Oncogenes and Angiogenesis: Down-regulation of Thrombospondin-1 in Normal Fibroblasts Exposed to Factors from Cancer Cells Harboring Mutant Ras

Wojciech Kalas, Joanne L. Yu, Chloe Milsom, Jack Rosenfeld, Robert Benezra, Paul Bornstein, and Janusz Rak

Abstract

The onset of angiogenesis in cancer often involves down-regulation of endogenous angiogenesis inhibitors, of which thrombospondin-1 (TSP-1) is a paradigm. As this effect is thought to occur under the influence of transforming genetic lesions (e.g., expression of the mutant ras oncogene), its nature is regarded as intrinsic to cancer cells themselves. Here, we show that ras-transformed cancer cells can also induce TSP-1 down-regulation in their adjacent nontransformed stromal fibroblasts, but not in endothelial cells, in a paracrine and distance-dependent manner. Indeed, several H-ras-expressing fibrosarcoma (528ras1, B6ras, and NIH3T3ras) and carcinoma (DLD-1 and IEC18Ras3) cells were found to release soluble factors capable of suppressing TSP-1 protein, mRNA, and promoter activity in nontumorigenic, immortalized dermal fibroblastic cell lines in culture (e.g., in fibroblasts expressing enhanced green fluorescent protein/TSP-1 reporter). This effect was abrogated in Id1−/− fibroblasts. At least two low molecular weight (<3 kDa), heat-labile, and trypsin-resistant mediators of TSP-1 suppression were found to be released from 528ras1 cells. Their effects on normal fibroblasts were inhibited (albeit to different extents) by pertussis toxin and, in one case, by dimethylsphingosine, none of which affected TSP-1 expression by 528ras1 cells. Collectively, our study suggests that the effect of mutant ras on tumor neovascularization is not limited to changes in angiogenic properties of cancer cells themselves. Rather, mutant ras, through a different signaling mechanism, may modulate the properties of the adjacent normal stroma, thus eliciting a proangiogenic field effect.

Introduction

The process of perpetual blood vessel formation and remodeling (angiogenesis) is now widely recognized as a hallmark of cancer (1) and a prerequisite for three-dimensional tumor growth, invasion, and metastasis (2). It is also increasingly clear that tumor angiogenesis is triggered, sustained, and continuously transformed by sequential genetic events (“hits”) that accompany and drive tumor progression (3, 4). In particular, various oncogenes and tumor suppressors influence expression of angiogenesis stimulators and/or inhibitors, thereby contributing to the angiogenic phenotype of cancer cells (3, 4).

Mutant-activated Ras proteins constitute an interesting paradigm for oncogene-dependent induction of tumor angiogenesis due to their widespread presence in human cancer (5), their central role in cellular signal transduction (5), and the ample evidence for their involvement in the regulation of key proangiogenic growth factors, notably vascular endothelial growth factor (VEGF)/vascular permeability factor/VEGF-A (6–8). Indeed, in some instances, up-regulation of VEGF may be a sine qua non of the “angiogenic switch” in cancer (9). However, in other cases, the role of VEGF may be far less essential (or even nonessential; ref. 10). For example, when mutant ras is expressed in adult VEGF-deficient (VEGF−/−) mouse dermal fibroblasts (MDF528), not only do such cells (528ras1) display features of malignant transformation in vitro but also they become overtly tumorigenic and angiogenic in vivo (10). The latter observation suggests that other (VEGF-independent) changes may contribute to angiogenesis in tumors driven by oncogenic ras (8), a notion that points (among other factors) to thrombospondin-1 (TSP-1), a well-recognized ras target and potent angiogenesis inhibitor frequently down-regulated in cancer (10–13).

TSP-1 down-regulation in cells expressing mutant ras is currently attributed to activation of the epistatic signaling cascade involving phosphatidylinositol 3-kinase (PI3K), small GTPase Rho, Rho kinase (ROCK), and Myc (13). Notwithstanding the major role of this pathway, TSP-1 expression is also under the control of several other regulatory elements of potential relevance to tumorigenesis (14, 15). For instance, in cells deficient in the transcriptional suppressor Id1, TSP-1 expression was reported recently to be constitutively elevated (16), a property consistent with the systemic impairment of tumor growth and angiogenesis observed in mice deficient for Id1 and Id3 transcriptional inhibitors (17). Curiously however, Id1 loss in animals hosting either Lewis lung carcinoma (16) or spontaneous tumors due to PTEN loss (18) does not result in elevated TSP-1 expression, suggesting that the regulation of TSP-1 in tumor stroma is influenced by factors other than Id1 expression. Nevertheless, enforced genetic or pharmacologic restoration of TSP-1 activity in ras-driven tumors often leads to hypovascularity, necrosis, and tumor growth retardation (10, 13) even in the presence of endogenous and elevated VEGF expression (13). Thus, TSP-1 status is of considerable importance in the context of tumor angiogenesis driven by this oncogene.
TSP-1 is a large, 450-kDa, trimeric protein that is expressed by various cell types and often present in their extracellular matrices (14). The matricellular nature and multidomain structure of TSP-1 are associated with a multiplicity of functions, of which angiogenesis inhibition is mainly ascribed to the second type 1 repeat of the central stalk of the protein (14). Interaction between this domain and the CD36 receptor on activated endothelial cells is thought to result in their Fas-mediated apoptosis, collapse of capillaries, and hypoperfusion-related tumor growth inhibition (19). In agreement with this role, TSP-1 expression is often down-regulated in certain aggressive human cancers (15).

