (0%) and proliferation at 72 h in the absence of TSP1 (100%). Results represent the mean  $\pm$  S.D. of at least three separate experiments.

both  $\beta_1$  integrins and  $\alpha_v$  integrins, other than  $\alpha_v\beta_1$ , mediate chemotactic responses stimulated by TSP1. Migration of VSMCs to the N-terminal domains of TSP1 and TSP2 is mediated by  $\beta_1$  integrins, whereas migration to the C-terminal domain of TSP1 is mediated by  $\alpha_v$  integrins. Because NoC2 lacks the  $\alpha_3\beta_1$  binding site of NoC1 (Calzada et al., 2003),  $\alpha_4\beta_1$  and  $\alpha_6\beta_1$  may mediate chemotaxis stimulated by this domain of TSP2.

### 3. Discussion

Several differences have been reported between vascular endothelial and VSMCs in their responses to exogenous

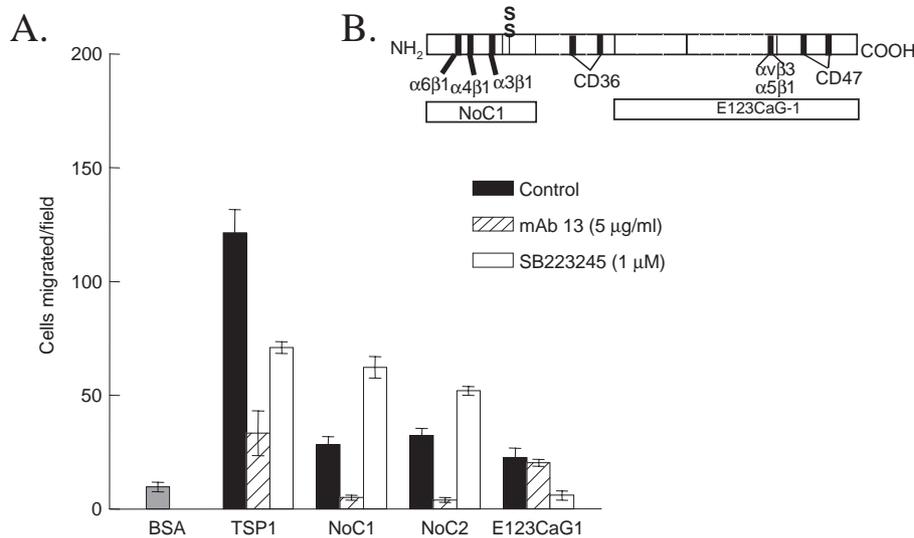


Fig. 10. N-terminal domains of TSP1 and TSP2 mediate  $\beta$ 1-dependent chemotaxis of vascular smooth muscle cells. TSP1, NoC1, NoC2 or E123CaG1 (20  $\mu$ g/ml) were added into the lower wells of Boyden chambers (A). In the upper chamber HSMC ( $0.3\text{--}0.5 \times 10^5$ /well) were added in the absence (control) or presence of the  $\beta$ 1-blocking antibody, mAb 13 (5  $\mu$ g/ml) or the  $\alpha_v$  inhibitor, SB223245 (1  $\mu$ M). Cells were allowed to migrate 5.5 h at 37 °C in 5% CO<sub>2</sub>. Medium with BSA in the lower chamber was used as a negative control. Migrated cells were counted microscopically after fixation. Results are presented as number of cells migrated/field  $\pm$  S.D.,  $n=2$ . (B) Schematic of TSP1 and TSP1 recombinant fragments showing positions of the known binding sites for integrins and integrin-associated proteins.

