

Tumor Escape from Endogenous, Extracellular Matrix – Associated Angiogenesis Inhibitors by Up-Regulation of Multiple Proangiogenic Factors

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Abstract Purpose: Thrombospondin-1 (Tsp1), endostatin, and tumstatin are extracellular matrix – associated proteins that inhibit angiogenesis. We examined the mechanisms by which tumor cells may bypass the antiangiogenic effects of these endogenous regulators.

Experimental Design: CT26 colon and RenCa renal carcinoma cells were stably transfected with *Tsp1*, *endostatin*, or *tumstatin* cDNA. Subcutaneous and metastatic tumor growth in syngeneic mice was analyzed. Expression of proangiogenic factors in resulting tumors was measured by quantitative real-time PCR. The combination of Tsp1 and vascular endothelial growth factor (VEGF) receptor-2 inhibition was also examined.

Results: There was significant suppression of angiogenesis in flank tumors and liver metastases formed from cells overexpressing Tsp1, endostatin, or tumstatin. However, all tumors ultimately escaped angiogenesis inhibition. The combination of all three angiogenesis inhibitors had no additive effect beyond overexpression of a single inhibitor. Using quantitative real-time PCR, we found that VEGF and platelet-derived growth factor (PDGF)-A levels were routinely up-regulated at least 5-fold in all CT26 tumors overexpressing any antiangiogenic protein, and there were variable increases in angiopoietin 2 (Ang2), basic fibroblast growth factor, and PDGF-B. In contrast, RenCa tumors, which have high baseline levels of VEGF and PDGF-B, relied on basic fibroblast growth factor, Ang1, and PDGF-A up-regulation to counteract Tsp1 overexpression. Growth of CT26 cells with Tsp1 overexpression was suppressed when anti-VEGFR-2 treatment was added.

Conclusions: Cancer cells with overexpression of three different endogenous angiogenesis inhibitor eventually escape angiogenesis inhibition by up-regulation of various proangiogenic factors. Tsp1, endostatin, and tumstatin may be functionally redundant in this system. These endogenous angiogenesis inhibitors are likely best used in combination with the blockade of proangiogenic pathways or with traditional chemotherapy or radiation therapy.

Primary colorectal and renal cell carcinomas and their metastases require angiogenesis, or new blood vessel formation, to grow beyond a few cubic millimeters (1, 2). It is well understood that tumor angiogenesis is regulated by a balance

between proangiogenic and antiangiogenic factors (3). Cancer cells interact with their host environment and must create a positive angiogenic balance to promote neovascularization and tumor growth (4). Tumors induce neovascularization in part by up-regulating a variety of proangiogenic factors, including vascular endothelial growth factor (VEGF), which is overexpressed by the vast majority of cancers, including colorectal and renal cell carcinomas (5, 6).

More than two dozen endogenous antiangiogenesis inhibitors have been identified and oppose the effects of VEGF and other proangiogenic factors (7). Many of these inhibitors are found in the extracellular matrix and some are derived from the breakdown of extracellular matrix proteins (8). Three of the best-studied endogenous, antiangiogenic proteins are thrombospondin 1 (Tsp1), endostatin, and tumstatin. Tsp1, the first endogenous angiogenesis inhibitor to be described, is a multifunctional extracellular matrix protein and one of a five-member family of extracellular calcium-binding proteins (9). Tsp1 exerts its effects by interacting with proteoglycans, other matrix proteins, growth factors such as transforming growth factor β , and membrane receptors such as CD36. Tsp1 blocks angiogenesis by inhibiting endothelial cell proliferation and

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migration and by increasing apoptosis (10). Synthetic peptides that include Tsp1 sequences (e.g., ABT-510) are in early clinical trials (11). Endostatin is a 20 kDa COOH-terminal fragment of collagen type XVIII (12), whereas tumstatin is a 28-kDa fragment of the α_3 chain of type IV collagen (13). Both peptides have been shown to have antiangiogenic properties both *in vitro* and *in vivo* (7). Whereas endostatin binds $\alpha_5\beta_1$ integrin on endothelial cells and inhibits mitogen-activated protein kinase signaling, tumstatin binds $\alpha_v\beta_3$ integrin, leading to activation of the mammalian target of rapamycin pathway and inhibition of Cap-dependent protein synthesis. Although Tsp1, endostatin, and tumstatin treatment have been shown to inhibit tumor growth in mouse models, translation of these results to patients with metastatic colorectal cancer and other cancers has been difficult (14).

In this study, we sought to determine how cancer cells overcome the actions of endogenous angiogenesis inhibitors and induce tumor neovascularization. We found that even with the high levels of expression following stable transfection of *Tsp1*, *endostatin*, or *tumstatin* cDNA, CT26 colorectal carcinoma cells and RenCa renal carcinoma cells eventually escape growth suppression and undergo logarithmic tumor growth. This growth occurs despite persistence of Tsp1, endostatin, or tumstatin overexpression. In response to overexpression of these three different endogenous inhibitors, CT26 cells up-regulate expression of a variety of proangiogenic factors, including VEGF and platelet-derived growth factor A (PDGF-A). In contrast, RenCa cells, which already have high levels of VEGF at baseline, up-regulate basic fibroblast growth factor (bFGF, FGF-2), angiopoietin 1 (Ang1), and PDGF-A. The combination of VEGF signaling blockade (using the monoclonal antibody DC101 targeting VEGFR-2) and overexpression of Tsp1 prevented CT26 tumors from attaining logarithmic growth for more than 5 weeks.

Materials and Methods

Plasmids. The plasmid pSecTag2.Endo was created by inserting mouse *endostatin* cDNA into pSecTag2/HygroB (Invitrogen) as previously described (15). Human *Tsp1* cDNA was purchased from Addgene and PCR amplified. The resulting PCR product was digested and ligated into pSecTag2/HygroB to create plasmid pSecTag2.Tsp1. The *Tsp1* cDNA introduced into the final plasmid was sequenced to confirm that no mutations were introduced. The expression plasmid α pcBFT with human *tumstatin* was generously provided by Dr. Raghu Kalluri (Beth Israel Deaconess Medical Center, Boston, MA). The *tumstatin* cDNA was ligated into pSecTag2/HygroB to create plasmid pSecTag2.Tum.

Cell lines and tissue culture. CT26 mouse colon carcinoma, B16F10 mouse melanoma, Lewis lung carcinoma, and RenCa renal carcinoma cell lines were obtained from the American Type Culture Collection. MC26 mouse colon carcinoma cells were obtained from the National Cancer Institute Tumor Repository. T241 cells were kindly provided by Dr. Judah Folkman (Children's Hospital, Boston, MA). These cell lines were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μ g/mL of streptomycin. Hygromycin (Invitrogen), 250 or 500 μ g/mL, was added to this medium for stably transfected CT26 and RenCa cell lines, respectively. DC101 hybridoma cells were obtained from American Type Culture Collection and maintained in BD Cell Mab serum-free medium (BD Biosciences). Human umbilical vein endothelial cells (HUVEC) were obtained from Cambrex and maintained in EGM-2-MV medium (Cambrex).

Generation of stable cell lines expressing Tsp1, endostatin, and tumstatin. Stably transfected cell lines were generated by transfecting CT26 or RenCa cells with pSecTag2.Tsp1, pSecTag2.Endo, pSecTag2.Tum, or the parent plasmid pSecTag2/HygroB using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. Hygromycin-resistant colonies were expanded and tested for secretion of the Tsp1-, endostatin-, or tumstatin-myc-His fusion protein by Western blot analysis as previously described (15).

