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**13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)**

METH1/ADAMTS1 is a metalloprotease with a disintegrin motif and three thrombospondin anti-angiogenic domains in the carboxy-terminal. METH1 was previously shown to inhibit angiogenesis in a variety of *in vitro* and *in vivo* assays. In the present study, I demonstrate that METH1 significantly blocks VEGFR2 phosphorylation with consequent suppression of endothelial cell proliferation. The effect on VEGFR2 function was due to direct binding and sequestration of the VEGF<sub>165</sub> by ADAMTS1. Binding was confirmed by co-immunoprecipitation and crosslinking analysis. Inhibition of VEGF function was reversible, as active VEGF could be recovered from the complex. The interaction required the heparin-binding domain of the growth factor, as VEGF<sub>121</sub> failed to bind to ADAMTS1. Structure/function analysis with independent ADAMTS1 domains indicated that binding to VEGF<sub>165</sub> was mediated by the carboxy-terminal (CT) region. ADAMTS1 and VEGF<sub>165</sub> were also found in association in tumor extracts. These findings provide a mechanism for the anti-angiogenic activity of ADAMTS1 and describe a novel modulator of VEGF bioavailability.

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## **Introduction**

Growth tumors and metastasis require neovascularization. The dependency of tumors on blood vessels has been clearly demonstrated by recent clinical trials in which suppression of tumor growth has been accomplished by inhibitors of angiogenesis. Consequently further investigation on novel inhibitors and a full understanding of their mechanism of action can bring new avenues of therapy for the treatment of tumors. Using the anti-angiogenic domain of thrombospondin-1 (TSP1), several years ago our laboratory cloned two novel proteins: METH-1/ADAMTS-1 and METH-2/ADAMTS-8 (Vazquez et al., 1999). In addition to several thrombospondin anti-angiogenic domains in the carboxy-terminus (three for METH-1 and two for METH-2), these proteins also contain an ADAM cassette, which includes a metalloprotease domain and a disintegrin motif. The proteins are in fact, active metalloproteases that are secreted as zymogens and require removal of the pro-domain to become active (Rodriguez-Manzaneque et al., 2000). Our laboratory has demonstrated that these proteins have anti-angiogenic properties in several in vivo and in vitro bioassays and more importantly for my project: overexpression of METH-1 in T47D mammary carcinoma cell lines significantly decreases tumor growth by an anti-angiogenic mechanism (Carpizo et al., manuscript in preparation).

## Key Research Accomplishments

**Annual Summary for Award Number DAMD17-02-1-0329**

**Proposal Title: Angiogenesis inhibitors in Breast Cancer**

**Principal Investigator: Alfonso Luque, Ph.D., UCLA**

In this Annual Summary I present the research accomplished for the period of March 25, 2002 - March 24, 2003 under the Training Grant number DAMD17-02-1-0329. These results partially address the 1<sup>st</sup> objective I proposed: "to determine the mechanism of action of METH1 that affects endothelial cell proliferation". I demonstrated that METH1, through its carboxy-terminal, binds and sequesters VEGF<sub>165</sub> inhibiting VEGFR2 phosphorylation and consequently suppressing endothelial cell proliferation. These findings have been accepted for publication in the Journal of Biological Chemistry with the acknowledgment of this grant. See the manuscript in the appendix.

### **Results**

**1) ADAMTS1 inhibits mitogenic effect of VEGF<sub>165</sub>.** Proliferation assays of bovine aortic endothelial cells (BAEC) stimulated with of FGF-2 and/or VEGF<sub>165</sub> were performed. Addition of recombinant METH1 decreased cell proliferation induced by FGF2 (49% +/- 16%) and FGF-2 + VEGF<sub>165</sub> (78% +/- 18%). Proliferative signals mediated by VEGF<sub>165</sub> were completely blocked in the presence of ADAMTS1 (Fig. 1A).