TSP1. TSP1 is generally a positive regulator of SMCs but an inhibitor of endothelial cell growth and motility. We have extended these studies to examine endogenous TSP1 using the respective cell types isolated from wild-type and TSP1 null mice and using 3D explant cultures, in which the combined roles of endothelial and perivascular SMCs can be quantified in a vascularization response. During outgrowth from the muscle explants, perivascular and endothelial cells exhibit the predicted opposite responses to loss of endogenous TSP1. Endothelial outgrowth is enhanced, and SMC outgrowth is inhibited. Using primary cell cultures, we show that the deficit of SMCs in the 3D cultures is probably due to positive effects of TSP1 on cell migration rather than an essential role for TSP1 in smooth muscle proliferation. The SMC migration deficiency is due to a specific requirement of TSP1 for PDGF-stimulated motility rather than a deficiency in motility to other attractants such as IGF1, serum, or exogenous TSP1. Endothelial cells show a similar dependence on endogenous TSP1 for some responses to PDGF, but in the explant assay, other mitogens and motogens presumably play more dominant roles in endothelial outgrowth.

Despite their differences, we also identified several unexpected similarities between endothelial and VSMC responses to TSP1. TSP1 inhibits proliferation of both cell types at high concentrations. Proliferation of both cell types is also inhibited by TSP1 antibodies, a response that requires endogenous TSP1. Endogenous TSP1 enhances chemotaxis and proliferative responses of both endothelial and SMCs to PDGF. PDGF is generally recognized as an attractant for SMCs, but its ability to induce chemotaxis of endothelial cells has also been reported (Phillips and Stone,

1994), and endothelial cell expression of PDGF receptor- $\beta$  is clearly required for some angiogenic responses (Crosby et al., 1999). However, we cannot strictly exclude the possibility that the endothelial cell chemotaxis to PDGF we observed was mediated by a minor perivascular contaminant in the endothelial cell cultures (Hirschi et al., 1999). Finally, both smooth muscle and endothelial cell chemotaxis (Calzada et al., 2004) are stimulated by N-terminal regions of TSP1 and TSP2 in a  $\beta$ 1 integrin-dependent manner.

Our present results indicate that TSP1 at low concentrations promotes VSMC proliferation, but it is not necessary unless PDGF is the only mitogen present. The hypothesis that TSP1 is necessary was based on the ability of several TSP1 antibodies to inhibit SMC proliferation (Majack et al., 1988; Patel et al., 1996, 1997). However, these studies could not distinguish whether the antibodies blocked a stimulatory activity of exogenous TSP1 or created an inhibitory signal by cross-linking TSP1 bound to a cell surface receptor. By comparing TSP1 null VSMCs to wild-type smooth muscle cells in the absence of exogenous TSP1, we found that cells lacking TSP1 do not have a proliferation defect, a TSP1 antibody inhibits proliferation only of cells expressing endogenous TSP1, and adding exogenous TSP1 at a concentration that does not directly perturb proliferation partially restores proliferation of the wild-type cells to that of null cells. Therefore, the antibody appears to have a direct inhibitory activity by cross-linking TSP1 bound to cell surface receptors rather than an antagonist activity for a net stimulation by exogenous TSP1. We also found that TSP1 antibodies inhibit endothelial cell proliferation under the same conditions. Therefore,

this phenomenon is neither specific for SMC nor predictive of an essential positive role for TSP1 in cell proliferation.

Several of the TSP1 receptors that are expressed on endothelial cells and mediate its effects on angiogenesis also function on SMCs. The CD36 binding motif in the type 1 repeats of TSP1 (Tolsma et al., 1993) mediates inhibition of endothelial cell chemotaxis by TSP1. Because a proteolytic fragment containing this site and a CD36 antibody stimulated SMC growth, CD36 probably plays a different role in SMC responses to TSP1. This domain of TSP1 may also regulate proliferative responses of the SMCs to collagen. Recognition of the N-terminal domain of TSP1 by  $\alpha 3\beta 1$  and  $\alpha 4\beta 1$  integrins stimulates endothelial cell proliferation (Chandrasekaran et al., 2000; Calzada et al., 2004). Our data shows that  $\alpha 3\beta 1$  ligation is also sufficient to stimulate SMC smooth muscle proliferation. N-terminal domains of TSP1 and TSP2 also stimulate chemotaxis in a  $\beta 1$  integrin-dependent manner, but in the case of TSP2, this probably involves  $\alpha 4\beta 1$  or  $\alpha 6\beta 1$ . These data are also consistent with the report that both TSP1 and  $\beta 1$  integrin blocking antibodies inhibit platelet-stimulated SMC proliferation (Ichii et al., 2002).