Cancer cell proliferation assays. To assay for cancer cell proliferation, 10^4 cells were plated onto 96-well flat-bottomed plates and maintained in medium overnight. A colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess cell number by absorbance after 1, 3, and 5 days as previously described (15). Data reflect the mean of six samples.

Endothelial cell assays. One million stably transfected cells were plated on 150-mm plates and incubated for 72 h. Cells were then washed with PBS, and 5 mL of DMEM were added per plate. Cells were incubated for an additional 24 h, and conditioned medium was collected and concentrated to 1 mL using Microcon YM-10 or YM-100 centrifugal filter devices (Millipore). HUVECs were plated at 1.25×10^4 cells per well in 24-well gelatin-coated plates in Opti-MEM with 1% fetal bovine serum and allowed to attach overnight. The next day, 250 μ L of concentrated, conditioned medium were then added to each well followed by addition of 250 μ L of Medium 199 supplemented with 10% fetal bovine serum, bFGF (5 ng/mL), and VEGF (10 ng/mL). Cells were incubated for 72 h, and the number of cells was quantified using a colorimetric MTT assay. Data reflect the mean of six samples.

HUVEC migration assays were done in a modified Boyden Chamber using a 48-well chemotaxis chamber (Neuroprobe, Inc.). Polycarbonate track-etch membranes (8- μ m pore size; Neuroprobe, Inc.) were coated with 65 μ g/mL Vitrogen 100 (3.1 mg/mL purified collagen; Cohesion) in 0.02 N acetic acid overnight and air dried. The membrane was placed over the bottom chamber containing Opti-MEM with 1% fetal bovine serum and VEGF (10 ng/mL). HUVECs were incubated in Opti-MEM with 1% fetal bovine serum overnight, resuspended at 8×10^4 cells/mL in Opti-MEM with 1% fetal bovine serum, and mixed 1:1 with concentrated, conditioned medium from stably transfected clones. The cells were incubated for 20 min at 37°C and added to the upper chamber. Aliquots (50 μ L) of cells were then added to each well in the upper chamber and allowed to migrate for 8 h at 37°C. Cells on the upper surface of the membrane that had not migrated were removed by scraping with a cotton swab. The filters were stained with Diff-Quick stain set (Dade Behring, Inc.). Migrated cells were counted under the microscope (magnification $\times 100$). Data reflect the mean number of cells counted in eight wells.

Animal studies. All mouse protocols were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care. To generate s.c. flank tumor, 10^6 cells were resuspended in 100 μ L HBSS and injected s.c. into the right flank of BALB/c mice following isoflurane anesthesia. For cell mixing experiments, 10^6 cells with the specified percentages of each clone were mixed in 100 μ L HBSS and injected s.c. Six 6- to 8-week-old male BALB/c mice were used for each group. Tumors were measured thrice per week for a maximum of 3 months, and tumor volume (TV) was calculated by using the following formula: $TV = \text{length} \times (\text{width})^2 \times 0.52$. After mice were sacrificed, tumors were excised and cut into thirds. One third of each tumor was fixed in 10% buffered formalin for 24 h, embedded in paraffin, and processed into 5- μ m sections. One third of each tumor was snap frozen in liquid nitrogen and processed into 5- μ m sections. The final third of each tumor was preserved in RNAlater (Qiagen). The delay in tumor growth was calculated by determining the day in which each tumor surpassed 200 mm³. The mean for each group with antiangiogenic protein expression was then determined and compared with the mean for the negative control group.

DC101 antibody was produced from hybridoma cells using the BD CELLline 1000 system (BD Biosciences) following the manufacturer's

instructions. ChromPure rat IgG (Jackson ImmunoResearch Laboratories) was used as control. To examine the effects of DC101 on flank tumors, Tsp1-6 or NC-5 cells were used to generate flank tumors as described above. When tumors reached at least 100 mm³, mice were randomized to receive either DC101 40 mg/kg i.p. or control IgG 40 mg/kg i.p. thrice per week.

To generate experimental liver metastases, mice were anesthetized using ketamine/xylazine anesthesia, and 1.5×10^6 cells resuspended HBSS in 100 μ L were injected intrasplenically. Five 6- to 8-week-old male BALB/c mice were used for each group. Mice were sacrificed after 2 weeks, and livers were harvested, fixed in formalin for 24 h, and photographed. Subsequently, the livers were embedded in paraffin and processed into 5- μ m sections. For the survival study, mice with experimental liver metastases were monitored daily beginning 10 days after injection and sacrificed when moribund. Six mice were used for each group.

Immunohistochemistry and Western blot analysis. CD31 immunohistochemistry was done on 5- μ m frozen sections as previously described (16). At low magnification ($\times 50$), regions of highest vessel density were identified. Then, under high power ($\times 200$), microvessel density was determined by a blinded observer by counting blood vessels in five fields.

For VEGF immunohistochemistry, paraffin sections were rehydrated in xylene followed by decreasing concentrations of ethanol. Slides were then placed in 10 mmol/L Tris-EDTA buffer (pH 9.0) and heated for 20 min at 95°C. Sections were then blocked with diluted rabbit serum, incubated overnight at 4°C with goat anti-VEGF primary antibody (1:10; R&D Systems) or isotype control antibody, followed by rabbit anti-goat biotinylated secondary antibody (1:500; Vector Laboratories). The remaining steps were performed using the Vectastain Elite ABC kit for goat IgG (Vector Laboratories), according to the manufacturer's instructions.

For myc immunohistochemistry, paraffin sections were rehydrated in xylene followed by decreasing concentrations of ethanol. Slides were then placed in 20 μ g/mL Proteinase K (Epicentre Biotechnologies) diluted in buffer [10 mmol/L Tris base, 1 mmol/L EDTA solution, 0.05% Tween 20 (pH 9.0)] and incubated for 45 min at 37°C. Sections were then blocked with diluted rabbit serum, and incubated overnight at 4°C with JAC6 rat anti-myc primary antibody (1:40; Abcam) or isotype control antibody followed by rabbit anti-rat biotinylated secondary antibody (1:500; Vector Laboratories). The remaining steps were performed using the Vectastain Elite ABC kit for rat IgG (Vector Laboratories), according to the manufacturer's instructions.

For analysis of PDGF-A and lamin protein levels, tumors were homogenized in radioimmunoprecipitation assay buffer (Boston Bio-Products) supplemented with Protease Inhibitor Cocktail (Roche Diagnostics), mixed with SDS buffer, separated on a 15% polyacrylamide gel, and transferred to a nitrocellulose membrane. After blocking in 5% nonfat milk, the membrane was incubated with anti-PDGF-A antibody (1:500, Santa Cruz Biotechnology) and anti-lamin antibody (1:10,000, Cell Signaling). Horseradish peroxidase-conjugated goat anti-mouse IgG (1:1,000, Zymed Laboratories) was used as secondary antibody.