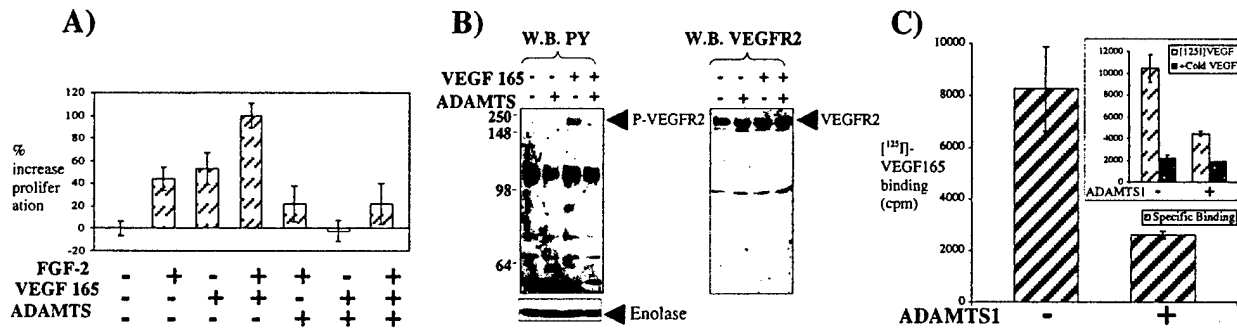
**2) VEGFR2 phosphorylation was strongly inhibited by ADAMTS1 in several endothelial cell cultures.** Fig 1B shows the effect observed on BAEC (Fig. 1B)

**3) METH1 reduced VEGF<sub>165</sub> specific binding to endothelial cells.** Binding assays of <sup>125</sup>I-VEGF<sub>165</sub> to HAEC was inhibited by 69% in presence of METH1 (Fig 1C).

**4) METH1 binds to VEGF.** The interaction of METH1 with VEGF<sub>165</sub> was evaluated by co-immunoprecipitation and crosslinking assays. The Fig 2A shows the complex generated by the association of METH1 with VEGF, crosslinked and analyzed by Western blot techniques. The 130 kD specie corresponds to the sum of ADAMTS1 (87kD) and VEGF (43kD).

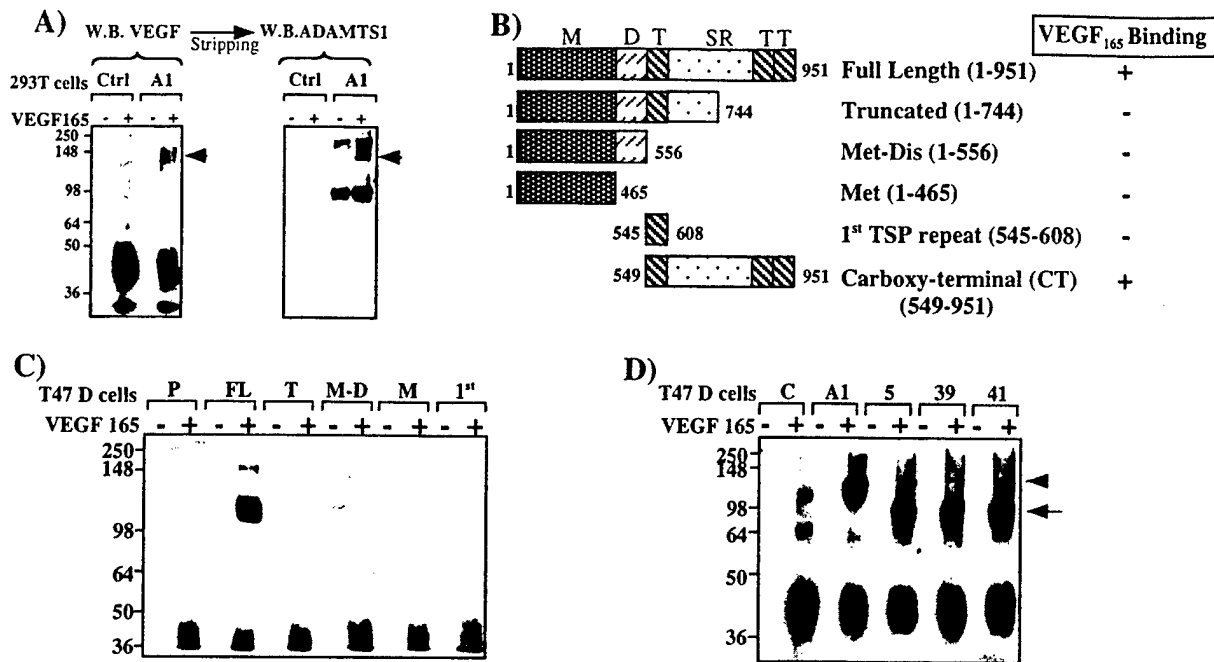
5) VEGF<sub>165</sub> binds to the carboxy-terminal (CT) region of ADAMTS1. To determine the domain(s) of ADAMTS1 that participate in the interaction with VEGF<sub>165</sub>, we used several deletion constructs (Fig. 2B). In these experiments, only the full length P87-ADAMTS1 was able to bind to VEGF<sub>165</sub> (Fig. 2C) indicating that the interaction of VEGF<sub>165</sub> to ADAMTS1 requires the two last TSP repeats and at least part of the spacer region. Therefore, we generated a deletion construct consisting of the CT of ADAMTS1 from the 1<sup>st</sup> TSP repeat to the last residue (including the spacer region). As anticipated, the CT region of ADAMTS1 binds to VEGF<sub>165</sub>, resulting in a 90 kD crosslinked complex (Fig. 2D).

