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# Thrombospondins in cancer

**S. Kazerounian, K. O. Yee and J. Lawler***

Division of Cancer Biology and Angiogenesis, Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, 99 Brookline Avenue, RN 270C, Boston, Massachussetts 02215 (USA), Fax: +1 617 667 3591, e-mail: jlawler@bidmc.harvard.edu

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**Abstract.** The thrombospondins (TSPs) are a family of five proteins that are involved in the tissue remodeling that is associated with embryonic development, wound healing, synaptogenesis, and neoplasia. These proteins mediate the interaction of normal and neoplastic cells with the extracellular matrix and surrounding tissue. In the tumor microenvironment, TSP-1 has been shown to suppress tumor growth by inhibiting angiogenesis and by activating transforming growth factor β. TSP-1 inhibits angiogenesis through direct effects on endothelial cell migration and survival, and through effects on vascular endothelial cell growth factor bioavailability. In addition, TSP-1 may affect tumor cell function through interaction with cell surface receptors and regulation of extracellular proteases. Whereas the role of TSP-1 in the tumor microenvironment is the best characterized, the other TSPs may have similar functions. (Part of a Multi-author Review)

**Keywords.** Thrombospondin, angiogenesis, cancer, transforming growth factor β, tumor dormancy.

## Introduction

Tumor progression involves a complex series of events that begins with mutations in tumor cells and ends with invasion and metastasis to distant sites. During this progression, the normal tissue architecture is disrupted and a response is initiated in the surrounding tissue that resembles wound healing. This response may be initiated by the hyperpermeable blood vessels that characterize the tumor vasculature. These vessels release plasma proteins that initiate fibrin generation. The tumor microenvironment also includes the activated fibroblasts, immune cells, extracellular matrix, and new capillaries that make up the desmoplastic response. Thus, tumors have been likened to 'wounds that don't heal' [1].

Thrombospondin-1 (TSP-1) and -2 (TSP-2) are highly expressed during the tissue remodeling that is associated with wound healing and tumor progression. Aberrant wound healing is observed in TSP-1- and -2-null mice [2]. In the TSP-1-null mice, the wounds heal more slowly and there is a decrease in the recruitment of macrophages. Whereas there is an increase in vessel density in the TSP-1-null wounds, TSP-2 appears to have a more significant effect on angiogenesis in cutaneous wounds. The role of the other members of the TSP gene family in tissue remodeling is less well understood. The use of gene array studies to characterize tumor tissue has revealed that these proteins are expressed in some cases. For example, TSP-4 is differentially expressed in invasive lobular breast carcinoma as compared to ductal breast carcinoma [3]. Furthermore, expression of TSP-3 reportedly stimulates osteosarcoma progression [4]. One of the ways that TSP-3, -4, and -5 (also referred to as cartilage oligomeric matrix protein or COMP) differ from TSP-1 and -2 is that they lack the type 1 repeats (TSRs). The TSRs of TSP-1 and -2, as well as other proteins, have been shown to mediate their anti-angiogenic activity. Thus, TSP-3, -4, and -5 would not be expected to inhibit angiogenesis in the tumor microenvironment.

Several recent reviews have discussed the role of TSP-1 as an inhibitor of angiogenesis and tumor progression [5–7]. In addition, the use of TSP-based therapies

* Corresponding author.

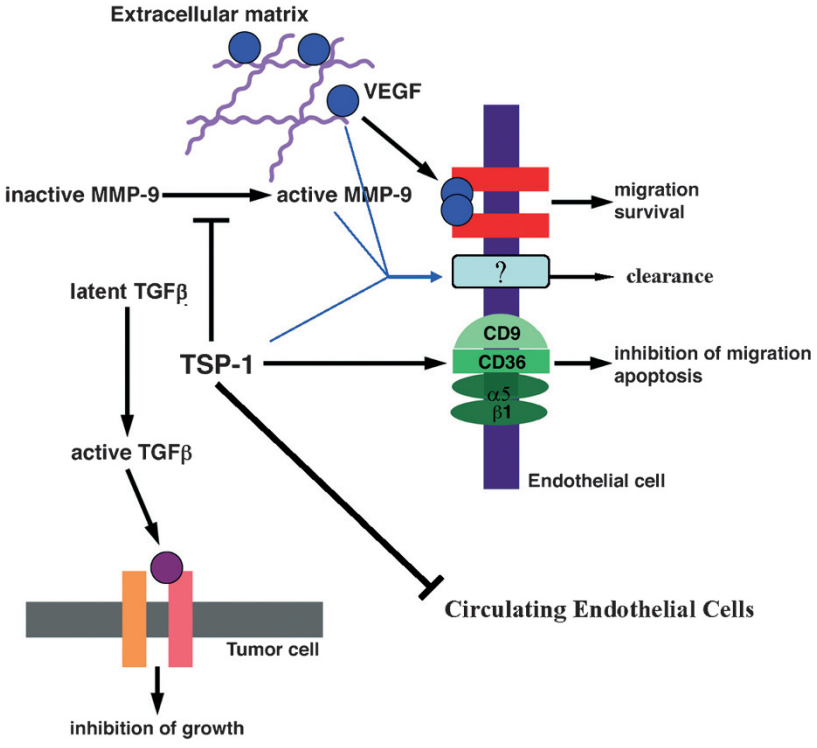


**Figure 1.** Schematic representation of the function of TSP-1 in tumors. TSP-1 directly affects endothelial cell function through interaction with CD36 and integrins. The downregulation of circulating endothelial cells may also be mediated by CD36. TSP-1 sequesters VEGF and inhibits mobilization of VEGF from the extracellular matrix by MMP-9. TGFβ that is activated by TSP-1 in the tumor microenvironment can inhibit angiogenesis and tumor cell growth in TGFβ-responsive tumor cells.

