Supporting Information

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SI Text

Supporting Materials and Methods

Cell Lines and *in Vitro* Conditions. The Lewis lung carcinoma (LLC), SCC-PSA1 teratocarcinoma (SP), human renal clear cell carcinoma (786-O) and bovine pulmonary arterial endothelial cell lines (C-PAE) were obtained from American Type Culture Collection and grown *in vitro*. The cell lines were maintained in DMEM (Life Technologies, Inc.) supplemented with 10% FCS (FCS), 100 units/ml of penicillin, and 100 mg/ml of streptomycin.

***In Vivo* Tumor Trials.** Five- to 6-week-old C57/BL6 and SV129 mice and 8-week-old athymic Nu/Nu mice were purchased from Jackson and Charles River Laboratories respectively. The nude mice were kept in autoclaved cages and given autoclaved water and laboratory rodent chow.

The various treatments commenced when the tumors had reached 4–6 mm in diameter, and the animals were stratified into groups according to tumor size. The tumors were measured every 3–4 days using Vernier calipers, and the volume was calculated using the standard formula (width^2 $\times$ length $\times$ 3,14/6). Tumor cell and i.v. drug injections were undertaken during isoflurane gas anesthesia. I.v. injections was given through the retrobulbar venous plexus.

For the SP tumor study, tumstatin peptide was resuspended in sterile PBS containing 10% dimethyl sulfoxide (DMSO), and the mice were injected i.v. with 30 μg/day from day 10 until sacrifice. DMSO was used because tumstatin peptide is poorly soluble in PBS due to a high percentage of hydrophobic amino acids (1). The control group received vehicle alone. From day 25, the tumstatin peptide-treated animals were given either preimmune rabbit serum or a tumstatin peptide-antibody, at a dose of 1,340 μg/mouse/day, together with the tumstatin peptide. The rabbit antibody against tumstatin peptide was prepared as previously described (2, 3). Calculated from the molecular weight of whole IgG and tumstatin peptide, the molar ratio of the preimmune serum or tumstatin peptide-antibody to tumstatin peptide was 1.3:1. Assuming that the effective fraction of IgG in the purified IgG is 10%, the ratio was 13:1.

For the LLC tumor study tumstatin peptide and tumstatin peptide mutant were resuspended in sterile PBS containing 10% DMSO, and the mice were injected i.v. twice a day at 5 mg/kg for 18 days. The control group received vehicle alone.

For the 786-O tumor response study the treatment schedules were: sham treatment: PBS 0.1 ml i.v. Mon-Fri and 0.1 ml i.p. Monday and Thursday, FITC-tumstatin peptide: 35 μg i.v. Monday–Friday, and/or bevacizumab (Avastin, Genentech): 100 μg i.p. Monday and Thursday. Treatment was given for a total of 5 weeks.

All animal studies were approved by the Institutional Animal Care and Use Committee of the Beth Israel Deaconess Medical Center and are in accordance with the guidelines of the Department of Health and Human Services.

Cell Proliferation Assay. A suspension of C-PAE cells (4,000 cells/well) in DMEM w/ 0.5% FCS was plated onto 96-well plates that had been precoated with fibronectin (10 μg/ml). Cells were left to attach overnight, and the following day the medium was removed and replaced with DMEM containing either 0.1% FCS (unstimulated negative control) or 20% FCS, 5 ng/ml vascular endothelial growth factor (VEGF, R&D Systems) and 10 ng/ml of basic fibroblast growth factor (bFGF, R&D). Tumstatin, tumstatin mutant protein, tumstatin peptide or tumstatin peptide mutants were added to the stimulated cells, and the cells were incubated for 48 h at 37°C. Thereafter the cell number was counted as described previously, using methylene blue staining to ensure that only living cells were included (4).