TSP1 binding to CD47 has been reported to have both stimulatory and inhibitory activities on endothelial cell responses (Kanda et al., 1999; Gao et al., 1996). Our data shows that the C-terminal domain of TSP1 and a CD47-binding peptide from this domain mediate an inhibitory activity for SMC proliferation. Notably, a CD47 antibody also inhibits IGF1 stimulated DNA synthesis (Maile and Clemmons, 2003). Finally, a larger C-terminal region of TSP1 has moderate chemotactic activity for SMCs mediated by recognition of the RGD sequence by an  $\alpha v$  integrin. Therefore, modulation of both vascular smooth muscle and endothelial cell responses involves multiple TSP1 receptors. Although the integrated response of SMCs to endogenous TSP1 in the explant assay is positive, these results demonstrate that TSP1 has both stimulatory and inhibitory effects on specific SMC responses through distinct receptors.

We demonstrated that trimeric N-terminal regions of TSP1 and TSP2 have chemotactic activities for VSMCs. A previous study found monomeric N-terminal domain of TSP1 to be less active than the C-terminal domain for stimulating chemotaxis (Nesselroth et al., 2001). The difference in valence may explain these results. Trivalent interactions with  $\beta 1$  integrins may enhance cell migration mediated by the N-terminal domain of TSP1. This interaction appears to be important for chemotaxis stimulated by native TSP1 because chemotaxis to TSP1 was blocked more effectively by a  $\beta 1$  integrin antibody than by an  $\alpha v\beta 3$  antagonist.

PDGF plays important roles in angiogenic responses and the overgrowth of VSMCs associated with atherosclerosis and mesangial cells in glomerulonephritis. PDGF is a VSMC mitogen and a potent chemotactic factor (Krishnaswami et al., 2002). We found that smooth muscle and lung-

derived endothelial cell chemotaxis induced by PDGF is dependent on endogenous TSP1. The supportive role of TSP1 in PDGF-mediated chemotaxis may result from TSP1 binding directly to PDGF and facilitating its binding to the PDGF receptor (Hogg et al., 1997). This may also involve lateral association of the PDGF receptor with the TSP1 receptor  $\alpha v\beta 3$  integrin (Borges et al., 2000).

The important role of endogenous TSP1 for motility responses to PDGF may account for the activity of a TSP1 antibody to reduce neointima formation in balloon-injured rat carotid artery (Chen et al., 1999). In angiogenesis, the significance of the synergism between TSP1 and PDGF is less clear, but our data imply that this plays a positive role in perivascular responses during angiogenesis. This should be considered as an additional mechanism for pro-angiogenic activities of TSP1.

Angiogenesis is a dynamic process that involves numerous cell types, cell signaling events, and certain stimulating and inhibiting factors. These events normally occur within a three-dimensional extracellular matrix. TSP1 generally inhibits endothelial motility and proliferation, but it also engages at least three receptors that elicit pro-angiogenic responses (Chandrasekaran et al., 2000; Calzada et al., 2003, 2004). Conversely, TSP1 is generally considered a positive regulator of VSMC proliferation and motility (Majack et al., 1987, 1988; Patel et al., 1997; Chen et al., 1999). However, we found that higher concentrations of TSP1 similarly inhibit proliferation of VSMCs, and TSP null SMCs grew to a higher density at confluence than did wild-type cells. Thus, TSP1 may be a bidirectional regulator of both vascular endothelial and SMCs. Efforts to understand the pathophysiological roles of TSP1 in cancer and cardiovascular diseases (Detmar, 2000; Zhao et al., 2001), therefore, should consider both positive and negative effects of TSP1 on both of these vascular cell types.