to inhibit tumor growth has been reviewed [8]. The similarity of the TSPs to other extracellular matrix proteins prompted many investigators to explore the ability of TSP-1 to support tumor cell adhesion and migration *in vitro* [9]. In 1990, Noel Bouck's laboratory identified TSP-1 as the first natural protein inhibitor of angiogenesis [10]. This group assayed the ability of culture supernatants derived from fibroblasts containing a mutation in a tumor suppressor gene to inhibit endothelial cell migration. N-terminal amino acid sequencing of a protein in the active fraction revealed that a proteolytic fragment of TSP-1 was the key component. Subsequent studies have revealed that multiple and varied mechanisms are involved in the inhibition of angiogenesis by TSP-1. In this review, we will provide an overview of the functions of the thrombospondins in cancer, with a particular focus on their role in the regulation of angiogenesis and the activation of transforming growth factor β (TGFβ) (Fig. 1).

## The TSP gene family

The five members of the TSP gene family can be divided into two groups on the basis of their molecular architecture. As mentioned above, the TSRs are present in TSP-1 and -2 (subgroup A), but not in the other family members (subgroup B, including TSP-3, -4, and -5). TSRs are compact, structurally stable and found in approximately 41 different proteins in the human genome (Fig. 2A). Carlson and co-workers provide a detailed description of the structures of the TSPs in the accompanying review. The TSRs of TSP-1 and -2 interact with the endothelial cell membrane protein CD36 to inhibit migration and induce apoptosis [11]. The TSRs of TSP-1 also bind and activate latent TGFβ, which, in turn, inhibits tumor growth if the tumor cells retain the ability to respond to this cytokine (see below). The TSRs also bind to integrins that contain the β1 subunit [12]. This interaction is involved in the inhibition of migration of large-vessel endothelial cells, which, in general, lack CD36 [13]. The subgroup A TSPs are distinct from the subgroup B TSPs in that the former are trimers and the latter are pentamers. It has been demonstrated that mixed multimers of subunits from each subgroup can form; however, the frequency and physiological significance of this property has not been established. The multimeric nature of the TSPs may enable them to cluster receptors and affect signal transduction pathways. TSP-1 has been reported to induce dimerization of one of its receptors, CD36 [14] . This dimerization may bring together CD36- associated proteins to initiate signal transduction. Fyn has been shown to associate with CD36 in the lipid raft fraction of the membrane and has been reported to mediate the anti-angiogenic effects of TSP-1 [15].

The hallmark of the TSPs is the 'signature domain', which represents the C-terminal half of the proteins.

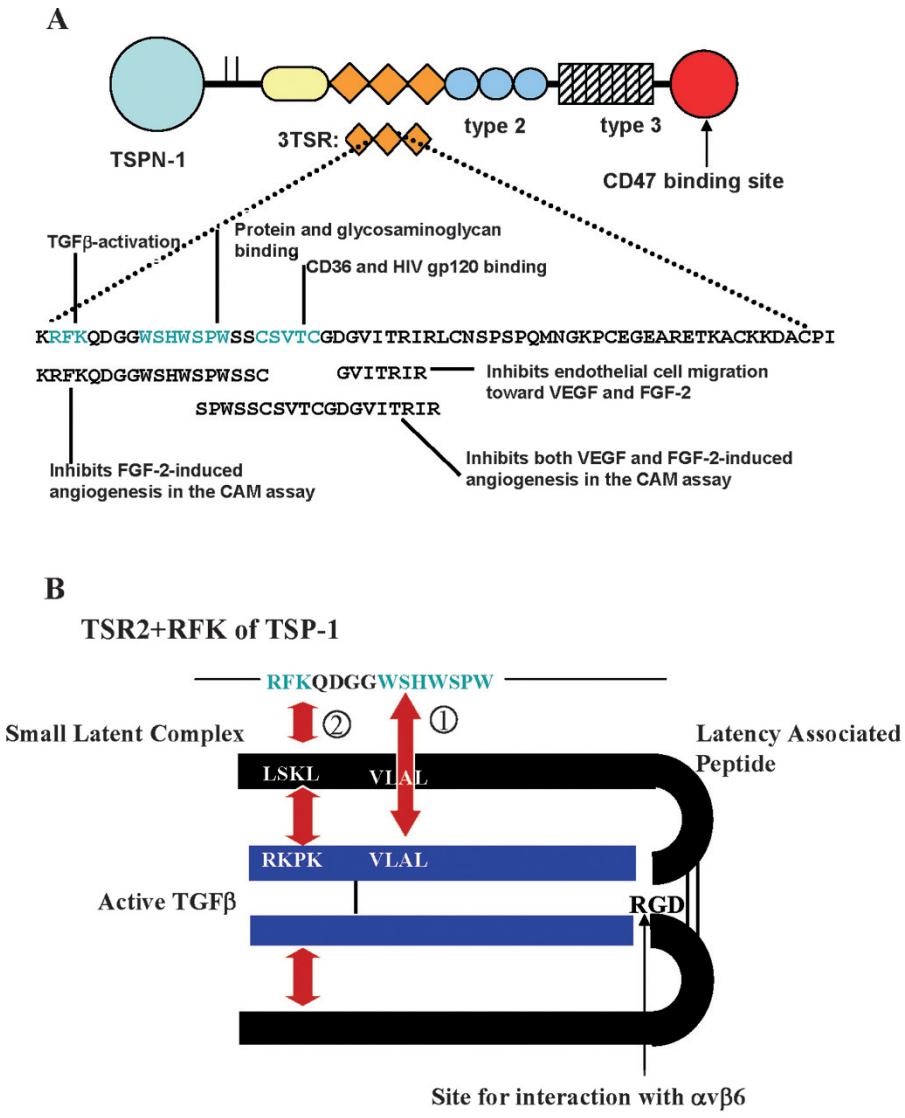


**Figure 2.** (*A*) Schematic representation of the domain structure of TSP-1. The amino acid sequence of the second type 1 repeat is shown with the sequences that are involved in the activation of TGFβ and the inhibition of angiogenesis highlighted. (*B*) Proposed mechanism for the activation of TGFβ by TSP-1. The LSKL sequence in the latency-associated peptide interacts with the RKPK sequence in latent TGFβ. When the WSHWSPW sequence binds to the VLAL sequence of active TGFβ, the RFK sequence of TSP-1 competes for LSKL binding and results in a conformational change that renders the active TGFβ able to bind to its receptor. The WSHW sequence of TSP-1 also binds to the VLAL sequence that is present in the LAP.