Although oncogene-dependent, cell-autonomous TSP-1 down-regulation by tumor cells provides an intuitively appealing explanation for the role of this protein in the tumor-related angiogenic switching, closer analysis of the events involved reveals several unanswered questions. First, mutations in ras proto-oncogenes would initially occur in single cells emerging during tumor progression. It is difficult to imagine how down-regulation of TSP-1 at this (unicellular) level could affect the global (multicellular) angiogenic balance and trigger angiogenesis amid most TSP-1-expressing host and tumor cells (4). Second, during our earlier gene profiling studies, we observed that TSP-1 transcripts are down-regulated not only in ras-transformed cells in culture but also to an even greater extent in corresponding tumors in vivo. This is puzzling because, in these tumors, "contamination with TSP-1-positive host cells would be expected to increase rather than decrease the TSP-1 signal intensity." (10). Third, tumor growth is reportedly accelerated in TSP-1-deficient mice, suggesting that host TSP-1 status may play a significant role in regulating tumor angiogenesis (15). Fourth, TSP-1 down-regulation in stromal cells is often a predictor of poor prognosis, including in cancers known for a high frequency of ras mutations (20). In light of these observations, we have postulated previously that cancer cells harboring certain types of genetic defects (e.g., ras mutations) may somehow trigger TSP-1 down-regulation in their adjacent host stroma (10).

Here, we show that ras-transformed murine fibrosarcoma cells release soluble mediators that can induce TSP-1 down-regulation in adjacent nontransformed, normal dermal fibroblasts. Responsiveness of the latter cells to these influences is blocked by abrogation of Id1 gene expression and by pharmacologic antagonists of Gi protein and sphingosine kinase/sphingosine-1-phosphate (SIP) pathway. These observations suggest a possible novel role for the mutant ras oncogene as a modulator of angiogenic properties of tumor stroma and a trigger of an angiogenic field effect.

Material and Methods

Cells and culture conditions. Derivation of the MDF528 (mouse dermal fibroblast) cells, including their V12-H-ras-transformed (528ras1) and HER-2/neu–transformed (528neu2) counterparts has been described in detail elsewhere (10). Briefly, MDF528 is a VEGF-deficient (VEGF−/−) non-tumorigenic fibroblastic cell line derived from dermal explants of adult chimeric (VEGF+/+ and VEGF−/−) mice. Despite the absence of a functional VEGF gene, oncogene-transformed sublines derived from MDF528 cells (528ras1 and 528neu2 cells) readily form angiogenic tumors in severe combined immunodeficient (SCID) mice in a manner that suggests oncogene-dependent down-regulation of TSP-1 as a contributing factor (10). Two additional dermal fibroblastic cell lines, MDF6b and MDF6b-Id1−/−, were established in a similar fashion from mouse dermal explants isolated from VEGF-competent C57BL/6 and C57BL/6-Id1−/−Id1−/− mice, respectively. Permanent cell lines were established from these primary cultures by transfection with pSV3neo vector encoding the SV40 large T antigen followed by expression of V12-H-ras oncogene (in MDF6b cells).

The latter entailed a cotransfection of Ras3 and pcDNA3.1Zeo (Invitrogen, Burlington, Ontario, Canada) vectors and selection in 150 μg/mL Zeocin (Invitrogen, Burlington, Ontario, Canada) as described elsewhere (10). MDF528 cells were also engineered to stably express a TSP-1 reporter gene that consisted of enhanced green fluorescent protein cassette (EGFP-F1, Clontech, Mississauga, Ontario, Canada) placed under control of the mouse TSP-1 (MTSP-1) promoter (HindIII/KpnI fragment containing 2,800 bp of the MTSP-1 5′-untranslated region; ref. 21). A stable cell line (MDF-EGFP/TSP-1) was generated by cotransfection of repcDNA3.1Zeo (Invitrogen, Burlington, Ontario, Canada) with the vector containing the EGFP reporter (EGFP/TSP-1) followed by selection in 150 μg/mL Zeocin. Mutant K-ras-positive human colorectal cancer cell line DLD-1 and its variant with disrupted K-ras oncogene were a gift from Dr. Senji Shirasawa (22), whereas IEC-18 and IEC-18Ras3 cells were generously supplied by Dr. Jorge Filmus (Sunnybrook Research Institute, University of Toronto, Toronto, Ontario, Canada). The angiogenic properties of these cells and of NIH3T3Ras cells line were described previously (6, 12). Before assay, all cell lines [except for human umbilical vein endothelial cells (HUVEC)] were cultured in DMEM (Hyclone) supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin, and 50 μg/mL streptomycin (Life Technologies, Grand Island, NY). HUVECs were cultured in complete Endothelial BME-2 medium (Mandel, Guelph, Ontario, Canada) as recommended by the supplier (American Type Culture Collection, Rockville, MD).