## 4. Experimental procedures

### 4.1. Proteins and antibodies

TSP1 was purified from human platelets obtained from the NIH blood bank as previously described (Roberts, 1996). The  $\beta 1$  integrin function-blocking antibody (mAb13) was provided by Dr. Ken Yamada (NIDCR, National Institutes of Health). Recombinant fragments expressed in insect cells containing the N-terminal and C-terminal regions of TSP1 (NoC1 and E123CaG1, respectively) and the N-terminal region of TSP2 (NoC2) were generously provided by Dr. Deane Mosher (University of Wisconsin). Recombinant C-terminal (G) domain was expressed in *Escherichia coli* (Gao et al., 1996) SB223245, a selective antagonist of the  $\alpha v$  integrins (Keenan et al., 1997) was provided by Dr. William Miller (Glaxo SmithKline). Polyclonal rabbit anti-TSP1 (R187) was produced from a

rabbit immunized with pooled fractions of human TSP1. Monoclonal murine antibody to TSP1 (clone A6.1) was supplied by NeoMarkers (Fremont, CA). Monoclonal CD36 antibody (clone FA6-152) was obtained from Immunotech (Marseille, France). To prepare the TSP collagen-binding domain (50/70 kDa chymotryptic fragment), TSP in Tris-buffered saline (TBS; 0.02 M Tris, 0.15 M NaCl, pH 7.6) with 5 mM EDTA added was digested with chymotrypsin as described (Dixit et al., 1986a). The digest was applied to a Sephadex G-100 column equilibrated with TBS, and the trimer of 70-kDa chains was eluted at the void volume. Monoclonal  $\alpha 3$  integrin antibody (clone PIB5) was obtained from Chemicon International (Temecula, CA). Monoclonal CD47 antibody (clone B6H12) was grown and purified from a hybridoma from American Type Culture Collection (Manassas, VA).

#### 4.2. Animals

C57/B16 wild-type (Taconic, Germantown, MD) and TSP1 null mice (Lawler et al., 1998) were housed five to a cage in a pathogen-free environment and handled in compliance with the guidelines established by the Animal Care and Use Committee of the National Cancer Institute.

#### 4.3. Human cells

Human aortic VSMC were purchased from Cambrex Biosciences (Wakersville, MD) and maintained in SmGM-2 (Clonetics, Wakersville, MD) containing 5% FCS, gentamicin/amphotericin B, insulin (5  $\mu\text{g}/\text{ml}$ ), hFGF-2 (2  $\text{ng}/\text{ml}$ ) and hEGF (1  $\text{ng}/\text{ml}$ ). Cells were harvested when approximately 80% confluent. MDA-MB-435 human breast cancer cells and TSP1-transfected MDA-MB-435 cells (clone TH26) (Weinstat-Saslow et al., 1994) were maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, penicillin and streptomycin.

#### 4.4. Murine cell culture

Primary VSMCs were isolated from the aortas of 8–12-week-old wild-type and TSP1 null mice. The cells were grown at 37 °C and 5% CO<sub>2</sub>. Animals were euthanized by cervical dislocation, and under loop magnification, the aorta was harvested and placed in iced culture medium (DMEM with 10% fetal calf serum, 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin and 2 mM glutamine) (Ray et al., 2001). With microsurgical instruments adventitia was excised, the vessel cut into 1-mm fragments and incubated with collagenase type II (Worthington, Lakewood, NJ) at 37 °C for 4 h, and the mixture was then aliquoted into 25-cm<sup>2</sup> culture flasks containing culture medium, with medium changed every 3 days.