This domain contains numerous calcium binding sites, and the conformation of the domain is profoundly affected by calcium. In some TSPs the signature domain reportedly mediates the interaction of the TSPs with cell surface proteins such as integrins and CD47. The signature domain of TSP-1 binds αvβ3 and that of TSP-5 binds α5β1 [16, 17]. In addition, the signature domains of TSP-1 and TSP-5 are able to bind to glycosaminoglycans [18, 19]. These interactions may be important in supporting the adhesion and migration of tumor cells and stromal cells during cancer progression.

## The role of TSP-1 and -2 in cancer progression

Tumor progression is a multistep process that requires that the tumor cells acquire (1) the ability to evade apoptosis, (2) self-sufficiency in growth signals, (3) insensitivity to anti-growth signals, (4) limitless replicative potential, (5) sustained angiogenesis, and (6) the ability to invade and metastasize [20]. In this process, angiogenesis is a key step that facilitates the transfer of nutrients to tumor cells and provides a transport system through which tumor cells can metastasize to distant organs. Therefore, angiogenesis and tumor progression are inextricably linked with angiogenesis being a rate-limiting step in tumor growth and metastasis. In fact, microscopic dormant breast, prostate, and thyroid tumors are commonly observed in autopsies of individuals who had not been diagnosed with cancer [21]. These and other observations imply the existence of an angiogenic switch [22]. Once the angiogenic phenotype has been acquired, the degree of angiogenesis within tumors depends on the balance between pro-angiogenic and anti-angio-

genic factors that are produced by both tumor and stromal cells. This balance is established through genetic alteration in tumor cells, hypoxia, and stromal cell recruitment. TSP-1 and -2 are important factors in regulating angiogenesis. Several reports have addressed the possible correlations between the expression level of TSP-1 in tumors and tumor growth and metastasis both *in vivo* and *in vitro* [5, 6, 9, 23–25]. These studies have also addressed the possible factors for regulating TSP-1 expression during tumor progression, which is important for modulating the balance between anti-angiogenic and pro-angiogenic stimuli.

Tuszynski et al. first documented the importance of TSP-1 in breast cancer in 1987 [26]. According to these studies, the i.v. injection of TSP-1 into mice prior to injecting tumor cells increased seeding of tumor cells in the lung. This was related to the ability of TSP-1 to stimulate tumor cell adhesion to vessel walls. Zajchowski et al. investigated the differential gene expression pattern between immortalized nontumorigenic mammary epithelial cells and MCF-7 tumor cells [27]. They demonstrated that fusion of these two cell types suppresses the tumorigenic activity of MCF-7 cells due to higher expression of intermediate filaments keratin 5, fibronectin, and anti-angiogenic factors such as TSP-1. Campbell et al also demonstrated an inverse correlation between the expression level of TSP-1 and malignancy stage of human lung, breast, and bladder cancer [28]. Brown et al. compared the expression level of TSP-1 at the mRNA and protein levels in normal breast tissue, in invasive ductal and lobular breast carcinoma, and in *in situ* breast carcinoma [29]. According to these findings, in normal breast tissue, TSP-1 mRNA is expressed in stromal fibroblast and epithelial and myoepithelial cells of ducts and lobes. The immunostaining of tissue sections also showed a weak expression of TSP-1 in the basement membrane of normal ducts, but no staining was detected in stroma [30]. Both groups also detected higher levels of TSP-1 mRNA and protein in stromal cells of ductal carcinoma and weak expression in some tumor cells. By contrast, in invasive lobular carcinoma, the more invasive tumor cells expressed high levels of TSP-1 mRNA and protein. A similar expression pattern was also detected in stromal cells surrounding the tumor cells. In *in situ* carcinoma, TSP-1 was detected in the basal layer of myoepithelial cells, and also stromal cells adjacent to tumor cells. These data suggest that as tumor cells become more invasive, they modify their surrounding stroma to a highly vascular environment. These changes may be a prerequisite for going from carcinoma *in situ* to invasive adenocarcinoma. One critical factor in this event is the changes in the distribution of TSP-1 and most probably its interactions with critical cell surface receptors. Bertin et al. also demonstrated a significant increase in TSP-1 mRNA in the stroma of invasive breast carcinoma compared to normal and benign breast tissue, and suggested fibroblasts were the source of TSP-1 in stroma [31]. The significance of stromal derived TSP-1 can be seen when tumor cells are implanted into TSP-1-null mice. In these experiments, the tumors grow approximately twice as fast in the TSP-1-null mice as they do in their wild-type counterparts [32]. Surprisingly, systemic treatment of wild-type tumor-bearing mice with an antibody to TSP-1 inhibits experimental tumor growth. Whereas the reason for this result is unclear, it is possible that the antibody, which was an immunoglobulin M (IgM), actually increased the activity or stability of endogenous TSP-1, rather than antagonized it.