Conditioned medium. Cells were incubated for 48 hours with either complete or serum-free growth medium as indicated. The resulting conditioned medium was then collected, centrifuged at 5,000 × g for 30 minutes, filtered through a 0.22-μm filter (Millipore, Nepean, Ontario, Canada), and used for treatments either immediately or after storage at −80°C. Conditioned medium was fractionated using Centriprep YM-3 filters (Millipore) with a 3-kDa molecular cutoff. This fractionated material was subjected to treatment with heat (85°C for 2 hours) or trypsin (5 mg/mL for 2 hours at 37°C). Target cells were incubated with various conditioned medium preparations or appropriate control medium for 48 hours before analysis unless otherwise indicated. Dimethylsphingosine (5 μmol/L) and pertussis toxin (0.005 μmol/L) were purchased from Biomol, Inc. (Plymouth Meeting, PA).

Analysis of the thrombospondin-1 expression gradient (field effect) in vitro. MDF528 fibroblasts expressing TSP-1/EGFP reporter gene (MDF-EGFP/TSP-1 cells) were plated onto the Lab-Tek chamber slides (Nalgene, Nepean, Ontario, Canada) and treated with 528ras1 conditioned medium or co-cultured (as indicated) with 528ras1 cells prelabeled with PHK26 red fluorescent dye (Sigma, San Diego, CA). To control diffusion of secreted factors, the cultures were embedded in SeaPlaque agarose (Cambrex, Nepean, Ontario, Canada) and treated with 528ras1 conditioned medium or its variant with disrupted K-ras oncogene (in MDFB6 cells). In this assay, conditioned medium was then collected, centrifuged at 5,000 × g for 30 minutes, filtered through a 0.22-μm filter (Millipore) with a 3-kDa molecular cutoff. Conditioned medium was then collected after either 24 hours before analysis unless otherwise indicated. Dimethylsphingosine (5 μmol/L) and pertussis toxin (0.005 μmol/L) were purchased from Biomol, Inc. (Plymouth Meeting, PA).

Analysis of the thrombospondin-1 expression gradient (field effect) in vivo. MDF528 fibroblasts expressing TSP-1/EGFP reporter gene (MDF-EGFP/TSP-1 cells) were implanted into the Lab-Tek chamber slides (Nalgene, Nepean, Ontario, Canada) and treated with 528ras1 conditioned medium or co-cultured (as indicated) with 528ras1 cells prelabeled with PHK26 red fluorescent dye (Sigma, San Diego, CA). To control diffusion of secreted factors, the cultures were embedded in SeaPlaque agarose (Cambrex, Guelph, Ontario, Canada) at concentrations varying between 0.5% and 2%. After 12, 36, or 72 hours, the layer of agarose was removed, and slides were washed with PBS and fixed in 4% paraformaldehyde. Images were taken using a Carl Zeiss LSM510 Confoacial Microscope System. Fluorescence intensity was analyzed using Scan Image software (Scion Corp.). In some experiments, MDF-EGFP/TSP-1 cells were harvested using 1% trypsin-EDTA (Life Technologies/Invitrogen) and analyzed in suspension using a FACS calibur (BD Bioscience, Mountain View, CA) flow cytometer equipped with WinMDI software.

High-performance liquid chromatography fractionation. The conditioned medium sample was transferred into vial suitable for injections into a Waters Wisp Model 712 autosampler (Waters, Mississauga, Ontario, Canada). An aliquot of 200 μL was injected onto a Beckman ultraharsh octadecylsilica column (5 μm particle size, inner diameter of 4.6 mm, and length of 25 mm). A guard column (1 mm inner diameter and length of 10 mm) was packed with the same phase. Compounds in the sample were separated by gradient elution chromatography at a flow rate of 2 mL/min. Initial composition of the mobile phase was 15% acetonitrile in water (both containing 0.2% trifluoroacetic acid) and the final composition was 85% acetonitrile, with the time of the linear gradient being 15 minutes. The pumps were Waters 600E system controlled by Millennium software to deliver the gradient. Samples were collected from the effluent in 3 minutes or 6-mL aliquots, solvent evaporated (nitrogen at 40°C). The samples were then lyophilized, dissolved in DMEM (10% FCS), and applied to bioassays.