Cell Attachment Assays. The assays were performed as described, with some modifications (5, 6); 96-well plates were coated with tumstatin peptide (50 μg/ml) or bovine serum albumine (BSA, 100 mg/ml) for 2 h. C-PAE cells (1.5×10^5 cells/ml in DMEM) were preincubated with 1, 5, 10, or 50 μg/ml of polyclonal anti-tumstatin peptide or anti-tumstatin antibody or control rabbit IgG (preimmune serum) for 15 min at room temperature. The rabbit antibodies against tumstatin and tumstatin peptide were prepared as previously described (2, 3). The cells were thereafter plated at 100 μl/well and incubated for 45 min at 37°C to allow cell attachment. Then the medium was removed, and the cells were washed once with warm DMEM, before adding warm DMEM containing 10% FCS and incubating for another 60 min. After a brief wash with warm PBS (PBS, Mediatech), the cells were fixed with 10% formalin in neutral buffered saline (Sigma) for 30 min at room temp. Then the formalin was removed and the cells were stained with 80 μl/well of 1% methylene blue (Sigma) in 0.01 M borate buffer (pH 8.5) for 30 min. After washing, the methylene blue was extracted with 100 μl of 0.1 N HCl/Ethanol (1:1 mix) for 30 min at room temp. The optical density was thereafter read at a wave length of 655 nm using a Bio-Rad microplate reader to determine the percentage of cells that were attached.

***In Vitro* Binding of Tumstatin Peptide and Dependence on Cell Proliferation Status.** C-PAE cells were grown to 40% or 100% confluency on 8-chamber slides (Lab-Tek). Then FITC-tumstatin peptide (30 μg/ml) was added and incubated with the cells for 20 min, before fixation with either acetone (4°C, 5 min) for surface integrin labeling or ethanol/acetic acid (50:50 vol/vol, -20°C, 20 min) for intracellular VE-cadherin staining. Cells were permeabilized with Tris buffered saline (TBS) supplemented with 0.1% Tween 20 and blocked with 1% BSA for 20 min at room temperature. The cells were then incubated with a monoclonal mouse anti-human αv integrin subunit (Chemicon) or a polyclonal goat anti-human VE-cadherin (Santa Cruz Biotechnology) primary antibody for 60 min at room temperature. Subsequently, a rhodamine-conjugated donkey anti-mouse or donkey anti-goat IgG secondary antibody (Jackson Immunoresearch, dilution 1:100) was applied for 60 min at room temperature. Sections were mounted with Vectashield (Vector Laboratories) and analyzed by confocal microscopy.

Distribution of Tumstatin Peptide in Mice With or Without Endogenous Collagen IV-α3 (Tumstatin Precursor Deficient). LLC tumors were implanted and grown in collagen IV-α3 (+/-) and collagen IV-α3 (-/-, tumstatin precursor deficient) mice as described previously (7). Ninety minutes before sacrifice 50 μg/g body weight of BrdU was injected i.v. and 45 min before death FITC-tumstatin peptide 0.5 mg/kg was injected i.v. Tumors and various organs were collected and processed for immunohistochemistry as described below.

Attachment of Tumstatin Peptide to Neovessels During Normal and Pathological Angiogenesis. Skin wound healing, liver regeneration and *in vivo* Matrigel plug assays were performed as previously

described (7, 8). Matrigel (BD Biosciences) was mixed with 20 units/ml of heparin (Pierce) and 50 ng/ml of VEGF and the Matrigel mixture was injected s.c. in five-six weeks old male C57/BL6 mice. Ten days after the skin wound infliction or 70% liver removal, or six days after the Matrigel injection, FITC-tumstatin peptide was injected i.v. at 0.5 mg/kg, and 45 min later the mice were killed. The Matrigel plugs were removed and fixed in 4% paraformaldehyde. The plugs were embedded in paraffin, sectioned and stained with HE or PAS. Sections were thereafter examined by light and fluorescence microscopy in the same visual fields.