Fontana et al. addressed the possible processes that control the angiogenic switch in tumor cells [33]. In their studies, they compared the expression level of TSP-1 in tumor cells and stromal fibroblasts in breast samples from patients with invasive ductal carcinoma. According to their findings, during early stages, the level of stromal TSP-1 is high enough to inhibit neovascularization and delay tumor growth. The prolonged exposure of cells to TSP-1 may promote hypoxia and an increase in VEGF secretion from tumor cells that overrides the effect of TSP-1 and therefore stimulates angiogenesis. In separate studies, Naumov et al. demonstrated that tumors remain dormant during the non-angiogenic stage, and only a small subset of tumor cells can switch to the angiogenic phenotype and become metastatic [5, 34]. They isolated the angiogenic and non-angiogenic tumor cells and demonstrated that the angiogenic switch is a multistep process. First, there is an increase in the expression level of TSP-1 in stromal fibroblasts that mediates anti-angiogenic events and inhibits tumor growth. These results also show that non-angiogenic tumor cells express higher levels of TSP-1 as compared to angiogenic tumor cells. It is possible that prolonged exposure of tumor cells to TSP-1 increases expression of angiogenic factors such as VEGF and bFGF and activates the PI3K/MYC signaling pathway. The presence of high levels of VEGF overrides the inhibitory effect of TSP-1 and initiates angiogenesis. Similar processes may occur in metastasis when circulating levels of TSP-1 are sufficient to suppress tumor growth. Rofstad and Graff performed similar studies on the effect of TSP-1 on melanoma progression and found that the growth of metastatic tumor cells, but not the seeding of cells into the lung, is inhibited by TSP-1 [35]. It is possible that the level of pro-angiogenic factors increases in the metastatic cells over time to promote growth. However, the exact timing and mechanism that

allows this switch is different among various tumor types and still remains a puzzle.

The dynamic nature of the interplay between TSP-1 and VEGF is further demonstrated by the studies of Filleur and co-workers, who showed that prolonged exposure to TSP-1 can foster the outgrowth of a population of tumor cells that are resistant to the inhibitory effects of TSP-1 and TGFβ through the genetic mutations that arise in tumor cells during rapid proliferation [36]. Rat fibrosarcoma cells and C6 gliomas expressing both murine TSP-1 and luciferase were injected subcutaneously into syngeneic Fisher rats. When doxycycline was withdrawn, TSP-1 and luciferase were expressed in the cells and an initial delay in tumor growth was observed. Once the tumors established themselves, TSP-1-expressing tumors grew at the same rate as the control tumors. Furthermore, if doxycycline withdrawal was commenced 18 days after tumor implantation, only a transient reduction in tumor volume was observed. Moreover, cells isolated from tumors that are resistant to TSP-1 growth inhibition formed lung metastasis when injected intravenously, even in the presence of doxycycline. This insensitivity to TSP-1 caused an increase in tumor vessel density *in vivo* and induced chemotaxis of capillary endothelial cells *in vitro*. An increase in pro-angiogenic factors, particularly VEGF, was detected and conferred resistance to TSP-1 anti-tumorigenic properties.

At the later stages of tumor progression, tumor cells reduce their focal adhesion, detach from the primary tumor site, cleave extracellular matrix to invade surrounding tissue, and access blood vessels, to metastasize. Therefore, proteolytic activity plays a crucial role in tumor cell invasion [37–41]. One of the major proteolytic enzymes that degrades the extracellular matrix in the cell environment with broad specificity is plasmin. Albo and co-workers have demonstrated that TSP-1 upregulates plasmin formation at the cell surface and mediates tumor cell invasion [39, 40, 42]. They have also suggested that TSP-1 has a duel role in promoting cell invasion. At higher concentration, it activates the plasminogen/plasmin system and therefore promotes cell invasion, but at a lower concentration, it promotes cell-cell and cell-matrix contacts that are necessary for tumor cells to grow. The adhesive property of TSP-1 has been extensively studied in platelets, endothelial cells, and fibroblasts, and it is considered one of the primary functions of TSP-1 [23, 43–47]. Wang et al. further demonstrated that the role of TSP-1 in mediating metastasis depends on its ability to regulate cell adhesion. According to these studies, TSP-1 mediates attachment of tumor cells to type IV collagen [48, 49].

Taken together, these findings suggest that there are two temporally distinct phases to the effect of TSP-1 on cancer progression. During the early stage (dormancy), stromal cells express a high level of TSP-1, which inhibits neovascularization and holds tumor growth in check. Later, the tumors acquire an angiogenic phenotype as a result of an increase in pro-angiogenic factors, and the tumors grow and eventually become invasive. In this latter step, TSP-1 may function as an adhesive protein or a modulator of extracellular proteases to promote tumor invasion (for review see [9, 50]). The data suggest that the presence of TSP-1 affects multiple aspects of tumor development through effects on angiogenesis and tumor cell phenotype. Additional studies of the effect of TSP-1 on the expression level of other cellular proteins within tumor cells or stromal cells, as well as the signaling pathways involved in regulating tumor progression, are required to fully understand the complex role of TSP-1 in the tumor microenvironment.

Recent studies have revealed the signaling events that regulate the expression level of TSP-1 in various stages of breast cancer progression. Watnick et al. demonstrated that Ras oncoprotein inhibits TSP-1 expression and stimulates VEGF expression, and therefore mediates cell proliferation and tumor growth [51]. According to these studies, tumor cells with low expression or no expression of Ras had high levels of TSP-1, and after injection into nude mice, they formed tumors that were 60 % smaller than those formed in cells expressing high levels of Ras. The tumor burden formed by cells expressing high levels of Ras and low levels of TSP-1 was also eight-fold larger than those with low levels of Ras and high levels of TSP-1. These authors further demonstrated that activated Ras increased the level of Myc phosphorylation through activation of the PI3K/Rho pathway to inhibit TSP-1 gene expression. By contrast, Ras increased the level of VEGF through activation of Raf/MEK 1/2/Erk1/2 pathway. Kalas et al. further suggested that the effect of Ras on TSP-1 was not limited to the tumor cells themselves [52]. Cells expressing mutant/activated Ras protein release soluble factors that act through GPCR/SIP and Id1 to induce downregulation of TSP-1 in adjacent normal cells such as fibroblasts. These data indicate that the tumor cells use paracrine factors to create a pro-angiogenic field in their immediate environment.