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Thrombospondin-1 Down-regulation in Normal Fibroblasts
Thrombospondin-1 promoter activity assays. The pHTSP1 vector containing 2,800 bp of the MTSP-1 promoter upstream of firefly luciferase pGLO2 reporter has been described in detail elsewhere (21). Cells were transfected using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) and after 12 hours pooled into 24-well plates for treatment. Following a 24-hour incubation, cultures were washed, lysed in cell culture lysis reagent (CCLR) containing 1% Triton X-100, 10% glycerol, 2 mM/L 2-diaminocyclohexane-N,N,N’,N’-tetraacetic acid, 2 mM/L DTT, and 25 mM/L Tris phosphate (pH 7.8; Promega, Madison, WI) supplemented with a protease inhibitor mix (Roche, Palo Alto, CA; ref. 23), and stored at −80°C until used. MTSP-1 promoter activity was measured in cell lysates using Luciferase Assay Reagent (Promega) and Luminometer TD20/20 (Turner Design). Results were normalized to protein content as determined by Bradford assay (Bio-Rad, Mississauga, Ontario, Canada).

Western blotting. Following treatment, cells were washed in PBS and lysed in CCLR, and protein (80 μg/lane) was resolved by SDSPAGE (8% or 12% gel) followed by transfer to Immobilon-P membrane (Millipore). Membranes were probed with a mixture of primary antibodies (anti-TSP-1 Ab1 and Ab4, each 2 μg/ml; Neomarkers, Fremont, CA). The signal was visualized using horseradish peroxidase–conjugated anti-mouse secondary antibody (1:5,000, Bio-Rad) followed by incubation with the enhanced chemiluminescence reagent (Amersham Biosciences, Baie d’Urfe, Quebec, Canada). To confirm equal loading, membranes were probed with antibody to extracellular signal-regulated kinase (ERK) 1/2 antibody (1:1,000, Upstate, Charlottesville, VA) followed by anti-rabbit secondary antibody (1:5,000). Signal intensity was measured and quantified using ImageQuant software (Amersham Biosciences).

Northern blotting. Excised tumors or cell pellets were snap frozen in liquid nitrogen and total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) as described by the manufacturer. To quantify expression of the TSP-1 transcript, RNA (40 μg) was resolved in a 1% agarose gel, transferred to Hybond N+ membranes (Amersham Biosciences), and probed as described previously (12). Gels were stained with ethidium bromide and photographed and the intensity of 28S rRNA band was used as an indicator of equal loading. The 250-bp TSP-1 probe was prepared from human TSP-1 cDNA (American Type Culture Collection).

Analysis of tumor stroma. Fibrosarcoma cells (2 × 106 per mouse) were injected s.c. (orthotopically) in 0.1 ml PBS into SCID/yellow fluorescent protein (YFP) mice and the tumors were allowed to form (10). Then, 80 to 120 days after injection, the animals were killed, tumors were excised and halves were analyzed by fluorescent confocal microscopy at an excitation wavelength of 488 nm. The ubiquitously expressed YFP transgene served as a marker to selectively visualize host stromal cells within nonfluorescent fibroblastic and vascular origin (Fig. 1B, lanes 3 and 4). Instead, we reasoned that this inconsistency might be explained if ras-expressing cancer cells could down-regulate TSP-1 expression in their adjacent stromal surroundings.

Down-regulation of thrombospondin-1 promoter activity in nontumorigenic dermal fibroblasts exposed to tumor cell–derived soluble mediator(s). Oncogene-driven changes in TSP-1 expression are detectable both at the level of mRNA (Fig. 1B) and promoter activity (16). To monitor such changes in individual cells and their populations, we generated a reporter variant of the MDF528 cell line (MDF-EGFP/TSP-1), in which EGFP was expressed under control of the MTSP-1 promoter. As shown in Fig. 2, the levels of GFP fluorescence emitted by MDF-EGFP/TSP-1 cells faithfully reflect changes in TSP-1 promoter activity and gene expression under a variety of experimental conditions. For instance, a decrease in serum (FBS) concentration from 5% to 0% resulted in a reduction in mean GFP fluorescence of MDF-EGFP/TSP-1 cells [as measured by flow cytometry (fluorescence-activated cell sorting)] along with comparable diminution in TSP-1 promoter luciferase activity in their parental MDF528 cells subjected to the same treatment (Fig. 2B). We also tested the effects of culturing these cells at increasing densities, as this is known to change TSP-1 expression in some tumor cell types (24). Although changes in cell density were inconsequential in the particular context of MDF528 fibroblasts, the levels of TSP-1 mRNA (data not shown) and protein in both MDF528 and MDF-EGFP/TSP-1 cells were consistent with intensities of GFP fluorescence under the corresponding culture conditions (Fig. 2B). Collectively, these experiments suggest that
GFP fluorescence of MDFEGFP/TSP-1 cells accurately reflects changes in TSP-1 promoter activity. Interestingly, exposure of MDF-EGFP/TSP-1 fibroblasts to conditioned medium derived from H-ras-expressing 528ras1 cancer cells (528ras1 conditioned medium) induced a near complete inhibition of GFP fluorescence as indicated by both confocal microscopy and flow cytometry (Fig. 2C, left). Growth of dermal fibroblasts at increasing cell density does not change TSP-1 expression and promoter activity. MDF528 fibroblasts and their GFP-expressing MDF-EGFP/TSP-1 counterparts manifested unchanged levels of TSP-1 protein expression (Western blotting; middle and right) and GFP fluorescence (FACS; left), respectively. C, TSP-1 down-regulation in MDF-EGFP/TSP-1 cells treated with conditioned medium (CM) of H-ras-expressing 528ras1 fibrosarcoma cells (528ras1 CM). TSP-1 down-regulation was detected simultaneously at the level of MTSP-1 promoter activity [GFP fluorescence (left): confocal microscopy (top) and FACS (bottom)], mRNA (middle), and protein (right). 28S rRNA and ERK1/2 were used as loading controls for Northern and Western analyses, respectively.