Immunostaining of Tumors and Normal Tissues. Tumors and normal tissues were fixed in 10% neutral buffered formalin (Sigma) or collected in OCT compound (Sakura) and snap frozen in liquid nitrogen. Formalin-fixed tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin (HE) or periodic acid Schiff (PAS). Sections were thereafter examined by light and fluorescence microscopy in the same visual fields. Frozen sections (4 μm) were made from OCT-embedded tissues, the sections were fixed in cold acetone for 5 min on ice, and blocked with 1% BSA for 20 min at room temp. The sections were then incubated with a monoclonal rat anti-mouse CD31 (BD PharMingen), anti-entactin (Chemicon) or a mouse anti-BrdU (Roche Diagnostics) primary antibody for 60 min at room temp, before rinsing with PBS and incubating with a rhodamine-conjugated anti-rat or anti-mouse IgG secondary antibody (Jackson Immunoresearch) for 60 min. The sections were thereafter mounted with Vectashield (Vector Laboratories) and analyzed using fluorescence microscopy. For CD31 quantification, in each tumor section, the number of CD31-positive blood vessels were counted in ten 200x magnification fields and an average was calculated for each mouse (9).

For CD31 staining of the Matrigel plugs, we used 4 μm sections from formalin fixed, paraffin wax embedded tissue. Briefly, sections were deparaffinized and rehydrated. The sections were immersed in 0.01 M citrate buffer (pH 6.0), brought to a boil in a microwave oven and incubated in a damp chamber (98°C) for 1 h. After blocking with diluted goat serum for 1 h, the slides were incubated overnight (+4°C) with a goat anti-mouse CD31 polyclonal antibody (dilution 1:150, Santa Cruz Biotechnology). After blocking endogenous peroxidase activity for 30 min with 3% hydrogen peroxide (Sigma), a biotinylated anti-goat secondary antibody was applied for 30 min (1: 200, Vector Laboratories). The antigen-antibody complex was revealed with avidin-biotin-peroxidase for 30 min according to the manufacturer's instructions (Vectastain® ABC Kit, Vector). Staining was done for 5 min with diamino-benzidine tetrahydrochloride (Sigma). The sections were then counterstained with haematoxylin, dehydrated and mounted with Entellan (Electron Microscopy Services).

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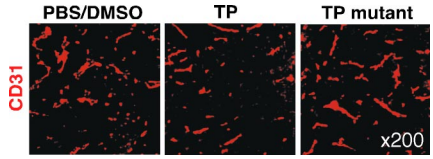

Fig. S1. The effect of tumstatin peptide (TP) and tumstatin peptide mutant on the number of blood vessels in LLC tumors on day 32. Red: anti-CD31.

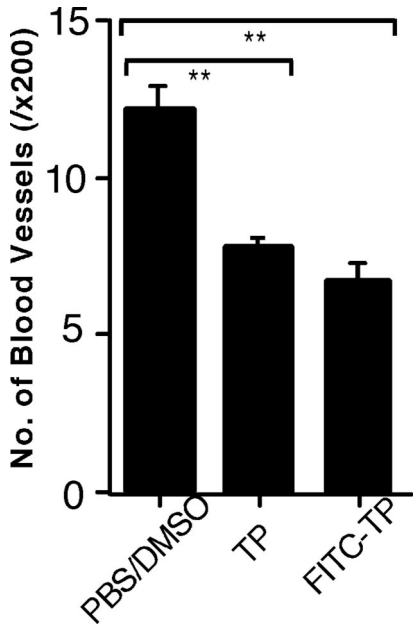

Fig. S2. The effect of tumstatin peptide and FITC-tumstatin peptide on the number of blood vessels (per ×200 high-power field) in LLC tumors on day 32. $^{**}P <$ 0.01 compared to PBS/DMSO.