Whereas TSP-1 expression can be downregulated by oncogenes, its expression can be induced by tumor suppressor genes (see [6] for a review). A positive correlation of p53 status with TSP-1 expression has been demonstrated in some tumor tissues. Loss of p53 function has been shown to correlate with reduction in TSP-1 expression and switch to a pro-angiogenic

phenotype in fibroblasts derived from a patient with Li-Fraumeni syndrome [53]. Over-expression of p53 increases TSP-1 expression and decreases VEGF expression, leading to decreased tumor growth [54]. Giuriato et al. also demonstrated the importance of p53 in suppressing angiogenesis and hematopoietic tumor growth [55]. Based on these findings, the inactivation of Myc is not sufficient to suppress tumor growth and angiogenesis in tumors that have lost p53 due to the fact that p53 induces the expression of TSP-1. This suggests that both p53 and TSP-1 have to be present for angiogenic switch in MYC-inactivated tumor cells. Whether or not p53 directly regulates transcription of the TSP-1 gene has not been established. Linderholm et al. suggest that the correlation between TSP-1 expression and p53 status is tumor-specific and is not present in breast cancer cells isolated from patient tissues [56]. Urquidi et al. also compared the gene expression pattern between non-metastatic and metastatic breast cancer cells to further address the differential expression pattern of proteins during cancer progression [57]. According to their studies, in addition to previously known Ras and Myc, osteopontin is also highly expressed in metastatic cancer cells where the expression of TSP-1 is very low. Another important factor in regulating TSP-1 expression in breast cancer cells is fibroblast growth factor 8 (FGF8). Mattila et al. proposed that an increase in FGF8 decreases the TSP-1 expression at the mRNA and protein levels, and therefore increases angiogenesis and tumor growth [58]. However, the precise mechanism of its regulation of TSP-1 is unknown.

A similar reciprocity between TSP-2 expression and tumor growth has been reported in several systems. In addition, the downregulation of TSP-2 in the tumor cells can be accompanied by TSP-2 expression in the stroma. Whereas TSP-2 expression is downregulated in chemically induced skin papillomas, strong TSP-2 expression is observed in the stromal cells, including fibroblasts [59]. The average latency period is reduced from 14 weeks in the wild-type mice to 9 weeks in the TSP-2-null mice. TSP-2-null mice develop more papillomas and display a significantly higher rate of tumor growth. The rate of conversion of the papillomas to carcinoma is equivalent in the wild-type and TSP-2-null mice in this model. However, TSP-2 appear to suppress lymph node metastasis. TSP-2 also suppress angiogenesis as measured by vessel size and density.

The growth of experimental squamous cell carcinomas, malignant melanomas and Lewis lung carcinomas is inhibited by overexpression of TSP-2 [60]. The tumors that grew in the presence of increased TSP-2 displayed a significant decrease in tumor vessel size and an increase in tumor cell apoptosis. A recombinant version of TSP-2 that contains the N-terminal domain half of the molecule, including the TSRs, has been used systemically to inhibit the growth of experimental squamous cell carcinomas [61]. Thus, TSP-1 and TSP-2 are potent endogenous inhibitors of angiogenesis and tumor growth. The next section describes the mechanisms by which these proteins may inhibit angiogenesis. Whereas the majority of studies have focused on TSP-1, the level of sequence identity in the active domains of TSP-1 and -2 suggests that they have similar activities.

## Molecular mechanisms for the inhibition of angiogenesis by TSP-1 and -2

As indicated above, the thrombospondins are complex multidomain molecules. Whereas the preponderance of *in vivo* evidence indicates TSP-1 is an endogenous inhibitor of angiogenesis in the tumor microenvironment, some studies have reported that TSP-1 or domains of TSP-1 can stimulate angiogenesis (see below). The inhibition of angiogenesis by TSP-1 involves direct effects on endothelial cell migration and apoptosis, as well as effects on the bioavailability of VEGF. TSP-1 influences VEGF activity in two ways. By inhibiting the activation of matrix metalloproteinase 9, TSP-1 suppresses the release of VEGF from the extracellular matrix [62]. In addition, TSP-1 binds directly to VEGF, and recent data indicate that TSP-1 can mediate the uptake and clearance of VEGF [63]. Similarly, TSP-2 reportedly binds to MMPs and mediates their uptake and clearance [64]. Thus, TSP-2 may also affect the bioavailability of VEGF. Several groups have mapped the anti-angiogenic activity of TSP-1 to the TSRs [24]. Studies with synthetic peptides have identified the WSHWSPW, VTCG, and GVITRIR sequences as active (Fig. 2A). Abbott Laboratories' therapeutic, designated ABT-510, is derived from the GVITRIR sequence [24]. The interaction of TSP-1 with CD36 plays a significant role in the anti-angiogenic activity of TSP-1 both *in vitro* and *in vivo* [11, 15, 65]. *In vitro*, CD36 mediates TSP-1 inhibition of endothelial cell migration and tube formation [11, 15, 65]. Both GST fusion proteins containing the TSP-1-binding region of CD36 and antibodies against CD36 block the inhibition of endothelial cell migration caused by TSP-1. Transfection of CD36 into human umbilical vein endothelial cells (HUVECs) renders them more sensitive to inhibition of migration by TSP-1. Furthermore, molecules that bind to CD36, including collagen, oxidized low-density lipoprotein (LDL), and an anti-CD36 IgM antibody, can also inhibit endothelial cell migration [11, 15, 65]. Whereas TSP-1 is a potent