Figure 2. TSP-1 promoter activity in fibroblasts expressing GFP reporter gene.

A, parallel regulation of GFP fluorescence (FACS) and luciferase activity in MDF-GFP/TSP-1 and MDF528 cells, respectively. The cells were incubated at different concentrations (0-5%) of FBS. MDF-EGFP/TSP-1 cells are a variant of MDF528 stably transfected with EGFP/TSP-1 reporter construct (left); MDF528 were transiently transfected with MTSP-1/pGL2/luciferase construct (right).

B, growth of dermal fibroblasts at increasing cell density does not change TSP-1 expression and promoter activity. MDF528 fibroblasts and their GFP-expressing MDF-EGFP/TSP-1 counterparts manifested unchanged levels of TSP-1 protein expression (Western blotting; middle and right) and GFP fluorescence (FACS; left), respectively.

C, TSP-1 down-regulation in MDF-EGFP/TSP-1 cells treated with conditioned medium (CM) of H-ras-expressing 528ras1 fibrosarcoma cells (528ras1 CM). TSP-1 down-regulation was detected simultaneously at the level of MTSP-1 promoter activity [GFP fluorescence (left): confocal microscopy (top) and FACS (bottom)], mRNA (middle), and protein (right). 28S rRNA and ERK1/2 were used as loading controls for Northern and Western analyses, respectively.
of between $10^5$ and $2 \times 10^6$ cells strongly affected GFP fluorescence of MDFEGFP/TSP-1 cells even at distances as large as 500 μm or greater (Fig. 3B). This distribution of the GFP signal across the monolayer of reporter fibroblasts is indicative of a concentration gradient formed by TSP-1-suppressing paracrine factors released from cancer cells into the common agarose matrix where they seem to propagate over considerable distances (Fig. 3C).

Production of soluble mediator(s) by a diverse panel of ras-expressing cancer cell lines. In addition to the aforementioned paracrine properties of 528ras1 cells, we observed that also conditioned medium of human colorectal cancer cells harboring a mutant K-ras oncogene (DKO-3 cells), efficiently suppressed the activity of the TSP-1 reporter gene in MDF-EGFP/TSP-1 cells (Fig. 4A). A similar effect was noticed in the soluble material secreted by IEC-18Ras3 and NIH3T3Ras cells, both expressing oncogenic H-ras and derived from rat intestinal epithelium and NIH3T3 fibroblasts, respectively (Fig. 4B; refs. 12, 22). However, 528neu2 cells, another transformed MDF528 variant harboring activated HER-2/neu oncogene rather than H-ras, did not efficiently suppress TSP-1 expression by nontransformed (MDF528) fibroblasts (Fig. 4C). Thus, as shown in Figs. 2C, 3A-C, 4A-C, and 5A, at least five different ras-expressing, VEGF-deficient or VEGF-proficient cancer cell lines (528ras1, B6ras, NIH3T3ras, IEC-18Ras3, and DLD-1) of either fibroblastic or epithelial origin were found capable of inducing paracrine suppression of TSP-1 levels in at least three different normal dermal fibroblastic cell lines (MDF528, MDF-EGFP/TSP-1, and MDFB6).

Requirement for Id1 gene expression during paracrine thrombospondin-1 down-regulation in nontransformed fibroblasts. Our panel of fibroblastic cell lines includes those obtained from dermal explants of C57BL/6 mice, from which immortalized MDFB6 cells and their H-ras-transformed B6ras counterparts were derived. Once again, we observed that expression of TSP-1 by the nontransformed MDFB6 fibroblasts was precipitously down-regulated when these cells were incubated with B6ras conditioned medium (Fig. 5A).