Quantitative real-time PCR. Total RNA was isolated from tumor tissue preserved in RNA Later using RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. RNA was isolated from cell lines *in vitro* using Trizol (Invitrogen) following the manufacturer's instructions. cDNA was synthesized using the Superscript First-Strand Synthesis System (Invitrogen) with random hexamers. Quantitative real-time PCR analysis was done using the LightCycler Detection System (Roche Diagnostics) using 500 ng of cDNA product and LightCycler FastStart DNA Master^{PLUS} SYBR Green I, per manufacturer's instructions. Primers for 18S RNA were obtained from QuantumRNA Classic 18S Internal Standard (Ambion), respectively. Primers for mouse VEGF, Ang1, Ang2, bFGF, PDGFA, and PDGFB were the following: VEGF (forward) 5'-ATCTTCAAGCCGTCCTGTGT-3', (reverse) 5'-GCATTCACATCTGCTGTGCT-3'; Ang1 (forward) 5'-

GGAACCGAGCCTACTCACAG-3', (reverse) 5'-TTAGATTGGAAGGGC-CACAG-3'; Ang2 (forward) 5'-CCGCTACGTGCTTAAGATCC-3', (reverse) 5'-ATTGTCCGAATCCTTTGTGC-3'; bFGF (forward) 5'-AGCGGCTCTACTGCAAGAAC-3', (reverse) 5'-GCCGTCCATCTTCCTT-CATA-3'; PDGF-A (forward) 5'-CAAGACCAGGACGGTCATTT-3', (reverse) 5'-ACTTTGGCCACCTTGACACT-3'; PDGF-B (for) 5'-GATCTCTCGGAACCTCATCG-3', (rev) 5'-GGCTTCTTTCCGCA-CAATCTC-3'. Of note, the VEGF primers were designed to measure all isoforms of VEGF.

The concentrations of 18S RNA and angiogenic factors were calculated from the crossing point using a standard curve. The relative value of each angiogenic factor was then determined by the dividing the calculated angiogenic factor level by the calculated 18S level for each sample and then normalizing the data so the lowest relative value was 1.

Results

Generation and characterization of cell lines overexpressing endogenous angiogenesis inhibitors. To compare the efficacy of three different endogenous angiogenesis inhibitors, cell lines of CT26 murine colon carcinoma cells stably overexpressing Tsp1, endostatin, or tumstatin as well as negative control cell lines were generated. Expression of the transgene was confirmed by examining conditioned medium from stable clones for the fused Myc tag by Western blot analysis (Fig. 1A). Two stable clones each were selected overexpressing Tsp1 (Tsp1-6, Tsp1-7), endostatin (Endo-6, Endo-8), and tumstatin (Tum-1, Tum-4) as well as one negative control clone (NC-5). To ensure these clones grew at equal rates *in vitro*, proliferation assays were performed for all clones comparing their growth rates to wild-type CT26 cells. Proliferation after 1, 3, and 5 days for all selected clones was equal to CT26 wild-type cells (Fig. 1B). The biological activity of the secreted antiangiogenic proteins was tested *in vitro* by adding concentrated, conditioned medium to HUVECs. HUVEC proliferation was inhibited 30% to 34%, 6% to 10%, and 38% to 43% with conditioned medium from Tsp1-, endostatin-, and tumstatin-secreting cells, respectively, compared with conditioned medium from control cells (Fig. 1C). The minor effect of endostatin on endothelial cell proliferation was not statistically significant, and prior studies have reported that endostatin has little or no effect on endothelial cell proliferation *in vitro* but rather inhibits migration (17). The biological activity of secreted endostatin in conditioned medium was assessed in a modified Boyden chamber assay, and endothelial cell migration was inhibited by 65% to 71% when compared with conditioned medium from NC-5 control cells (Fig. 1D and E). In this assay, secreted Tsp1 and tumstatin had minimal effect on HUVEC migration.

Effect of overexpression of endogenous angiogenesis inhibitors on tumor growth. The *in vivo* growth of cell lines secreting various antiangiogenic factors was next determined in syngeneic BALB/c mice using a s.c. flank tumor model. After 15 days, growth of CT26 clones overexpressing Tsp1, endostatin, or tumstatin was significantly decreased compared with growth of NC-5 control tumors (Fig. 2A). Tumors were harvested after 15 days and microvessel density was quantified by CD31 immunohistochemistry. Microvessel densities were reduced by 45% to 56% in tumors from Tsp1-, endostatin-, or tumstatin-secreting cells compared with tumors from NC-5 cells (Fig. 2B and C). At the time of tumor harvest at 15 days, Tsp1-6, Endo-6, and Tum-1 tumors were an average of 101, 54, and 199 mm³ in size, respectively, whereas NC-5 tumors averaged 917 mm³