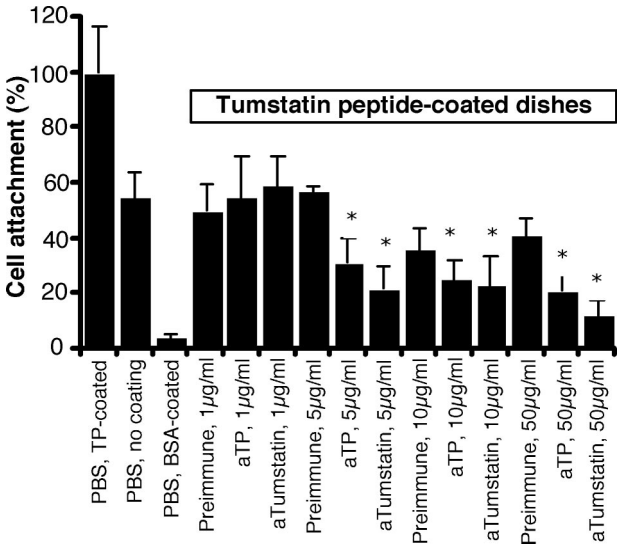

Fig. S3. The attachment of C-PAE cells to tumstatin peptide (TP)-coated plates. The cells were preincubated with a preimmune, anti-tumstatin peptide (aTP) or anti-tumstatin (aTumstatin) antibody. Bars: mean ± SEM. *, $P < 0.05$ compared to preimmune/control antibody of same concentration.

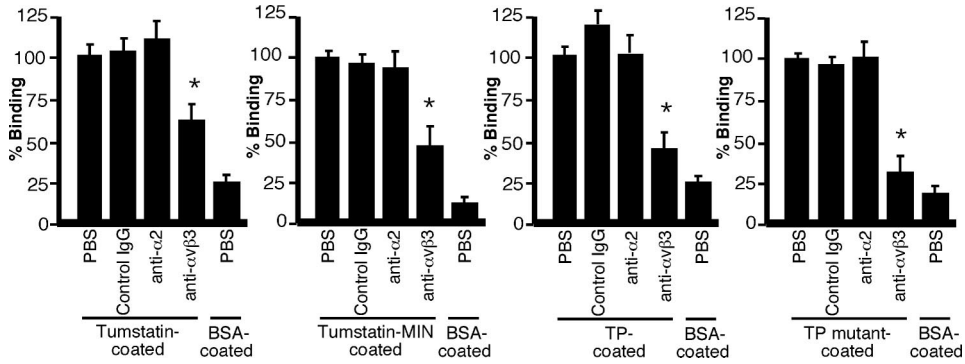

Fig. S4. The importance of $\alpha v\beta 3$ integrin receptor for endothelial cell binding to tumstatin and tumstatin peptide. Plates were coated with tumstatin, tumstatin mutant protein (tumstatin-MIN), tumstatin peptide or tumstatin peptide mutant, and C-PAE cell attachment was assessed. The cells were preincubated with either a control, $\alpha 2$ integrin subunit or $\alpha v\beta 3$ integrin-antibody. *, $P < 0.05$ compared to control or $\alpha 2$ antibody.

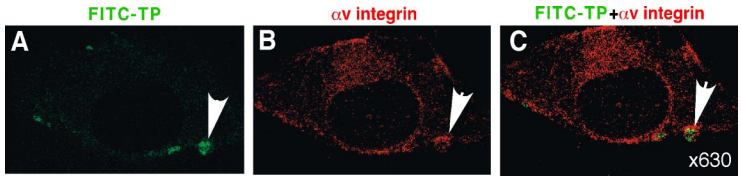

Fig. S5. The binding characteristics of tumstatin peptide to the αv integrin subunit on endothelial cells. FITC-tumstatin peptide binds to the endothelial cell surface via αv(β3) integrin. Green: FITC-tumstatin peptide (FITC-TP). Red: anti-αv integrin subunit.

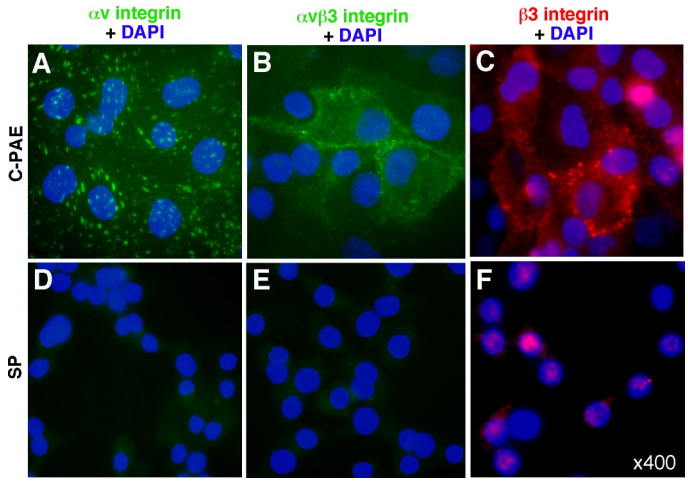

Fig. S6. The expression of $\alpha v\beta 3$ integrin on C-PAE endothelial cells and SP teratocarcinoma cells. Apart from slight nuclear staining of SP nuclei for the $\beta 3$ integrin subunit, there was positive immunostaining only on the endothelial cells. Green/red: integrin. Blue: DAPI.