Primary lung endothelial cells were likewise grown in pure culture. Briefly, lungs from wild-type and TSP1 null mice were harvested sterilely, cut into 1-mm fragments and

incubated with collagenase in endothelial growth medium (EBM with 10% FCS, 1.0  $\mu\text{g}/\text{ml}$  hydrocortisone, 10  $\text{ng}/\text{ml}$  EGF, 12  $\mu\text{g}/\text{ml}$  bovine brain extract, 30  $\mu\text{g}/\text{ml}$  gentamicin sulfate and 50  $\text{ng}/\text{ml}$  amphotericin-B) (Cambrex, San Diego, CA) for 1 h and the solution plated in 25-mm culture flasks containing endothelial growth medium with 300  $\mu\text{M}$  rotterlin ( $\delta$  protein kinase C inhibitor) (Calbiochem, San Diego, CA). The medium was changed every third day.

Purity of the primary endothelial and VSMC cultures was checked periodically via immunohistochemistry using a commercial kit (Vector Labs MOM Immunodetection Kit, Burlington, CA) with primary monoclonal antibody to CD31 (PECAM-1, BD Biosciences, San Jose, CA) or  $\alpha$ -smooth muscle actin (Sigma, St. Louis, MO). Endothelial cell cultures were also stained as per the manufacturer's recommendations with acetylated low-density lipoprotein (Dil-Ac-LDL, Biomedical Technologies, Stoughton, MA). Only cultures of >90% purity were used. To minimize fibroblast, macrophage and other cell contaminant overgrowth, primary cultures without passage were harvested for each experiment.

To eliminate exogenous TSP1, media used in cell culture and assays was stripped of TSP1 by passing through a heparin–agarose affinity column (Sigma). A TSP1 ELISA verified no detectable amounts of intact TSP1, though some fragments of the molecule may remain. Additionally, other heparin-binding growth factors should be depleted, but the depleted serum promoted SMC growth.

#### 4.5. Co-culture of muscle explants with human breast cancer cells

0.5% noble agar (Difco) in 0.5 ml of RPMI1640 medium containing and 10% FBS was solidified in 24-well plates and 5000 MDA-MB-435 cells or TSP1-transfected MDA-MB-435 cells (clone TH-26) in 0.5 ml of RPMI 1640 medium, containing 0.3% soft agar and 10% FBS, were added to each well. After the agar solidified, 1.5-mm muscle explants from TSP1-null mice were placed on top of the soft agar and covered with 0.5 ml of neutralized Vitrogen solution as described above. After the gel formed, 0.5 ml of EBM medium supplemented with 20% FBS, 30  $\mu\text{g}/\text{ml}$  of gentamicin sulfate, 50  $\text{ng}/\text{ml}$  of amphotericin-B was added to each well. The vascular cells emerging from the muscle explants were quantified by phase contrast microscopy.

#### 4.6. Growth assay

VSMCs from aortas of wild-type and TSP1 null mice were plated in TSP1 deficient medium (500  $\mu\text{l}$ ) in 12-well culture plates (Nunc, Denmark). Plates were incubated at 37 °C with 5% CO<sub>2</sub> until confluent. VSMCs were harvested from duplicate wells on culture days 6, 9 and 11 and counted on a hemocytometer.

#### 4.7. Muscle explant/cell migration assay

The pectoralis major muscle was harvested from 8- to 10-week-old wild-type and TSP1 null mice. The fascia was excised and the muscle cut into fragments 3×3 mm in size. Fragments were plated in 24-well culture plates (Nunc) in polymerized collagen gel (Vitrogen, Cohesion Technologies, Palo Alto, CA) as described previously (Calzada et al., 2004). Endothelial cell invasion was calculated after days 3, 6, 9 and 14 of incubation and measured as the maximum distance of cell migration in each of four quadrants. Vascular outgrowth from implants was quantified using a standard commercial software package allowing determination of total area and vessel length (Image-Pro Plus, Media Cybernetics, Silver Spring, MD). Area and path length of the capillary-like tubes were measured using the program after processing the raw image using the morphological Erode filter and deleting the muscle and optical artifacts from the object list. All assays were repeated in triplicate.