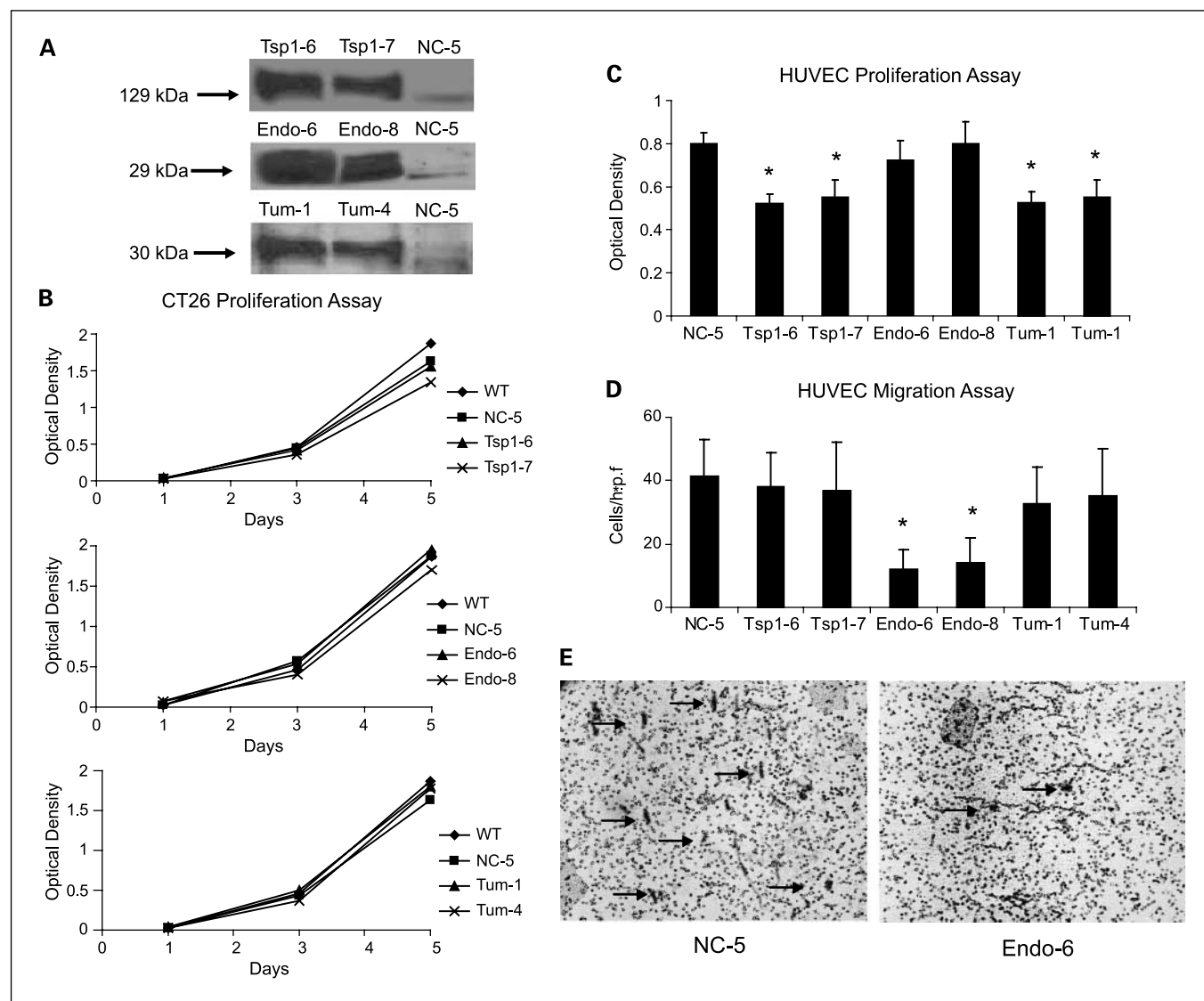


Fig. 1. Analysis of CT26 colon carcinoma cell lines with stable overexpression of Tsp1, endostatin, or tumstatin. *A*, Western blot analysis for Myc tag of conditioned medium from CT26 cell lines stably overexpressing Tsp1 (Tsp1-6, Tsp1-7), endostatin (Endo-6, Endo-8), or tumstatin (Tum-1, Tum-2) as well as CT26-negative control cell line (NC-5). *B*, *in vitro* proliferation assay at 1, 3, and 5 d using MTT colorimetric assay. CT26 wild-type cells (WT) are included. Inhibition of HUVEC proliferation (*C*) and migration (*D*) with conditioned medium from CT26 stably expressing Tsp1, endostatin, or tumstatin. *E*, representative photos of HUVEC migration. Columns, mean; bars, SD. *, $P < 0.05$ compared with NC-5. Arrows, examples of migrated cells (purple).

in size. CT26 cells with Tsp1, endostatin, or tumstatin overexpression formed significantly smaller tumors after 15 days compared with control cells. In a subsequent experiment, the clones with the slowest growth after 15 days were selected from each group (Tsp1-6, Endo-6, and Tum-1), and flank tumors were grown beyond 15 days. Tumors from Tsp1, endostatin, or tumstatin-secreting cells ultimately escaped growth inhibition and began logarithmic growth after ~25 days (Fig. 2D). There was an ~10-day delay in tumor mass expansion to 200 mm³ in size for all colon cancer cell lines regardless of which specific antiangiogenic protein was secreted.

It is unclear if the inhibitory effect of endogenous antiangiogenic protein overexpression is attenuated if only a minority of cell secrete the antiangiogenic protein. We previously reported that flank tumor growth inhibition for

renal carcinoma cells persisted when only 10% of cells overexpressed endostatin (15). When Tsp1-, endostatin-, or tumstatin-secreting cells were mixed at a ratio of 1:2 with NC-5 cells, the delay in flank tumor growth was equivalent to tumors derived when all the cells overexpressed Tsp1, endostatin, or tumstatin (Fig. 2E). To determine if the escape from tumor growth inhibition could be prevented by the overexpression of a combination of antiangiogenic factors, we injected combinations of Tsp1-7, Endo-6, or Tum-1 cells into the flanks of mice. Combination therapy did not significantly increase the delay in tumor growth over that of single inhibitor overexpression (Fig. 2F). Indeed, even overexpression of all three endogenous angiogenesis inhibitors did not result in a greater delay in tumor growth than any of the inhibitors alone. Microvessel density was determined in tumors that had escaped growth inhibition and reached ~1,000 mm³ in size. Microvessel

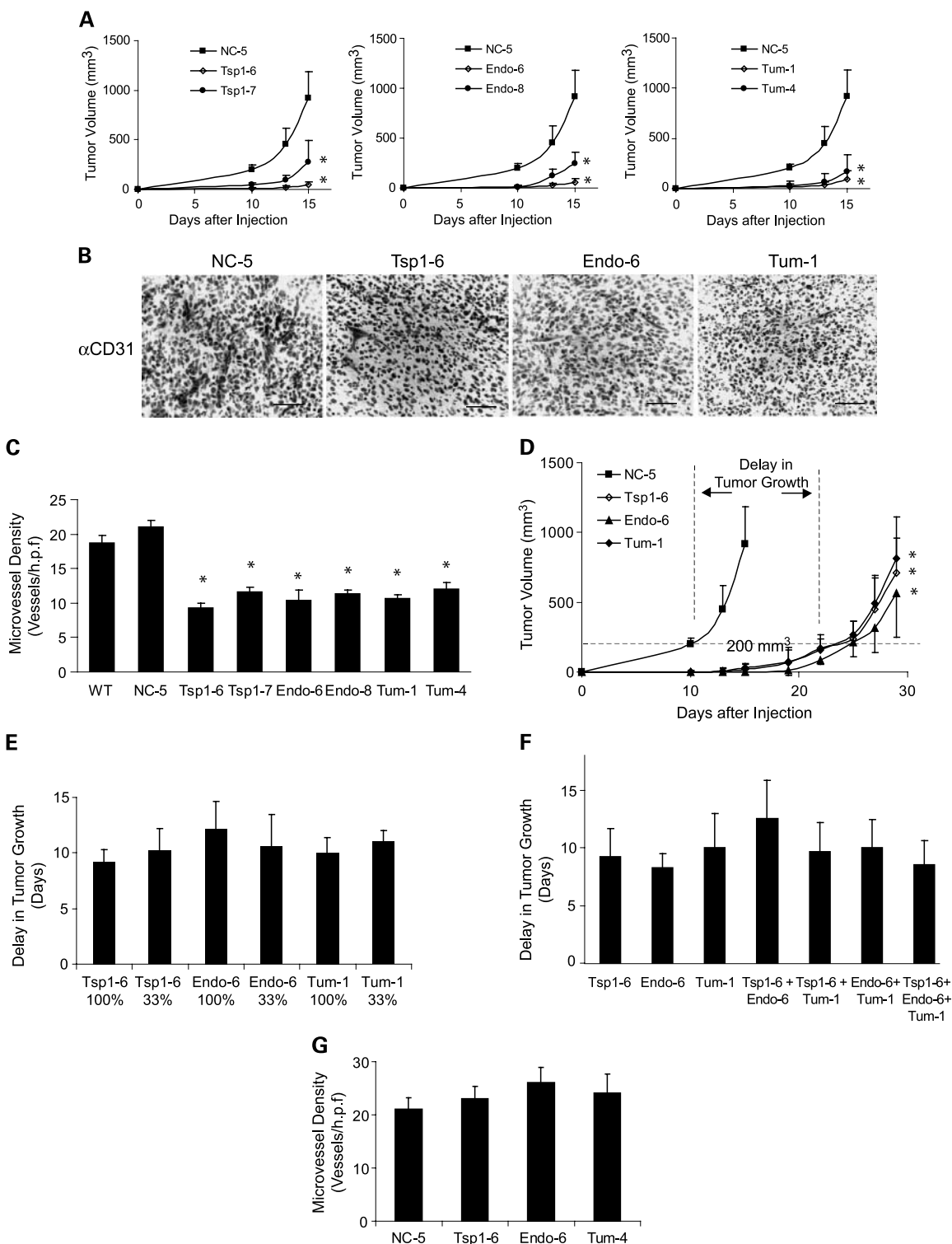


Fig. 2. Subcutaneous flank tumor growth. *A*, flank tumor growth of CT26 cells overexpressing Tsp1 (Tsp1-6, Tsp1-7), endostatin (Endo-6, Endo-8), or tumstatin (Tum-1, Tum-4) and negative control cells (NC-5) over 15 d. Six mice were used per group. *B*, CD31 immunohistochemical staining of tumors harvested after 15 d. Scale bar, 40 μ m. *C*, microvessel density of CD31-positive blood vessels per high-power field (*h.p.f.*) in tumor harvested after 15 d. CT26 wild-type tumors are included. *D*, flank tumor growth of cells stably overexpressing Tsp1 (Tsp1-6), endostatin (Endo-6), or tumstatin (Tum-1) and negative control cells (NC-5) over 30 d. Delay in tumor growth represents number of days that tumors took to grow beyond 200 mm^3 compared with control tumors. Six mice were used per group. *E*, delay in tumor growth beyond 200 mm^3 for tumors composed of 100% cells overexpressing Tsp1, endostatin, or tumstatin, or tumors with 33% cells overexpressing Tsp1, endostatin, or tumstatin (and 67% NC-5 cells). *F*, delay in tumor growth beyond 1,000 mm^3 for tumors with overexpression of one, two, or three endogenous angiogenesis inhibitors. *G*, microvessel density per high-power field in tumors harvested after reaching 1,000 mm^3 in size. Columns, mean; bars, SD. *, $P < 0.05$ compared with NC-5.