Figure 1



**Figure 1. A) METH1 inhibits VEGF<sub>165</sub> stimulated endothelial cell proliferation.** BAEC were synchronized in G<sub>0</sub> by serum starvation (48 h) post confluence. Cells were seeded and stimulated with VEGF<sub>165</sub> (25 ng/ml), FGF-2 (2 ng/ml) or a combination of both, in the presence or absence of recombinant ADAMTS1 (5 μg/ml). Treatment was performed for 24 h. During the last 8 h, a pulse of 1 μCi/well of [<sup>3</sup>H]thymidine was added. Incorporated counts were determined by liquid scintillation. All counts were normalized to maximum stimulation (VEGF<sub>165</sub> and FGF-2). **B) METH1 blocks VEGFR2 phosphorylation in VEGF<sub>165</sub> stimulated BAEC.** BAEC were stimulated with VEGF<sub>165</sub> (50 ng/ml) for 6 minutes in the presence of conditioned media from 293T cells expressing ADAMTS1 or vector alone. Phosphorylated proteins were visualized using the anti-phosphotyrosine polyclonal Ab. Anti-enolase was used as loading control. Insert shows VEGFR2 levels of the same samples resolved in a parallel Western blot. **C) Purified ADAMTS1 decreases the specific binding of <sup>125</sup>I-VEGF<sub>165</sub> to the endothelial cell surface.** Human aortic endothelial cells (HAEC) were incubated with <sup>125</sup>I-VEGF<sub>165</sub> (16 ng/ml) in binding buffer in presence (+) or absence (-) of ADAMTS1. After incubation (1h at 4°C), cells were washed, solubilized in lysis buffer and counted in a gamma counter. Non-specific binding was calculated in the presence of 20-fold excess of cold VEGF<sub>165</sub>. Specific binding was determined by subtracting nonspecific binding(+cold VEGF) from total binding (<sup>125</sup>I-VEGF) shown in the insert.