On the same genetic background (C57BL/6), we also generated another nontransformed fibroblastic cell line MDFB6-Id1−/−, which is deficient for the helix-loop-helix transcriptional inhibitor Id1. As mentioned earlier, embryonic fibroblasts isolated from the angiogenesis-impaired Id1−/− mice were reported previously to manifest increased constitutive expression of TSP-1 (16); hence, it was of interest to determine whether this effect could be abrogated by factor(s) produced by cancer cells expressing oncogenic ras. To address this question, we assayed TSP-1 protein expression by Id1-deficient dermal fibroblasts (MDFB6-Id1−/−) cultured in the presence or absence of conditioned medium from various ras- or neu-transformed fibrosarcoma cell lines (e.g., B6ras, 528ras1, and 528neu2). Unlike in their wild-type syngeneic counterparts (MDFB6

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**Figure 3.** Distance-dependent down-regulation of TSP-1 in nontransformed fibroblasts in agarose-embedded coculture with ras-expressing cancer cells. A, diminution of GFP fluorescence in dermal fibroblasts stably expressing the MTSP-1 reporter gene (MDFEGFP/TSP-1 cells) surrounding a single 528ras1 cancer cell (red fluorescence, arrow). The cells were cocultured in monolayer for 18 hours in 0.6% agarose, which was introduced to enable formation of soluble factor gradients. B, change in GFP fluorescence in MDFEGFP/TSP-1 fibroblasts as a function of distance from the cancer cell (528ras1) spheroid (confocal microscopy (top) and fluorescence intensity versus distance (bottom); red bar, 500 μm). Confluent monolayers of MDF-EGFP/TSP-1 cells and 10^7 of prelabeled (with PKH26 dye) and preaggregated 528ras1 cells were cocultured for 38 hours in 1% agarose matrix. The respective spheroids and monolayers were best visualized at different focal planes due to their respective distinct geometries (thickness). Therefore, separate images of the same coculture are presented. C, TSP-1 down-regulation during exposure of fibroblasts to the influence of large masses of 528ras1 cancer cells. Large 528ras1 spheroids (2 × 10^6 cells) were incubated with MDF-EGFP/TSP-1 reporter fibroblasts for 72 hours before high-magnification (>100) images were taken at various distances. Apparent decreases in GFP fluorescence of MDF-EGFP/TSP-1 cells occurred as a function of proximity to 528ras1 cell spheroids (top). Bottom, experimental design of the coculture system: 528ras1 cells were placed in 2.5% agarose and layered over a monolayer of MDF-EGFP/TSP-1 cells. Chamber slides were filled with medium containing 1.5% agarose and incubated for 72 hours before collection of images.
expression of TSP-1 in Id1-deficient nontransformed fibroblasts (MDFB6-Id1−/−) remained undiminished in the presence of the cancer cell–derived soluble material (Fig. 5B). This finding suggests that Id1 expression is required to maintain the responsiveness of normal cells to the paracrine influences that regulate TSP-1.

It is noteworthy that we did not observe any striking differences in constitutive TSP-1 expression between unstimulated MDFB6 and MDFB6-Id1−/− cells (data not shown). This is at variance with a prior report (16), and the reasons for this discrepancy are presently unknown. It is conceivable that differences between adult dermal fibroblasts used in our study and embryonal cells studied by others (16) are the underlying cause of these differential findings. Nonetheless, our results are consistent with the idea that loss of Id1 does not, by itself, affect TSP-1 levels in stroma but that instead Id1 is required for competence of the stroma to respond to factor(s) secreted by certain tumors.

Unresponsiveness of endothelial cells to thrombospondin-1-suppressing ras-dependent paracrine factors. Endothelial cell–associated TSP-1 may influence the viability and response of these cells to proangiogenic stimuli in an autocrine manner (16).
Therefore, we examined the effect of ras-dependent paracrine factor(s) on expression of TSP-1 in HUVECs. The latter cells were treated for 48 hours with conditioned medium from 528ras1, 528neu2, and B6ras cells and assayed for TSP-1 protein production by Western blotting. Although no discernable effect on endogenous TSP-1 levels in HUVEC was observed with these treatments, with the possible exception of weak down-regulation in the presence of 528neu2 conditioned medium (Fig. 5C), further studies with other types of endothelial cells, including those derived from the tumor vasculature, are warranted.

**Molecular characterization of ras-dependent mediator(s) involved in paracrine thrombospondin-1 down-regulation.** Paracrine effects of various ras-driven cancer cells were detected at the level of TSP-1 protein, mRNA, and promoter activity (Fig. 2C).

We have taken advantage of the rapidity and sensitivity of the latter assay (21) to further characterize the properties of the soluble factor(s) involved. First, high molecular weight species were removed from serum-free conditioned medium of 528ras1 cells (528ras1 conditioned medium) by ultrafiltration, and the activity of the resulting fractions was tested against MDF528 fibroblasts (Fig. 6A). This analysis clearly indicated that TSP-1-suppressing activity was mainly contained in the small molecular weight (<3 kDa) fraction, which induced an effect comparable in magnitude with that of unfractonated 528ras conditioned medium. Combining both high and low molecular weight fractions (<3 + >3 kDa) at the ratio corresponding to their original volumes recapitulated the activity of 528ras1 conditioned medium, suggesting that no major losses, changes in composition, or activity occurred during preparation of this material (Fig. 6A). Moreover, TSP-1-suppressing activity remained stable during long-term cold storage (at −80°C and −20°C) or incubation at 37°C for up to 2 hours, resisted trypsin digestion, but was highly sensitive to heating at 85°C (Table 1).