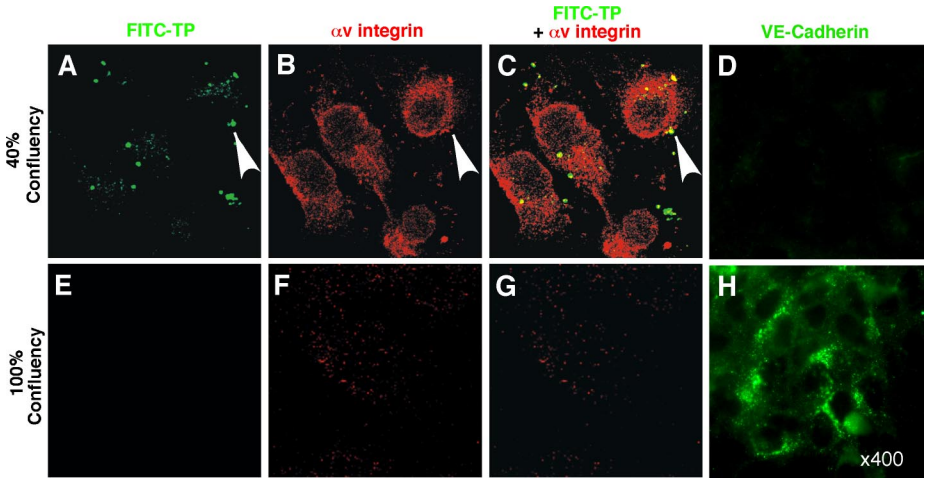

Fig. S7. (*A–H*) The importance of cell proliferation status for tumstatin peptide binding. VE-cadherin staining was used to demonstrate the confluency state of the cells. As expected VE-cadherin expression was low when the cells were non-confluent, whereas it was strongly up-regulated at confluency. Green: FITC-tumstatin peptide (FITC-TP). Red: anti-αv integrin subunit or VE-cadherin. Confocal microscopy.

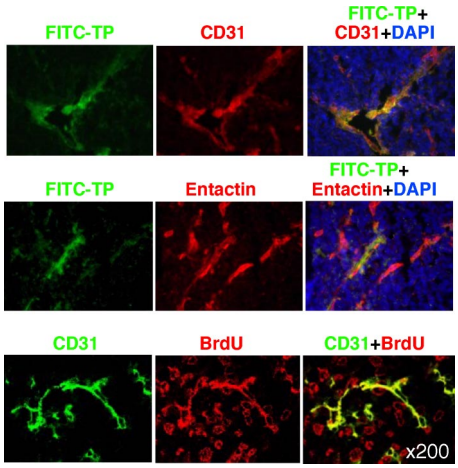

Fig. S8. Specific binding of FITC-tumstatin peptide to $CD31^+$ and $entactin^+$ blood vessels in LLC tumors, and the $BrdU^+$ proliferative status of the tumor endothelium.