Figure 2



**Figure 2.** A) ADAMTS1 binds to VEGF<sub>165</sub>. 293T cells expressing ADAMTS1 or pCDNA were incubated for 24 h at 37 °C in presence or absence of VEGF<sub>165</sub>. Conditioned media was crosslinked and the formation of complexes was evaluated by Western blot with anti-VEGF antibodies. Levels of ADAMTS1 were evaluated by reprobing the same membrane with MAb 5D4E11B5. Arrow indicates the species recognized by both antibodies. B) Schematic representation of ADAMTS1 constructs and binding properties to VEGF<sub>165</sub>. Structural motifs include: (M) Metalloprotease domain, (D) Disintegrin domain, (T) TSP repeat and (SR) Spacer region. Numbers correspond to amino-acids considering Methionine in position #1. C) VEGF<sub>165</sub> binds to full length ADAMTS1. Subconfluent T47 D cells expressing ADAMTS1 deletion constructs (Full length, FL; Truncated, T; Met-Dis, M-D; Met, M; and 1<sup>st</sup> TSP repeat, 1<sup>st</sup>) or vector alone (P) grown on 24 well plates were incubated in serum-free media in presence or absence of VEGF<sub>165</sub> and heparin. Conditioned media was crosslinked and subjected to SDS-PAGE and VEGF<sub>165</sub> patterns were evaluated by Western blot. D) VEGF<sub>165</sub> binds to the CT region of ADAMTS1. T47 D cells expressing vector alone (C), ADAMTS1 full length (A1) or the CT construct of ADAMTS1 (5, 39 and 41) were incubated in presence or absence of VEGF<sub>165</sub> and heparin. After crosslinking, VEGF<sub>165</sub> patterns were evaluated by Western blot. Arrow indicates shifting of VEGF<sub>165</sub> produced by the binding to the CT protein and the arrowhead indicates the shifting due to binding to the full length.

### **Reportables Outcomes**

\* "ADAMTS1/METH1 inhibits endothelial cell proliferation by direct binding and sequestration of VEGF<sub>165</sub>." A. Luque, D.R. Carpizo and M.L. Iruela-Arispe. Journal of Biological Chemistry. In press.

\* Attending to the Gordon Research Conference: Vascular Cell Biology (January 26-31, 2003)  
Poster title: "ADAMTS1 inhibits endothelial cells proliferation by direct binding and sequestration of VEGF<sub>165</sub>" Alfonso Luque, Darren Carpizo and Maria Luisa Iruela-Arispe.

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\* Rodriguez-Manzaneque JC, Milchanowski AB, Dufour EK, Leduc R, Iruela-Arispe ML. Characterization of METH-1/ADAMTS1 processing reveals two distinct active forms. *J Biol Chem.* 2000 Oct 27;275(43):33471-9.

\* Carpizo et al., manuscript in preparation.

**ADAMTS1/METH1 inhibits endothelial cell proliferation by direct binding and  
sequestration of VEGF<sub>165</sub>\***

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**Running title: ADAMTS1 binds to VEGF<sub>165</sub>**

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## Summary

ADAMTS1<sup>1</sup> is a metalloprotease previously shown to inhibit angiogenesis in a variety of *in vitro* and *in vivo* assays. In the present study, we demonstrate that ADAMTS1 significantly blocks VEGFR2 phosphorylation with consequent suppression of endothelial cell proliferation. The effect on VEGFR2 function was due to direct binding and sequestration of the VEGF<sub>165</sub> by ADAMTS1. Binding was confirmed by co-immunoprecipitation and crosslinking analysis. Inhibition of VEGF function was reversible, as active VEGF could be recovered from the complex. The interaction required the heparin-binding domain of the growth factor, as VEGF<sub>121</sub> failed to bind to ADAMTS1. Structure/function analysis with independent ADAMTS1 domains indicated that binding to VEGF<sub>165</sub> was mediated by the carboxy-terminal (CT) region. ADAMTS1 and VEGF<sub>165</sub> were also found in association in tumor extracts. These findings provide a mechanism for the anti-angiogenic activity of ADAMTS1 and describe a novel modulator of VEGF bioavailability.

<sup>1</sup>The abbreviations used are: ADAMTS1, a disintegrin and metalloproteinase with thrombospondin motifs; BAEC, bovine aortic endothelial cells; BSA, bovine serum albumin; CT, carboxy-terminal region of ADAMTS1; DSS, decanesulfonic acid sodium salt; EDTA, ethylenediamine tetraacetic acid; HAEC: human aortic endothelial cells; HPLC, high pressure liquid chromatography; MAb, monoclonal antibody; METH1, metalloprotease and thrombospondin; Met, metalloproteinase construct; Met-Dis, metalloproteinase and disintegrin truncated construct; MMP, matrix metalloproteinase; PAE, porcine aortic endothelial cells; PCR, polymerase chain reaction; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TGFbeta, transforming growth factor beta; TSP, thrombospondin; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

## Introduction

Extracellular matrix proteins can significantly modulate growth factor signaling. This occurs to a large extent, but not exclusively, from direct non-covalent interactions that mediate selective anchorage of growth factors to the extracellular milieu (1-3). Several extracellular matrix molecules have been shown to bind and sequester growth factors, as well as to enhance signaling by altering presentation to receptor binding sites (4-10). Angiogenesis is particularly sensitive to this type of regulation due to the critical role of paracrine growth factors in endothelial cell migration and proliferation.