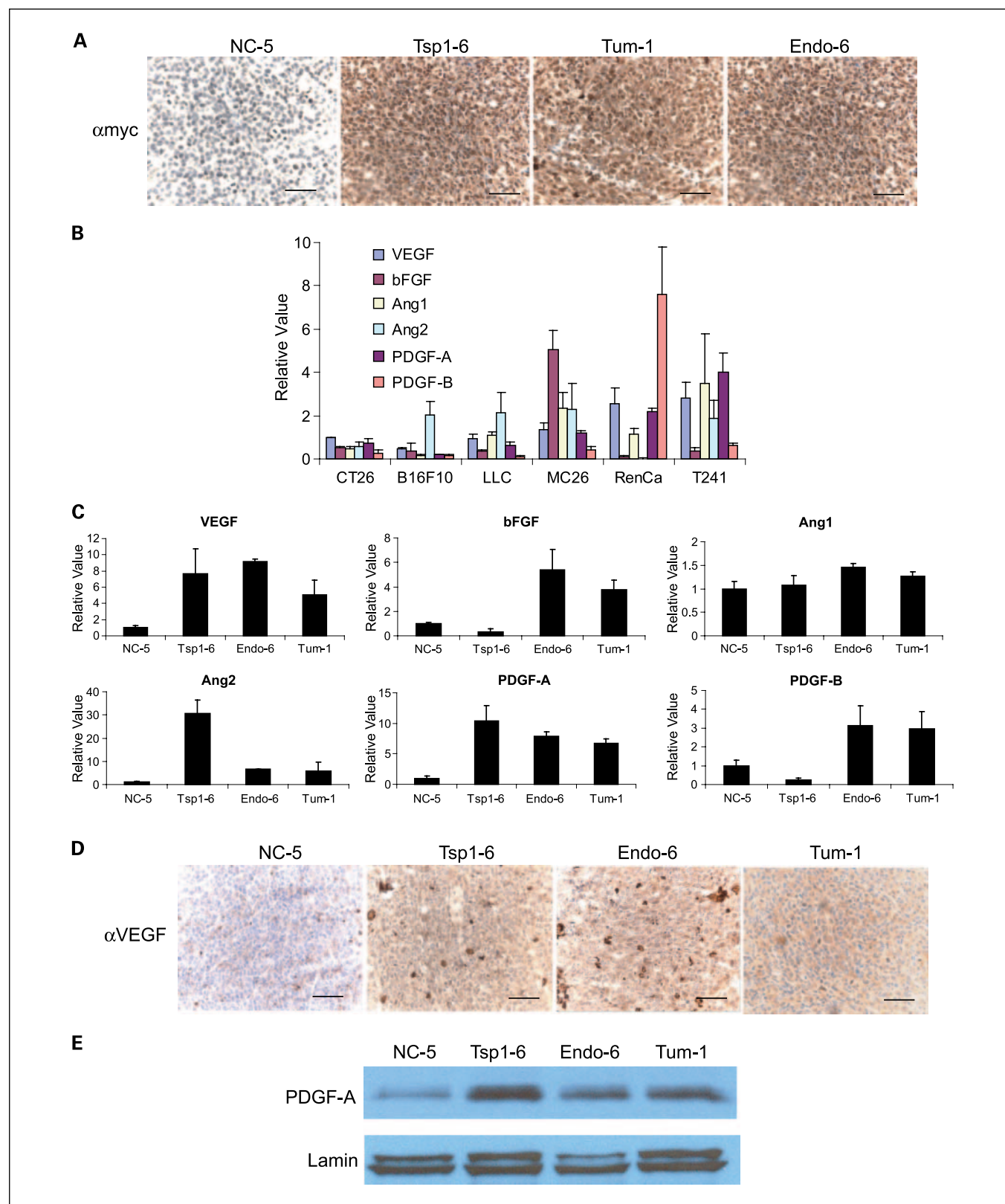
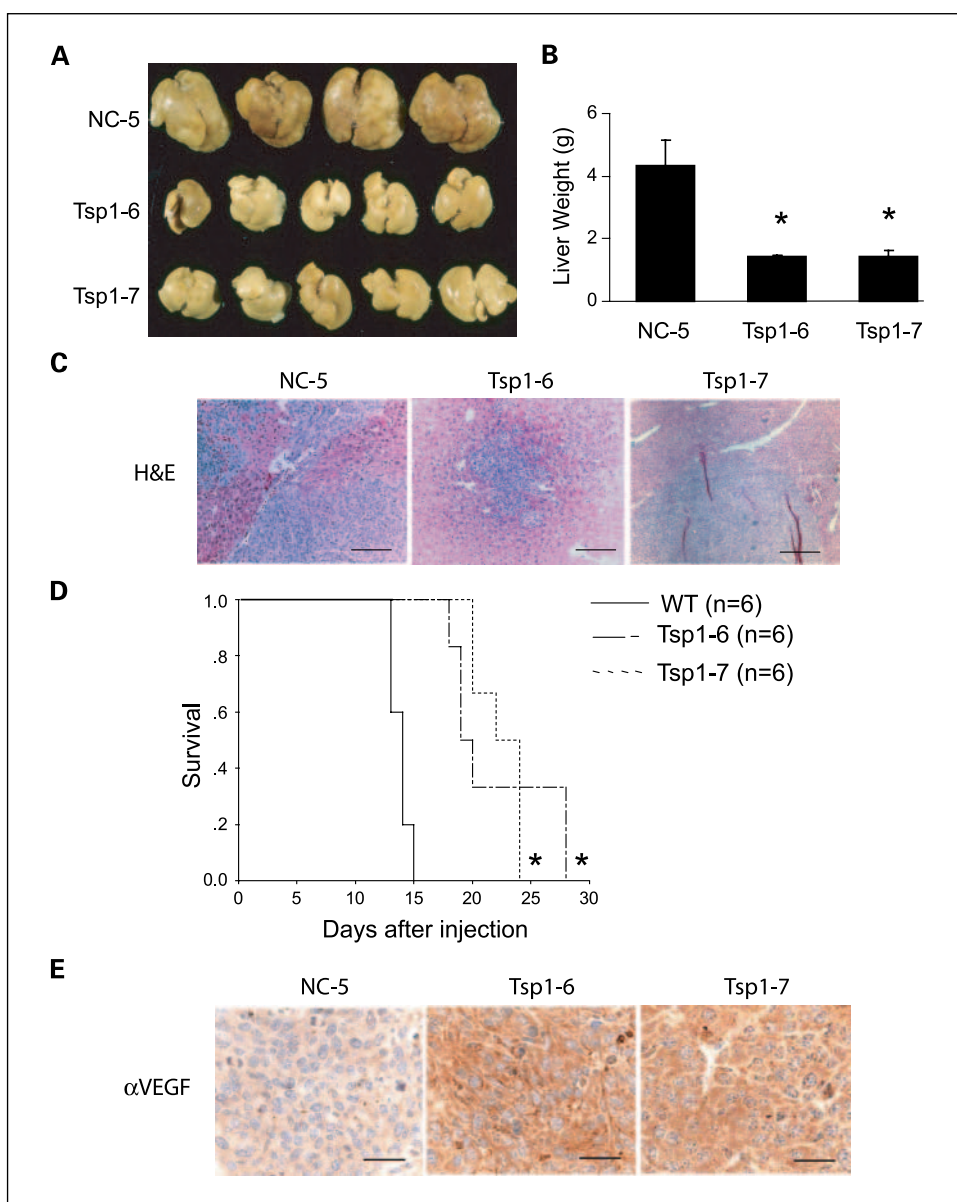