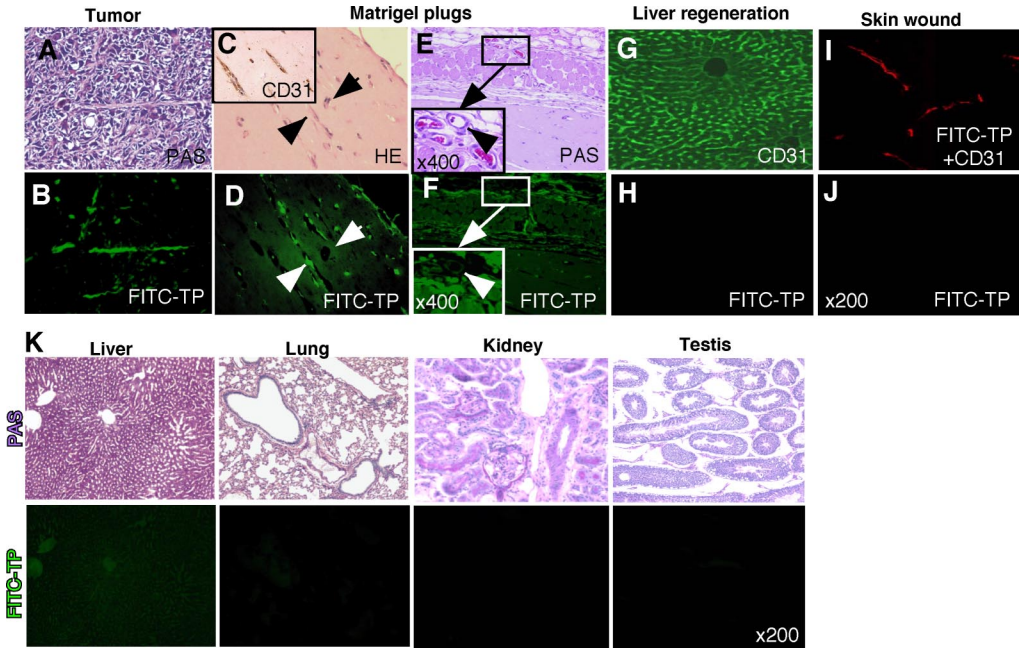

Fig. S9. Binding of FITC-tumstatin peptide (FITC-TP) to blood vessels during pathological and physiological, repair-associated angiogenesis. (*A–F*) FITC-tumstatin peptide binds to malignant (LLC tumor, *A* and *B*) and nonmalignant, pathological neovessels (Matrigel plugs, *C–F*). Arrowhead (*C* and *D*): FITC-tumstatin peptide binding to neovessels in the Matrigel. Arrow (*C* and *D*): Some blood vessels were not labeled by FITC-tumstatin peptide. (*C Inset*) CD31 vessel staining (brown, diamino-benzidine tetrahydrochloride). Arrowhead (*E* and *F Inset*): FITC-tumstatin peptide was not incorporated in the normal blood vessels around the Matrigel plug. (*G–J*) Lack of binding of FITC-tumstatin peptide to blood vessels during normal angiogenesis, in regenerating liver after 70% liver removal (*G* and *H*) and in a skin wound (*I* and *J*). In the liver regeneration experiment, images for CD31-staining (green) and FITC-tumstatin peptide (green) binding are from consecutive sections. (*K*) Lack of binding of FITC-tumstatin peptide to blood vessels in normal tissues.

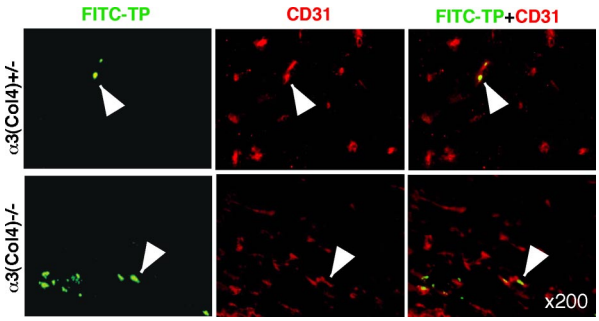

Fig. S10. FITC-tumstatin peptide binding to LLC tumor blood vessels was compared between collagen IV-α3 (−/−, tumstatin precursor deficient) and -α3 (+/−) mice. Arrowheads: binding of FITC-tumstatin peptide to blood vessels.

Table S1. Endogenous angiogenesis inhibitors and their endothelial integrin receptors

Integrin	Ligand (ref.)
$\alpha1\beta1$	Arresten (1)
$\alpha2\beta1$	Endorepellin (2)
$\alpha5\beta1$	Endostatin (3)
$\alpha v\beta3$	Tumstatin, canstatin, angiostatin (4–6)
$\alpha v\beta5$	Canstatin (5)

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