The vascular endothelial growth factor (VEGF) gene produces several splice variants critical for capillary morphogenesis and tumor angiogenesis (11-13). Haploinsufficiency of this gene is incompatible with development due to major vascular abnormalities, as demonstrated by inactivation of the gene through homologous recombination (14, 15). Isoforms of VEGF are secreted by diverse cell types including smooth muscle, fibroblasts and epithelial cells. These proteins function by activation of two tyrosine kinase receptors, VEGFR1 and VEGFR2, as well as binding to non-receptor tyrosine kinases coreceptors such as neuropilin 1 and 2 on endothelial cells (16, 17).

ADAMTS1 was the first member of a growing family of ADAMTS extracellular proteases characterized by the presence of disintegrin-like, metalloprotease and a variable number of thrombospondin-like domains (18, 19). Once secreted, ADAMTS1 activation requires furin cleavage and removal of the pro-domain. The active protease can undergo a secondary processing event that separates the catalytic subunit from the thrombospondin (TSP) repeats (20, 21). These TSP motifs in TSP1 and TSP2 have been shown to block angiogenesis by several, and likely not independent, mechanisms (22-24).

## ADAMTS1 binds to VEGF<sub>165</sub>

We demonstrated previously that ADAMTS1 is able to suppress capillary growth using multiple *in vivo* and *in vitro* assays (18). Interestingly, the ability of ADAMTS1 to inhibit neovascularization *in vivo* was greater than that of endostatin and TSP1 at the same molar ratio. The findings are intriguing and appear in marked contrast to the current paradigm that MMPs are pro-migratory and pro-angiogenic (25-28). In an effort to determine the mechanism(s) responsible for the angiostatic properties of ADAMTS1, we investigated its effects on endothelial cell growth and found that ADAMTS1 was able to drastically decrease VEGFR2 phosphorylation by a mechanism that involved direct binding and sequestration of VEGF<sub>165</sub>. The interaction was also verified *in vivo* using xenograft assays engineered to express ADAMTS1. Our results demonstrate that ADAMTS1 binds to VEGF<sub>165</sub>, and that this impacts the bioavailability of VEGF with consequences to receptor phosphorylation, endothelial proliferation and angiogenesis.

### Experimental Procedures

**Cells.** Bovine aortic endothelial cells (BAEC); human embryonic kidney 293T cells expressing full length human ADAMTS1 or vector alone (18); and breast tumor-derived T47D cells expressing deletion ADAMTS1 constructs were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Human aortic endothelial cells, (HAEC, provided by Dr Judith Berliner, Department of Pathology, UCLA) were grown in medium 199 containing 20% FCS, endothelial cell growth supplement (20 µg/ml) and heparin (90 µg/ml) (Sigma). Porcine aortic endothelial cells (PAE) transfected with VEGFR2 or vector alone (provided by Dr. Gera Neufeld, Technicon, Israel) were grown in Ham's F-12 medium supplemented with 10% FCS.

**Generation of monoclonal antibodies against ADAMTS1.** Balb/c mice were immunized by intraperitoneal injections of purified ADAMTS1 (10µg) (21) and complete Freud adjuvant (300 µl). Additional immunizations were done with incomplete Freud adjuvant at days 15 and 33 (intraperitoneal injection), and intravenously at day 45. At day 48, splenocytes were obtained from immunized mice and fused with SP2 myeloma cells at a 4:1 ratio following established techniques (29). Conditioned media from hybridoma cultures were screened by ELISA against purified ADAMTS1 coated to plastic. Further characterization of positive clones was performed by immunoprecipitation, Western blot analysis and immunohistochemistry. From 101 positive wells, twelve hybridomas were cloned by repeated limiting dilution based on epitopes and specific properties. Table I summarizes the selected monoclonal antibodies (MAbs).