Fig. 3. Up-regulation of VEGF and other proangiogenic factors. *A*, immunohistochemical staining for Myc of tumors with overexpression of Tsp1 (Tsp1-6), endostatin (Endo-6), or tumstatin (Tum-1) compared with negative control tumors (NC-5) to examine transgene expression in tumors harvested after reaching 1,000 mm³ in size. *B*, relative expression levels by qRT-PCR of proangiogenic factors in CT26 cells and five other cell lines grown *in vitro*. *C*, relative expression levels by qRT-PCR of proangiogenic factors in flank tumors with overexpression of Tsp1, endostatin, and tumstatin. *D*, immunohistochemical staining for VEGF expression in flank tumors. *E*, PDGF-A and lamin expression by Western blot analysis. Scale bar, 100 μ m. Bars, SD.

Fig. 4. Tsp1 overexpression and experimental liver metastases. **A**, livers harvested from mice 14 d after intraportal injection of NC-5, Tsp1-6, and Tsp1-7 cells. Five mice were used per group. **B**, graph showing mean liver weights at 14 d. **C**, low-power view of H&E sections of livers at 14 d, demonstrating large metastases in NC-5 liver and micrometastases in Tsp1-6 and Tsp1-7 livers. Scale bar, 100 μ m. **D**, Kaplan-Meier survival curve of mice after intraportal injection of tumor cells. Six mice were used per group. **E**, high-power view of immunohistochemical staining for VEGF in macroscopic (NC-5) and microscopic (Tsp1-6, Tsp1-7) liver metastases. Scale bar, 30 μ m. Columns, mean; bars, SD. *, $P < 0.05$ compared with NC-5.



density in large tumors overexpressing Tsp1, endostatin, or tumstatin was equal to NC-5 control tumors of similar size (Fig. 2G). These data suggest that tumors escaped inhibition by these endogenous angiogenesis inhibitors, resulting in up-regulation of blood vessel density and subsequent expansion of tumor mass.

VEGF expression in tumors overexpressing endogenous angiogenesis inhibitors. We next explored possible mechanisms by which tumors could escape growth inhibition by endogenous angiogenesis inhibitors. To ensure that the transgene was still expressed in large tumors, tumor sections were stained by immunohistochemistry for the fused Myc tag, and transgene expression was found in nearly all tumor cells (Fig. 3A).

To analyze the relative baseline levels of secreted angiogenic factors from CT26 cells compared with other cell lines, RNA from CT26 cell and five other cell lines was harvested and analyzed by quantitative real-time PCR (qRT-PCR) for

expression of six proangiogenic factors (Fig. 3B). CT26 cells secrete relatively low levels of all six factors compared with other cell lines. To determine if up-regulation of proangiogenic factors triggered the growth of tumor cells overexpressing Tsp1, endostatin, or tumstatin, qRT-PCR on reverse transcribed RNA from flank tumors between 100 and 200 mm³ in size was performed for a panel of six proangiogenic factors. VEGF and PDGF-A were up-regulated in all CT26 tumors with Tsp1, endostatin, or tumstatin overexpression. VEGF up-regulation ranged from 5- to 9-fold whereas PDGF-A up-regulation was between 6- and 10-fold compared with control tumors (Fig. 3C). Up-regulation of other angiogenic factors were variable. For example, bFGF and PDGF-B was up-regulated in endostatin and tumstatin-expressing tumors but not in Tsp1-expressing tumors. Ang2 was only up-regulated in Tsp1-expressing tumors, and Ang1 was not up-regulated in any tumor. Immunohistochemistry for VEGF (Fig. 3D) and Western blot analysis for PDGF-A

(Fig. 3E) confirmed that both these factors were more highly expressed in Tsp1-7, Endo-6, and Tum-1 flank tumors compared with NC-5 tumors.

Overexpression of Tsp1 in a liver metastasis model. Antian-angiogenic therapies are most commonly used for metastatic disease, with the liver being the most common metastatic site for colorectal cancer (18). To determine if the delay in flank tumor growth we previously described was similar in a more physiologically relevant organ environment, we compared the growth of Tsp1-overexpressing colon carcinoma cells and control cells in an experimental liver metastasis model. Two weeks after injection of cells into the portal venous circulation, macroscopic liver metastases were evident in mice injected with NC-5 cells but not in mice injected with Tsp1-6 or Tsp1-7 cells (Fig. 4A). Gross liver weights were markedly decreased for Tsp1-6 and Tsp1-7 livers compared with NC-5 livers (Fig. 4B). However, histologic examination of livers from mice injected with Tsp1-6 or Tsp1-7 cells revealed microscopic metastases (Fig. 4C). To determine if these micrometastases escaped Tsp1-mediated growth inhibition, a survival experiment was performed. Mice injected with NC-5 cells all died by day 15, whereas all mice injected with Tsp1-6 or Tsp1-7 cells died by day 24 and 28, respectively (Fig. 4D). Thus, similar to s.c. flank tumors, liver metastases also escape growth inhibition. VEGF was highly expressed in Tsp1-6 or Tsp1-7 micrometastases at 2 weeks status after intraportal injection compared with macroscopic NC-5 tumors (Fig. 4E), confirming that VEGF is up-regulated in the liver environment in response to Tsp1 overexpression.

Combination of Tsp1 overexpression and anti-VEGFR-2 therapy. Although up-regulation of VEGF was observed in tumors as they escaped inhibition from endogenous angiogenesis inhibitors, this up-regulation could be an epiphenomenon and not the true mechanism of escape. To rule out this possibility, we treated mice bearing flank tumors as they escaped Tsp1 inhibition with DC101, an antibody to VEGFR-2 (19). Tumors from Tsp1-overexpressing cells or NC-5 control cells were randomized once they reached 100 mm³ to either DC101 (40 mg/kg) or control IgG (40 mg/kg) i.p. thrice per week. Blockade of VEGF signaling with DC101 was able to suppress tumor growth similar to Tsp1 overexpression alone (Fig. 5A). However, the combination of Tsp1 overexpression and DC101 treatment resulted in a synergistic effect. Indeed even 5 weeks after tumor cell inoculation, tumor volumes on mice bearing Tsp1-overexpressing tumors and treated with DC101 were on average only ~200 mm³ in size. In comparison, mice with either DC101 treatment or Tsp1 tumor cell overexpression alone had tumor volumes that were >1,000 mm³ and had to be euthanized by day 35. Microvessel density was significantly decreased in Tsp1-producing tumors treated with DC101 compared with Tsp1-producing tumors treated with control IgG (Fig. 5B). Tsp1-overexpressing tumors treated with DC101 or IgG were analyzed for expression of angiogenic factors by qRT-PCR (Fig. 5C). Tsp1-expressing tumors were previously found to up-regulate VEGF, Ang2, and PDGF-A compared with control tumors (Fig. 3C). With the addition of DC101 treatment to Tsp1 overexpression, VEGF was further up-regulated and Ang1 expression was up-regulated (Fig. 5C). These data suggest that VEGF up-regulation is indeed one mechanism by which tumors thwart angiogenesis inhibition by Tsp1 and other antiangiogenic proteins. Further,

these data imply that treatment with both Tsp1 recombinant protein together with VEGF inhibition may delay or prevent tumor escape.