inhibitor of bFGF-induced corneal neovascularization in wild-type mice, it is not active in CD36-null mice [11, 15, 65]. In addition, upregulation of CD36 *in vivo* with ligands for peroxisome proliferator-activated receptor gamma (PPAR γ) [66] increases the anti-angiogenic activity of ABT-510. Jimenez et al. [15] have shown that TSP-1 activation of a CD36-Fyn-caspase-3-p38 MAPK cascade is essential for TSP-1's anti-angiogenic effect, as well as its induction of endothelial cell apoptosis. Activation of this pathway leads to increased endothelial cell expression of Fas ligand, which sensitizes the cells to the increased levels of Fas that are induced by factors that initiate angiogenesis [67]. The binding of TSP-1 to CD36 also blocks the uptake of myristate, which in turn can affect the myristolation and activity of eNOS and Fyn as well as membrane localization of Fyn [68]. Nor et al. [69] have reported that TSP-1 inhibits angiogenesis by decreasing expression of Bcl-2 and increasing expression of Bax. Taken together, the data indicate that the anti-angiogenic effect of TSP-1 depends, at least in part, on its ability to induce apoptosis of endothelial cells [15, 69].

CD36-independent mechanisms for the induction of endothelial cell apoptosis have also been identified [70–72]. Guo and co-workers reported that TSP-1, or peptides that comprise TSR sequences, induce apoptosis of bovine aortic endothelial cells [71]. Recent data indicate that the TSRs can bind to β1 integrins and this interaction can inhibit endothelial cell migration [12]. We have found that CD36, CD9, and β1 integrins form a complex on platelets and endothelial cells [73]. Primo et al. [74] have reported that mutation of cysteine 464 in the cytoplasmic domain of CD36 significantly reduces the ability of CD36 to associate with β1 integrins. This mutation also abrogates the ability of TSP-1 to inhibit endothelial cell migration. These authors also observed that treatment with TSP-1 reduced the phosphorylation of VEGFR2. These data suggest that the TSPs may engage a multiprotein complex to inhibit endothelial cell function and that these multiprotein complexes may form a platform for cross-talk between pro- and anti-angiogenic signal transduction pathways in the plane of the membrane.

Increased expression of CD47 has also been reported to correlate with increased endothelial cell apoptosis [70–72]. In addition, a peptide that contains the CD47 binding sequence of the C-terminal domain of TSP-1 has been reported to inhibit angiogenesis [70–72]. This peptide inhibits *in vitro* tube formation of brain capillary endothelial cells and bFGF-induced angiogenesis in the cornea. Whereas the role of CD47 in TSP-1-induced apoptosis has not been fully determined in endothelial cells, it has been shown that under mechanical stimulus, these proteins can induce apoptosis in HUVECs This occurs by initiating an autocrine loop that includes TSP-1, CD47, and αvβ3 complex, which results in downregulation of TSP-1 and CD47 [75]. Antibodies to CD47 induce apoptosis of B cells derived from patients with B cell chronic lymphocytic leukemia [76]. CD47 is also an important mediator of the effect of TSP-1 on nitric oxide (NO) signaling [7]. Binding of TSP-1 to CD47 inhibits the activation of soluble guanylyl cyclase and suppresses NO-dependent pro-angiogenic pathways [68].

In some experimental models, the inhibition of endothelial cell function by TSP-1 can be overcome by stimulation of angiogenesis by other cell types, including inflammatory cells or myofibroblasts. In addition, the N-terminal domain of TSP-1 (TSPN-1), prepared by proteolysis or as a recombinant protein, stimulates angiogenesis. In a study performed in the rabbit cornea, the authors conclude that the stimulatory effect of TSP-1 is due to increased activation and chemotaxis of polymorphonuclear cells [77]. In another study, rat aortic rings have been cultured in collagen or fibrin gels in the presence or absence of TSP-1 [78]. These authors conclude that TSP-1 stimulates the growth and migration of myofibroblasts, which, in turn, stimulate angiogenesis. This conclusion is consistent with other studies showing that TSP-1 stimulates proliferation and migration of smooth muscle cells.

Varying effects of the isolated TSPN-1 (amino acids 1–242) on endothelial cell behavior have been reported in the presence and absence of growth factors. Vogel et al. [79] reported that a recombinant version of TSPN-1 (amino acids 1–242) inhibits endothelial cell migration toward basic FGF-2. This protein also inhibits mitogenesis and proliferation of endothelial cells and the binding of FGF-2 to heparin. Based on these data, the authors conclude that the TSPN-1 competes with FGF-2 for binding sites on cell surface proteoglycans. By contrast, Taraboletti et al. [80] have found that TSPN-1 stimulates angiogenesis in the rabbit cornea in the absence of FGF-2. TSPN-1 also stimulates endothelial cell invasion through Matrigel by upregulating MMP-2 and -9, and reducing TIMP-2 expression [81]. Four receptors for TSPN-1 have been reported to mediate the effect of this domain on endothelial cell function. An antibody to syndecan-4 specifically blocks the binding of TSPN-1 to HUVECs [82]. In addition, recombinant proteins or synthetic peptide studies indicate that short sequences in the TSPN-1 mediate binding to the integrin α3β1 (amino acids 190–201), α4β1 (amino acids 151–164), and α9β1 [83–85]. The interaction with α3β1 and α4β1 reportedly stimulates endothelial cell proliferation and angiogenesis.