**Endothelial cell proliferation.** Quiescent BAEC were trypsinized and plated onto 24-well plates in DMEM supplemented with vascular endothelial growth factor (VEGF<sub>165</sub>, R&D Systems) (25 ng/ml), basic fibroblast growth factor (FGF-2) (2 ng/ml) (provided by Dr. Gera Neufeld, Technion, Israel) or a combination of both, in the presence or absence of recombinant ADAMTS1/METH-1 protein (5 µg/ml). A pulse of 1 µCi/well of [<sup>3</sup>H]thymidine (Amersham Pharmacia Biotech) was applied during the last 8 h prior to harvesting. Cells were washed and fixed in 10% trichloroacetic acid. Incorporation of [<sup>3</sup>H]thymidine was determined by scintillation counting, as described previously (30).

**Phosphorylation assays.** Subconfluent cells were incubated overnight in serum-free medium and subsequently preincubated for 5 minutes with 0.1 mM Na<sub>3</sub>VO<sub>4</sub> to inhibit phosphatase activity. Cultures were then washed once and pretreated with 1.5 ml of conditioned media from 293T cells expressing ADAMTS1 or vector alone for 15 minutes at 37°C. The concentration of ADAMTS1 in the conditioned media ranges between 5.85 µg/ml (66 nM) and 16.7 µg/ml (191 nM). These values were obtained by ELISA against a standard curve of purified ADAMTS1 (data not shown). Cells were stimulated for 6 minutes at 37°C with specified concentration of VEGF<sub>165</sub>. The incubation was terminated by removal of the medium and washes with cold PBS/0.2 mM Na<sub>3</sub>VO<sub>4</sub>. Cells were solubilized in lysis buffer (1% Triton X-100, 10 mM tris-HCl, pH 7.6, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 2.1 mM sodium orthovanadate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 2 µg/ml of aprotinin) at 4°C for 15 minutes. Insoluble material was removed by centrifugation at 4°C for 30 minutes at 14,000 x g. Equal amounts of cell lysate were separated by SDS-PAGE and transferred to nitrocellulose membranes. Phosphorylated proteins were detected by immunoblotting using

## ADAMTS1 binds to VEGF<sub>165</sub>

antiphosphotyrosine antibodies (polyclonal or MAb PY20, BD Transduction Laboratories) followed by secondary antibodies coupled with horseradish peroxidase and visualized by chemoluminescence (ECL kit, Pierce). Protein loading control was assessed by Western blot using anti-VEGFR2 (A-3, Santa Cruz, Biotechnology) and or anti-enolase antibodies.

**Immunoprecipitation.** Cell lysates were precleared with 40  $\mu$ l of protein G agarose (Roche) for 1 h at 4°C. Beads were discarded by centrifugation and the supernatant was incubated with 1  $\mu$ g/ml of the antiphosphotyrosine antibody (PY20) overnight at 4°C followed by addition of protein G agarose for 1 h under continuous agitation. Immunoprecipitates were washed three times with lysis buffer and extracted in 2X SDS-PAGE sample buffer by boiling 5 minutes, fractionated by one-dimensional SDS-PAGE, and further analyzed by Western blot with antiphosphotyrosine antibodies.

ADAMTS1 was immunoprecipitated from tumor lysates (450  $\mu$ g) as described above using the MAb 5C6D5. The presence of coimmunoprecipitated VEGF was assessed by Western blot using polyclonal anti-VEGF, #375 (generous gift from Don Senger, Beth Israel Deaconess Medical Center, Boston). Levels of ADAMTS1 were determined by Western analysis (MAb 5C6D5).