Escape from Tsp1 inhibition in renal carcinoma cells. To ensure that the up-regulation in proangiogenic factors in response to overexpression of endogenous angiogenesis inhibitors was not specific to CT26 cells, we created stable cell lines of RenCa renal carcinoma cells with Tsp1 overexpression. As shown in Fig. 3B, CT26 cells have relatively low basal levels of six different proangiogenic proteins whereas RenCa cells at baseline have relatively high levels of some of these same proangiogenic proteins (Fig. 3B). In our RenCa clones, we confirmed Tsp1 secretion (Fig. 6A), equal growth rates of our clones *in vitro* (Fig. 6B), and biological activity of secreted Tsp1 on HUVEC proliferation (Fig. 6C). When these cell lines were grown as flank tumors, Tsp1-overexpressing cells grew significantly more slowly than the control cell line but ultimately escaped Tsp1 inhibition (Fig. 6D). When analyzed by qRT-PCR, Tsp1-overexpressing RenCa tumors harvested around 200 mm³ in size had significantly higher levels of bFGF, Ang1, and PDGF-A compared with similarly sized control tumors (Fig. 6E). Minor elevations in VEGF, Ang2, and PDGF-B did not reach statistical significance.

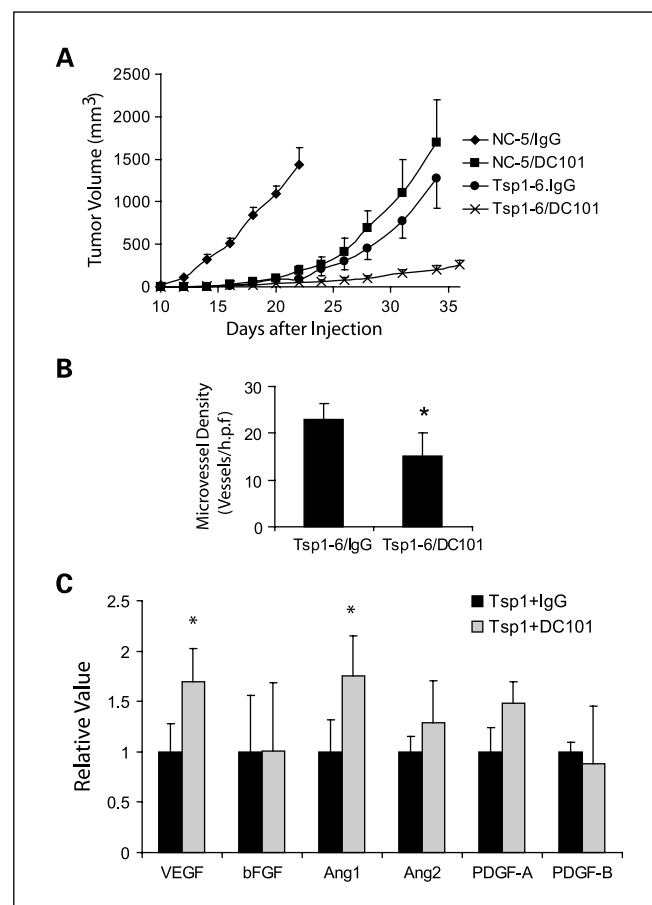


Fig. 5. Tsp1 overexpression and DC101 treatment. **A**, flank tumor growth of CT26 cells overexpressing Tsp1 (Tsp1-6) and negative control cells (NC-5) treated with DC101 40 mg/kg (Tsp1-6/DC101) or control IgG 40 mg/kg (Tsp1-6/IgG) thrice per week. Six mice were used per group. **B**, microvessel density of CD31-positive blood vessels per high-power field in tumor harvested after 35 d. **C**, quantitative RT-PCR examination of flank tumors for expression of VEGF, Ang1, Ang2, bFGF, PDGF-A, and PDGF-B. Bars, SD. *, $P < 0.05$ compared with Tsp1-6/IgG.