#### 4.8. Cell Proliferation

Proliferation of VSMCs was measured with a non-radioactive colorimetric assay (CellTiter 96, Promega, Madison, WI). Briefly, to each well of a 96-well culture plate (Nunc, Denmark)  $5 \times 10^3$  cells suspended in 100  $\mu$ l of culture medium were added and incubated for 72 h. Appropriate zero time controls were run for all assays and the optical density readings obtained then subtracted from those obtained at 72 h.

#### 4.9. Chemotaxis

Cultures of endothelial cells and VSMCs from wild-type and TSP1 null mice were grown for 2 weeks before use. Modified Boyden chambers containing gelatin-coated 8- $\mu$ m pore polycarbonate membranes (Neuro Probe, Gaithersburg, MD) were used as described (Calzada et al., 2004). The murine cells or human VSMCs were resuspended at  $0.8\text{--}1 \times 10^6$  cells/ml in assay medium (M199 containing 0.1% BSA) and added to the upper chamber. Migration to PDGF – BB was assessed microscopically after 5.5 h.

#### 4.10. Immunohistochemistry

VSMCs from murine aortas were cultured at a density of  $5 \times 10^3$  cells per well on sterile chamber slides (Lab-Tech, Nalge Nunc International) in 50  $\mu$ l of culture medium and incubated for 48 h. Cells were then fixed with acetone and labeled with monoclonal anti-mouse CD31 or anti  $\alpha$ -smooth muscle cell actin antibody (Sigma, St. Louis, MO) and stained using the Vector MOM Immunodetection Kit (Vector Laboratories, Burlingame, CA). Positive staining cells were counted in 10 randomly selected fields. Lung-derived endothelial cells were similarly cultured on sterile chamber slides and incubated in the presence or absence of

PDGF-BB (10 ng/ml) and labeled with CD31 and  $\alpha$ -smooth muscle actin antibody and stained using the Vector MOM kit. In another series, plated cells were incubated with acetylated low-density lipoprotein (10  $\mu$ g/ml) (Biomedical Technologies, Stoughton, MA).

Vascular cells from muscle explants in three-dimensional Vitrogen gels were collected by digesting the gels with 0.2% collagenase (type 2, Sigma) in Hank's balanced salt solution (HBSS) for 10 min, washed three times with HBSS and attached to slides using a Cytospin centrifuge. The slides were fixed with and stained with CD31 or  $\alpha$  smooth muscle cell actin antibodies. Human aortic VSMCs and human umbilical vein endothelial cells were stained as positive controls using a peroxidase-based kit (Zymed Labs, San Francisco, CA).

#### 4.11. TSP1 immunoassay

Levels of TSP1 in media were determined by ELISA. Briefly, 96-well plates (Nunc) were coated with heparin-BSA (5 ng/well), blocked with Tris-BSA (50 mM Tris, 1% BSA, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 150 mM NaCl and 1 mM CaCl<sub>2</sub> at pH 7.8) for 30 min, and 50  $\mu$ l of standard or samples added to each well and incubated at 37 °C for 2 h. Wells were again aspirated, and 50  $\mu$ l/well of TSP1 antibody (1:500) in Tris-BSA was added and incubated for 2 h at 37 °C. The wells were then washed three times with PBS containing 0.2% BSA, 0.2 mM PMSF and 0.05% Tween 20. Peroxidase-conjugated goat anti-rabbit IgG (1:1000 in wash buffer) was added to each well and incubated for 1 h at room temperature. The plate was again washed three times with wash buffer, and bound antibody conjugate was detected with *o*-phenylenediamine dihydrochloride (Sigma).

#### 4.12. Statistics

All studies were repeated in triplicate, and results are expressed as the mean  $\pm$  S.D. To compare results between treatment groups, Student's *t*-test was used with a *p*<0.05 being taken as significant.

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