### TSP-1 as an activator of TGFβ in the tumor microenvironment

The phenotype of the TSP-1-null mice is, at least in part, related to its ability to activate TGFβ [86]. These mice develop a patchy, acute, and organizing pneumonia that is similar, but less severe, to the TGFβ-null mice [87]. Since the integrin αvβ6 is a second mechanism for the activation of TGFβ, this pneumonia phenotype is also observed in integrin β6-null mice and is most severe in TSP-1 and β6 double-null mice [88, 89]. Several sequences in TSP-1 reportedly mediate the activation of TGFβ (Fig. 2B). The WSHWSPW and RFK sequences in the second TSR of TSP-1 mediate binding and activation of TGFβ, respectively [90, 91]. In this model, the L (54) SKL sequence in the N-terminal region of the latency-associated peptide (LAP) binds to the sequence R (94) KPK in the active portion of TGFβ [90, 91]. As a first step in activation by TSP-1, the WSHWSPW sequence binds to the sequence (VLAL) in the active portion of TGFβ. This interaction positions the RFK sequence of TSP-1 so that it can compete with the RKFK sequence for LSKL binding. The binding of the RFK sequence to the LSKL sequence induces a conformational change that renders the TGFβ able to bind to its receptor and initiate signaling. Since only the second TSR of TSP-1 has the RFK sequence, TSP-2 is not able to activate TGFβ.

Several lines of evidence indicate that TSP-1 activates TGFβ in the tumor microenvironment. These studies involve systemic treatment of tumor-bearing mice or expression of TSR-based peptides in tumor cells. Systemic treatment of mice that have been injected with B16F10 melanoma tumor cells with recombinant versions of TSR2 (does not activate TGFβ) and TSR2+RFK (does activate TGFβ) results in a significant inhibition of tumor growth [92]. In this model, the TSR2+RFK peptide is more effective at lower concentrations than the TSR2 peptide. When either a soluble form of the TGFβ receptor or an antibody against TGFβ is co-injected with the TSR2+RFK peptide, tumor volume increases by about threefold indicating that a portion of the inhibitory activity is directly attributable to activation of TGFβ. Furthermore, mutation of the RFK sequence to QFK resulted in a level of tumor inhibition that is comparable to TSR2 with regard to tumor volume and levels of active TGFβ. Tumor vessel density is decreased by all three peptides at 1.0 mg/kg/day. Finally, tumor extracts and cell culture supernatant from TSR2+RFK-treated B16F10 cells contain elevated levels of active TGFβ, while TSR2-treated cells have levels of active TGFβ that are comparable to saline-treated cells. In this model, the anti-angiogenic effect is not dependent on TGFβ since the inhibitors did not abrogate the decrease in vessel density with TSR2+RFK treatment. Thus, the anti-angiogenic effect is probably due to direct effects on endothelial cells or the modulation of other pro-angiogenic factors, as described above. The data indicate that TSR2+RFK is inhibiting tumor growth through two mechanisms, inhibition of angiogenesis and inhibition of tumor cell proliferation through activation of TGFβ.

We have shown that overexpression of full-length TSP-1 in A431 cells inhibits tumor growth in a xenograft mouse model by 70–80 % [93]. A decrease in vessel size and number is observed, consistent with the anti-angiogenic effect of TSP-1. To determine whether the ability of TSP-1 to activate TGFβ influences tumor growth in this model, the TGFβ-activating sequences of TSP-1, TSR2+RFK, and TSR2 were overexpressed in A431cells and the cells were injected subcutaneously into nude mice [41]. As in the case of overexpression of full-length TSP-1, tumors are significantly smaller in the TSR2+RFK-expressing tumors (20 % of control) as compared to control tumors. Interestingly, the TSR2-expressing tumors are also smaller than control (50 % of control). However, a decrease in tumor vessel size is only observed in the TSR2+RFK-expressing tumors. Furthermore, the levels of active and total TGFβ are considerably higher in the TSR2+RFK tumors as compared to the control tumors. As indicated above, the LSKL peptide binds to TSP-1 or TSR2+RFK and blocks TGFβ activation. Co-injection of the LSKL peptide with TSR2+RFK suppressed the ability of TSR2+RFK to inhibit tumor growth and decrease vessel size. The data suggest that in this model, a portion of the anti-angiogenic effect of TSP-1 is a direct result of the ability of the TSR2+RFK peptide to activate TGFβ.

Harpel and co-workers reported that TGFβ activation depends on TSP-1, the integrin αvβ3, and the integrin-associated protein CD47 [94]. In this study, enhanced activation of TGFβ (predominantly TGFβ3) by the human breast carcinoma cell line T47D is observed after treatment with tamoxifen or deprivation of estrogen [94]. The increase in TGFβ activation is dependent on TSP-1 since antibodies to TSP-1 or the GGWSHW peptide blocked TGFβ activation by 40–50 % [94]. The increase in TSP-1-dependent activation of TGFβ is also seen in tamoxifen-treated MCF-7 breast cancer cells, and antibodies against TSP-1, αvβ3, and CD47 decreased TGFβ activation [95]. In both cell lines, decreases in TSP-1 protein and mRNA levels (10-fold) were observed, suggesting that TSP-1 physically associated with its receptors (αvβ3 and/or CD47) to activate TGFβ. Indeed, there is a twofold increase in cell surface-associated TSP-1, αvβ3, and

CD47 in estrogen-depleted cells as compared to controls with no change in total TGFβ.

In some tumor cells, a positive feedback loop between TSP-1 and TGFβ may exist in that active TGFβ induces TSP-1 expression. TGFβ has been shown to increase expression of TSP-l through several different pathways. In an osteosarcoma cell line [96], increased TSP-1 mRNA stability accounted for increased TSP-1 expression (2.5 h vs. 10 h with treatment), and this occurred partially via p38 MAPK. Increased mRNA expression correlated with an increase in protein expression. In some cases, this positive feedback loop may contribute to a more aggressive phenotype. For example, elevated levels of TGFβ were associated with high-grade osteosarcomas [97, 98], which is consistent with the observation that fewer osteosarcomas occur in p53-deficient mice that lack TSP-1 as compared to those that express TSP-1 [32].