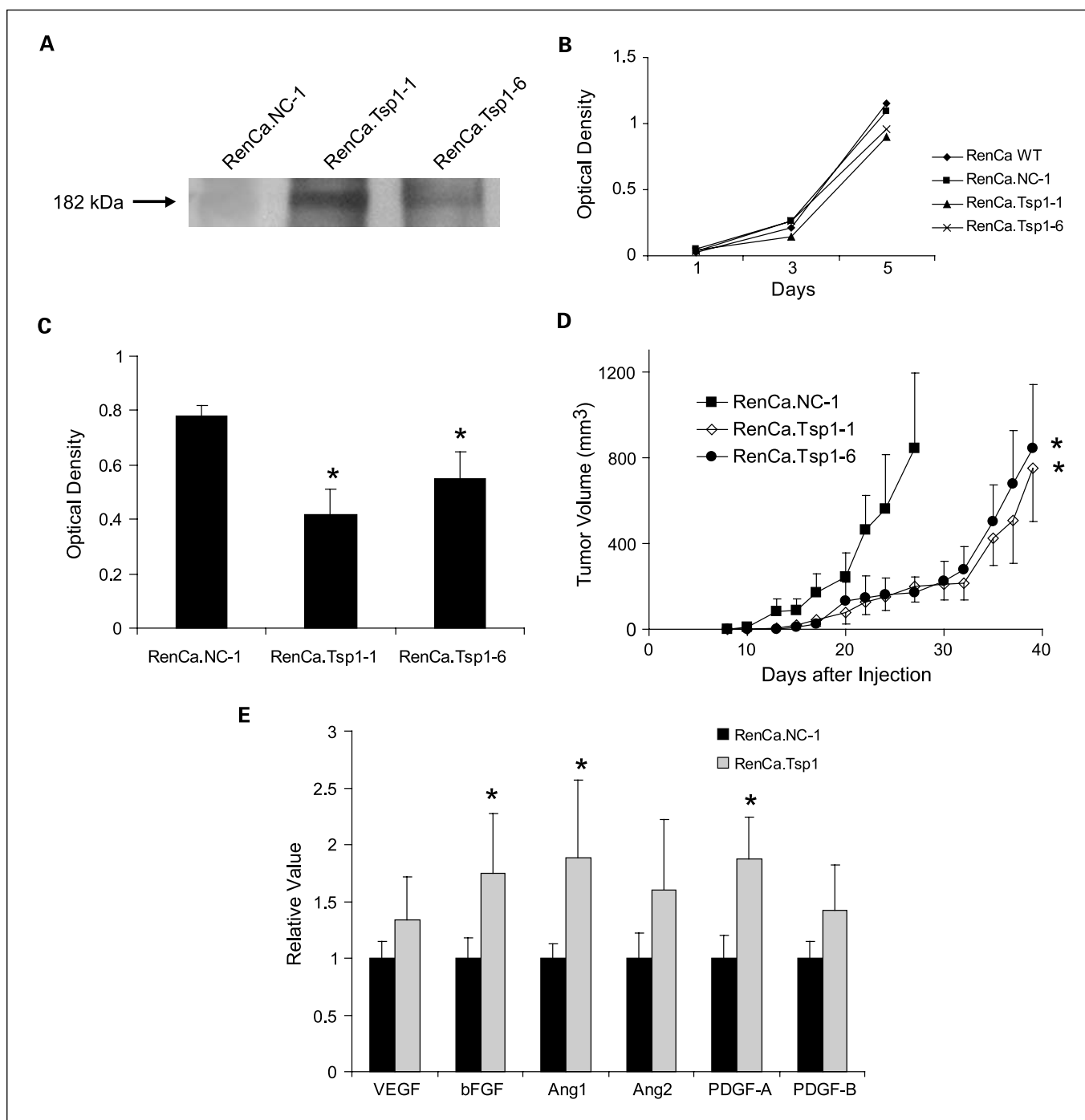


Fig. 6. Tsp1 overexpression in RenCa renal carcinoma cells. *A*, Western blot for Tsp1-Myc fusion protein of concentrated conditioned medium from RenCa cells stably transfected with Tsp1 (RenCa.Tsp1-1, RenCa.Tsp1-6) or control vector (RenCa.NC-1). *B*, *In vitro* proliferation over 5 d and HUVEC proliferation assay after 72 h in conditioned medium (*C*) as measured by colorimetric MTT assay. *D*, flank tumor growth of selected clones. *E*, relative value of six proangiogenic factors in RenCa flank tumors as measured by qRT-PCR. Bars, SD. *, $P < 0.05$ compared with RenCa.NC-1.

Discussion

This study explores mechanisms by which colorectal and renal carcinoma cells induce neovascularization and tumor growth in the presence of high local concentrations of endogenous angiogenesis inhibitors. We stably overexpressed Tsp1, endostatin, or tumstatin in CT26 colon carcinoma cells and derived clonal cell lines. Although tumors derived from cells

overexpressing these angiogenesis inhibitors initially grew slowly, they ultimately escaped angiogenesis inhibition and entered into logarithmic growth. The combination of all three antiangiogenic proteins (Tsp1, endostatin, and tumstatin) did not prevent tumor growth and, in fact, was no more effective than overexpression of any single angiogenesis inhibitor. Examination of flank tumors arising from cells overexpressing these angiogenesis inhibitors led to the novel finding that

up-regulation of certain proangiogenic factors is a common mechanism by which tumors escape angiogenesis inhibition by Tsp1, endostatin, or tumstatin.

Lack of efficacy of antiangiogenic proteins through systemic delivery has been attributed to problems with protein production, subtherapeutic levels following systemic administration, or inability of protein to reach the target tumor (20). Delivery of antiangiogenic genes introduces issues regarding efficiency of transduction and level of gene expression (21). Tumors may down-regulate the production of antiangiogenic factors such as Tsp1 as has been described for colorectal cancers (22). To avoid these issues, we created stable cell lines with antiangiogenic genes driven by the strong cytomegalovirus promoter. Tumor escape in the presence of overexpression of endogenous angiogenesis inhibitors did not occur due to silencing of the transgene. Expression of the stably transfected transgenes was maintained even as tumors reached large sizes. Therefore, whereas overexpression of endogenous angiogenesis inhibitors may initially suppress tumor growth, high levels of negative angiogenesis regulators are not sufficient to maintain long-term suppression of angiogenesis.

There are a variety of mechanisms by which tumors may escape angiogenesis inhibition. Casanovas et al. (23) showed that blocking VEGFR-2 in a transgenic model of spontaneous pancreatic islet tumors led to an increase in hypoxia and up-regulation of not only VEGF but also bFGF, Ang1, and other angiogenic factors. We found that the specific proangiogenic factors up-regulated in response to overexpression of endogenous angiogenesis inhibitors is variable among different tumor types. CT26 colon carcinoma cells at baseline express relatively low levels of VEGF, PDGF-A, and other proangiogenic factors. VEGF and PDGF-A up-regulation was an early event in tumor growth in response to any of the three different antiangiogenic factors and was seen in small flank tumors as well as liver micrometastases. The significance of increased VEGF expression in CT26 cells was shown by our studies treating mice bearing Tsp1-overexpressing tumors with anti-VEGFR-2 antibody to block the major receptor for VEGF signaling in endothelial cells. This combination produced a synergistic effect on the inhibition of tumor growth, implicating the up-regulation of VEGF as one essential mechanism by which tumor cells overexpressing Tsp1 escape angiogenesis suppression. In contrast, RenCa renal carcinoma cells at baseline express relatively high levels of proangiogenic factors. In response to Tsp1 overexpression, these cells do not increase VEGF expression but rather increase expression of bFGF, Ang1, and PDGF-A.

Colorectal cancers usually do not metastasize to s.c. soft tissues and so the mouse s.c. flank tumor model represents an uncommon host organ environment. There are significant differences in tumor angiogenesis depending on the host site in which tumor cells are inoculated (24). The liver is the most common site of colorectal cancer metastasis (25), and so a liver metastasis model was examined in this study. We found that

Tsp1 overexpression was effective in delaying growth of liver macrometastases. However, as seen in the flank tumor model, liver micrometastases from Tsp1-overexpressing cells ultimately overcame Tsp1 inhibition and grew to lethal sizes. Similar to the flank tumors, VEGF levels were higher in Tsp1-overexpressing metastases compared with control metastases. Thus, the findings in the flank tumor model also apply in more relevant host organ environments.

Blockade of VEGF signaling by DC101 in tumors from Tsp1-secreting cells prevented escape from Tsp1 inhibition. Other studies have found that antiangiogenic agents combined with VEGF inhibitors resulted in an additive or synergistic effect. Systemic administration of anti-VEGF short interfering RNA into tumor-bearing mice along with overexpression of Tsp1 delayed the emergence of Tsp1-resistant tumors (26). Combination of endostatin and SU5416 (a VEGFR-2 inhibitor) reduced tumor growth in mice bearing prostate, lung, and glioma tumors better than either agent alone (27). Tsp1 and endostatin are in clinical trials whereas tumstatin is in preclinical development for the treatment of various cancers. Our data predict that the combination of endogenous angiogenesis inhibitors with bevacizumab (28) or other anti-VEGF agents may better prevent or delay tumor growth in these cancer patients compared with use single antiangiogenic agent therapy.

When antiangiogenic therapies were initially conceived, the development of resistance was felt to be unlikely given that the therapies targeted genetically stable endothelial cells. However, recent clinical studies have found that tumors of patients given anti-VEGF therapies eventually grow despite continuous administration of angiogenesis inhibitors (29, 30). Several mechanisms by which tumors become resistant to antiangiogenic strategies have been elucidated. First, the genetic stability of tumor endothelial cells has been questioned (31), but the extent to which genetic alterations in endothelial cells contribute to resistance is unknown. Second, evidence indicates that there is a redundancy of angiogenic factors produced by the tumor cells. As reviewed recently (32), advanced breast cancer cells secrete nearly half a dozen angiogenic factors. Third, tumors can switch to alternative pathways to promote angiogenesis when one pathway is inhibited. This study suggests that up-regulation of specific proangiogenic factors is a common mechanism for colorectal and renal carcinoma cells to evade inhibition by endogenous angiogenesis inhibitors. Current research is relatively sparse in elucidating the mechanisms of escape or evasion from angiogenesis inhibition, but such research is vital in moving forward with new antiangiogenic strategies.

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