Although such molecular characteristics exclude the role of cytokines, chemokines, and large polypeptide mediators in the paracrine effect exerted by 528ras1 cells, they do not permit positive identification of the relevant (single or multiple/interacting) low molecular species. Therefore, standard liquid chromatographic resolution of 528ras1 conditioned medium was done and revealed biological activity within two different fractions of this material (Fig. 6B, fractions 2 and 5). This finding suggests that 528ras1 cells release at least two (or more) low molecular weight TSP-1-suppressing factors. Although establishing a complete molecular inventory of these multiple factors presently represents a considerable challenge and is a focus of our long-term studies, examining their mode of action on target fibroblasts could be informative as a way of exploring possible targets for pharmacologic intervention to inhibit the angiogenic properties of tumor stroma.

**Involvement of G-protein and lipid signaling pathways in paracrine thrombospondin-1 regulation by ras-dependent factors.** TSP-1 down-regulation by ras-dependent low molecular weight mediators could be either "nonspecific" or involve signaling by defined molecular pathways. With regard to the latter possibility, several entities in the molecular weight range of <3 kDa (lipids, nucleotides, amino acids, oligosaccharides, or oligopeptides) exert their biological effects through activation of G-protein-coupled receptors (GPCR) and in a manner often dependent on G-proteins, which are sensitive to pertussis toxin (25). Therefore, we tested the activity of the MTSP-1 promoter in MDF528 fibroblasts exposed to 528ras1 conditioned medium in the presence or absence of pertussis toxin. As shown in Fig. 6C, even very low concentrations of pertussis toxin (0.005 μg/mL) completely blocked TSP-1 suppression by 528ras1-derived factor(s). This result strongly

![Figure 6](https://example.com/figure6.png)
suggests the involvement of GPCR(s) in TSP-1 down-regulation. We also tested some of the putative effectors that might mediate biological effects downstream of GPCRs and found that pharmacologic inhibition of neither the mitogen-activated protein kinase (MAPK)/ERK kinase/MAPK pathway (by PD98059) nor the PI3K/Akt pathway (by LY294002) was able to specifically affect the response of MDF528 cells to 528ras1 conditioned medium (data not shown). In contrast, abrogation of TSP-1 suppression was observed (Fig. 6C) when MDF528 cells were incubated with dimethylsphingosine, a specific inhibitor of sphingosine kinase. The latter family of enzymes is involved in intracellular generation of SIP, an important intracellular and intercellular second messenger (25). Interestingly, both sphingosine kinase and SIP have recently been identified as essential downstream effectors of ras-dependent cellular transformation, although the activity of SIP in this context is confined to the cellular interior (26). In addition, SIP plays a well-established role in regulation of cellular interactions and angiogenesis, a function linked with its extracellular release (e.g., from platelets) and interaction with a series of distinct membrane GPCRs (S1P1-S1P5; ref. 25). The growing appreciation for the importance of this pathway in several cellular processes, including angiogenesis, is reflected by ongoing studies involving novel pharmacologic SIP receptor inhibitors, such as FTY720 (27). It is therefore interesting that inhibition by dimethylsphingosine of the ability of fibroblasts to generate SIP abrogated their proangiogenic responses to cancer cell–derived factors. It is noteworthy that addition of dimethylsphingosine or pertussis toxin alone (Fig. 6C and D) to MDF528 fibroblasts had a negligible effect on constitutive MTSP-1 promoter activity; hence, these inhibitors seem to block the activity of mediators present in the 528ras1 conditioned medium specifically.

As the material derived from 528ras1 conditioned medium contains at least two active fractions (Fig. 6B, fractions 2 and 5), we examined whether they act in a similar (i.e., GPCR- and SIP-dependent) or different manner. Interestingly, the latter seems to be the case. Thus, suppression of TSP-1 promoter activity by fraction 2 of 528ras1 conditioned medium was moderately sensitive to pertussis toxin but was completely abolished by dimethylsphingosine. In contrast, fraction 5 was relatively insensitive to dimethylsphingosine (i.e., did not require SIP synthesis but remained highly sensitive to pertussis toxin treatment; Fig. 6D). Collectively, our results suggest that at least two different low weight molecular species are involved in the paracrine down-regulation of TSP-1 in fibroblasts exposed to material secreted by tumor cells harboring mutant ras. Furthermore, this TSP-1 down-regulation is achieved in a manner that involves pathways acting through Id1, GPCR, and SIP.