One downstream signaling molecule of the TGFβ receptor is Smad4/DPC4. This gene is lost in many cancers of the gastrointestinal organs, including the pancreas. Restoration of Smad4 expression in the human pancreatic adenocarcinoma cell line Hs766T suppressed tumor growth *in vivo* such that the tumors grew to only 3–5 mm in diameter [32]. Whereas restoration of Smad4 expression did not restore the cells' ability to respond to TGFβ (e.g. decrease in cell proliferation), it did decrease VEGF mRNA and protein levels by two- to threefold and increased TSP-1 mRNA and protein levels by threefold. Interestingly, in light of the studies mentioned above, these cells formed tumors that had fewer large-size vessels (68% decrease of vessels that were 10–50 μm and 50% decrease in vessels that were >50 μm as compared to control tumors). The two studies suggest that TGFβ activation can influence endothelial cell biology by altering the ratio of pro-and anti-angiogenic factors.

Due to their anti-angiogenic properties, there has been an interest in the roles of TSP-1 and TGFβ in endothelial cell biology. In a study using murine cell lines, TGFβ increased the steady-state level of TSP-1 by twofold in normal, capillary endothelial cells, while in the tumorigenic endothelial cells (bEND.3), which had no steady-state TSP-1 expression, TGFβ increased TSP-1 to low levels. The increase in TSP-1 was dependent on TGFβ signaling because only a slight increase in TSP-1 expression was observed in Py-4-1 cells, which are not responsive to TGFβ [99]. Because tumorigenic, endothelial cells develop large, malformed blood vessels when injected into nude mice, it was hypothesized that TSP-1 contributed to the development and stabilization of vascular structures and may influence vessel diameter. To test the hypothesis that increased expression of TSP-1 by TGFβ contributes to vessel stability, TSP-1 expression levels were measured in large vessels (rat aortic endothelial cells) and small vessels (rat capillary endothelial cells) [99]. The capillary endothelial cells expressed significantly higher TSP-1 mRNA levels than the aortic endothelial cells. Furthermore, TGFβ treatment for 3 days increased expression of TSP-1 in the aortic endothelial cells and the extracellular matrix, and decreased cell proliferation in both normal and bEND.3 cells. Moreover, it was previously shown that smooth muscle cells/pericytes co-cultured, and physically in contact with endothelial cells, would give rise to active TGFβ, and the supernatant would inhibit endothelial cell growth [100, 101]. To test this effect *in vivo*, TSP-1 was overexpressed in bEND.3 cells and injected subcutaneously in nude mice [102]. The TSP-1-overexpressing cells failed to form tumors (hemangiomas), and the cells grew more slowly in culture and formed cords in Matrigel. Interestingly, TGFβ levels were similar in the parental cell line, but net fibrinolytic activity was decreased as a result of increased PAI-1 activity and a corresponding decrease in urokinase-type plasminogen activator (uPA) secretion.

As mentioned above, overexpression of TSP-1 can affect the fibrinolytic activity of a cell [102], but the direction depends on the cell type. In a number of other studies, TSP-1 or TGFβ was added to various cells and an increase in plasmin generation was observed [38, 103, 104]. This increase was a direct result of a decrease in PAI-1 protein levels. Human lung A549 carcinoma cells exhibited a twofold decrease [103]; human breast cancer cells MDA-MB-231, a five- to sixfold decrease [104]; and human pancreatic cell lines ASPC-1 and COLO-357, a dose-dependent decrease in PAI-1 levels [38]. The decrease in PAI-1 could be inhibited by anti-TSP-1 antibodies and by neutralizing antibodies against TGFβ, suggesting a direct role for these proteins.

## Conclusion

The TSPs function in a wide range of settings that involve tissue remodeling, including angiogenesis and neoplasia. They are pleotropic proteins that regulate matrix structure and function, as well as cellular phenotype. Through interaction with other proteins, including TGFβ, they regulate dynamic processes that are inherently complex, interwoven, and context-dependent. As a result, it is sometimes difficult to reconcile apparently contradictory experimental results. During the early stages of tumor development, the surrounding normal tissues secrete TSP-1 and -2 where they may contribute to tumor dormancy by

acting as an anti-angiogenic fence around the tumor. The ability of TSP-1 to activate TGFβ may also contribute to holding tumor growth in check during dormancy. In many instances, it appears that the tumor does not proceed beyond this stage. The specific type of tumor and the anatomic location probably contribute to dormancy. In those cases where the switch to an angiogenic phenotype occurs, the TSPs have distinct effects on the various cell types that comprise the tumor tissue. An understanding of the important receptors and signal transduction pathways that mediate the responses of each cell type to the TSPs will help to elucidate how the response to the growing tumor is orchestrated. In some ways, the TSPs may act to support tumor progression to metastasis through their effects on the degradation of the extracellular matrix and the ability of tumor cells to invade the surrounding tissues. Whereas the ability of the TSPs to support adhesion and migration of tumors cells has been studied *in vitro*, few studies have addressed the role of the TSPs in metastasis *in vivo*.

In the tumor microenvironment, the endothelial cells receive pro- and anti-angiogenic signals concurrently that they must integrate to determine biological response. There are probably several points of cross-talk between the anti-angiogenic signals that are initiated by CD36 and the pro-angiogenic signals that are initiated by VEGFR2. Interactions between the Akt survival pathway and the apoptosis pathways may be critical in this regard. Recent data indicate that CD36, integrins, and VEGFR2 may be organized into supermolecular complexes in the endothelial cell membrane, where they may facilitate positive and negative signal integration. Whereas the characterization of these complexes has just begun, an understanding of how they work may be critical for the design of anti-angiogenic therapeutic strategies. Since breast cancer cells also express CD36, these supermolecular complexes may also be involved in tumor cell migration and invasion.


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