### Table 1. Characterization of the ras-dependent paracrine mediator of TSP-1 down-regulation

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<th>Medium</th>
<th>Treatment</th>
<th>Mean MTSP-1 promoter activity (MDF528 cells), %</th>
<th>P (t test)</th>
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</thead>
<tbody>
<tr>
<td>Control medium (serum free)</td>
<td>None</td>
<td>100 ± 12*</td>
<td>—</td>
</tr>
<tr>
<td>528ras1 conditioned medium fraction &lt;3 kDa</td>
<td>None</td>
<td>30 ± 13 †</td>
<td>&lt;0.000001*</td>
</tr>
<tr>
<td>528ras1 conditioned medium fraction &lt;3 kDa</td>
<td>37°C for 2 h</td>
<td>42 ± 20 †</td>
<td>&lt;0.0005 †</td>
</tr>
<tr>
<td>528ras1 conditioned medium fraction &lt;3 kDa</td>
<td>85°C for 2 h</td>
<td>104 ± 9</td>
<td>&lt;0.0005 †</td>
</tr>
<tr>
<td>528ras1 conditioned medium fraction &lt;3 kDa</td>
<td>Trypsin for 2 h</td>
<td>14 ± 14</td>
<td>&lt;0.000001*</td>
</tr>
</tbody>
</table>

*Compared with control medium.
†Highly significant.
‡Compared with 528ras1 conditioned medium fraction <3 kDa.

Figure 7. Oncogene-dependent angiogenic field effect. This model proposes that as individual cancer cells acquire transforming genetic alterations (e.g., activating mutations of ras genes), such mutations induce expression of proangiogenic properties, including down-regulation of TSP-1. The resulting “intrinsic” angiogenic phenotype of cancer cells would remain inconsequential if confined to their small subset located amid a plethora of other (ras-nonexpressing) TSP-1-expressing tumor and stromal cells. This study shows that, in addition to intrinsic changes in TSP-1 expression, ras-transformed cells also release low molecular weight paracrine factors that induce TSP-1 down-regulation in a wider population of adjacent stromal cells. Therefore, we postulate that even a relatively small number of tumor cells can affect the local levels of TSP-1 and cause propagation of a proangiogenic “field,” an effect that may contribute to and accelerate the angiogenic switch, particularly in micrometastases, and small incipient tumor nodules.
Conclusions. We propose that the effect of ras on tumor angiogenesis may not, as hitherto believed, be restricted to intrinsic changes in expression of angiogenesis inhibitors and stimulators (e.g., by down-regulation of TSP-1 or up-regulation of VEGF in cancer cells themselves; ref. 4). Instead, cells harboring mutant ras may also release soluble factor(s) capable of widespread TSP-1 down-regulation (Fig. 7) among their adjacent normal counterparts as exemplified by dermal fibroblasts in our study. This mechanism could effectively translate genetic changes within single cancer cells into a much larger, amplified effect involving the heterogeneous multicellular micromilieu of tumor parenchyma and stroma. Such local mobilization and cooperation of multiple, rather than single, tumor and host cells (a proangiogenic field effect; refs. 4, 10) could greatly accelerate or enable the angiogenic switch in incipient cancers or their metastases (Fig. 8; ref. 2).

Our study presents experimental evidence that ras-dependent paracrine factors, at least within the dermal fibroblast lineage, act through GPCR, sphingosine kinase/SIP, and Id1, a set of effectors that is considerably different from the intrinsic cascade (PI3K/Rho/ROCK/Myc) described by Watnick et al. as a mechanism of TSP-1 down-regulation in ras-transformed cancer cells (13). In other words, these results suggest the possibility that TSP-1 may be regulated differently in transformed cells themselves versus their related or adjacent nontransformed counterparts. One implication of this finding is that restoration of TSP-1 expression in these respective tumor compartments would require two-pronged pharmacologic approaches. Furthermore, a similar dichotomy (parenchyma versus stroma) may apply more broadly to targeted and antiangiogenic agents.

We also observed that ras-expressing but not neu-expressing sister cell lines produce TSP-1-suppressing paracrine activities. The reasons for this apparent oncogene specificity and the influence of cellular background on the release, nature, and composition of TSP-1-down-regulating factors remain unclear at the moment. It should be mentioned in this context that the ability of ras to trigger such a paracrine effects is not unique. For instance, cells expressing activated c-jun were recently shown to produce a soluble factor capable of inducing TSP-1 down-regulation in either a paracrine or an autocrine manner (28, 29). In addition, Watnick et al. have also recently identified a paracrine, TSP-1-suppressing, factor secreted by transformed kidney epithelial cells.5

Again, the nature of these various mediators and regulatory mechanisms involved remain to be established. Nevertheless, our present study reinforces the need to consider more carefully another level of tumor angiogenesis regulation (i.e., the one related to the onset and amplification of proangiogenic properties in nontransformed cells exposed to paracrine influences of their genetically altered malignant counterparts).

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References

Oncogenes and Angiogenesis: Down-regulation of Thrombospondin-1 in Normal Fibroblasts Exposed to Factors from Cancer Cells Harboring Mutant Ras

Wojciech Kalas, Joanne L. Yu, Chloe Milsom, et al.


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