**Radiolabeling of VEGF.** VEGF<sub>165</sub> was labeled with <sup>125</sup>I-Na using iodogen as coupling agent. Briefly, VEGF<sub>165</sub> (2 $\mu$ g) was incubated in 200  $\mu$ l of borate buffer (0.01 M Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>, 0.14 M NaCl, pH 8.2) with 0.3 mCi of <sup>125</sup>I-Na (Amersham Pharmacia Biotech) in IODO-GEN pre-coated iodination tubes (Pierce) 5 minutes at R.T. The reaction was stopped by transferring it to a fresh tube containing 40  $\mu$ l of 0.4 mg/ml tyrosine in borate buffer for 1 minute at RT. and adding 200  $\mu$ l of 1 mg/ml IK in PBS/1% BSA. <sup>125</sup>I-VEGF<sub>165</sub> was separated from free iodine

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using size exclusion chromatography on Sephadex-G25 columns (Amersham Pharmacia Biotech). The specific radioactivity of the purified iodinated VEGF<sub>165</sub> was 18,886 cpm/ng. Quality and integrity of the labeled VEGF was assessed by SDS-PAGE followed by autoradiography of the dried gel.

**Binding assays.** Subconfluent cells were incubated at low serum (2% serum for HAEC and 0.1% for PAE and PAE-VEGFR2 cells) for 5 h at 37°C, rinsed in binding buffer (media containing 20 mM Hepes, 0.2% gelatin) and equilibrated at 4°C for 15 minutes. Indicated concentrations of <sup>125</sup>I-VEGF<sub>165</sub> were added to the wells in a final volume of 350 µl of binding buffer in the presence or absence of ADAMTS1. After 1h incubation at 4°C, cells were washed four times in binding buffer and solubilized in 500 µl of lysis buffer (2% Triton X-100, 10% glycerol, 1 mg/ml BSA). Bound protein was assessed by liquid scintillation on a gamma counter. Non-specific binding was calculated in the presence of 20-fold excess of cold VEGF<sub>165</sub>. Specific binding was determined by subtracting nonspecific binding from total binding.

**Purification of ADAMTS1 and evaluation of VEGF levels.** Recombinant ADAMTS1 protein was purified from conditioned media of stable 293T cells expressing ADAMTS1 by heparin and Zn<sup>2+</sup>-chelate affinity chromatography (21). Conditioned media from 293T cells transfected with the vector alone was used as control. Presence of VEGF in the ADAMTS1 fractions was assessed by Western blot (Chemicon). Levels of ADAMTS1 were tested using the MAb 5D4E11B5 after stripping of the same membrane.

**Gel Permeation Chromatography.** ADAMTS/VEGF complexes purified by heparin chromatography were concentrated to 1ml and dialyzed into 10mMHEPES, 150mMNaCl, 25 mM 1-decanesulfonic acid sodium salt (DSS). Sample was loaded onto a Superdex 75 HR 10/30, previously equilibrated in 10 mM HEPES, 150 mM NaCl, and 0.3% Zwittergent 3-12. Chromatography was carried out at 4°C with a flow rate was 0.3 ml/min (at 1100 psi). Fractions of 300 µl were collected and evaluated by Western blots.

**Binding of VEGF to immobilized ADAMTS1.** ADAMTS1 was immunoprecipitated from conditioned media of stably transfected 293T cells using the MAb 5C6D5. Protein A beads were washed three times with PBS, 1% Triton X-100, 1% BSA, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> and equilibrated in binding buffer (50 mM Hepes, 150 mM NaCl, 0.5% NP40, 0.5% BSA, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>). Soluble ligands: VEGF<sub>165</sub> (50 ng) (R&D Systems) and/or heparin (50 ng) (Sigma), were added to the pellets in a final volume of 500 µl of binding buffer, and incubated for 30 minutes at 4°C. The beads were subsequently washed three times with binding buffer. Proteins bound to the immunoconjugates were subjected to SDS-PAGE and the presence of coimmunoprecipitated VEGF was analyzed by Western blots. Immunoprecipitated ADAMTS1 was tested reproving the same membrane with the MAb 5D4E11B5.

**Crosslinking experiments. A) Using purified proteins.** Purified ADAMTS1 (1.25 µg) was incubated with VEGF<sub>165</sub> (25 ng) in presence or absence of heparin (50 ng) in 75 µl of binding buffer (50 mM Hepes, 100 mM NaCl, pH 7.2) for 2 h at R.T. Bound proteins were crosslinked with disuccinimydil suberate (1.5 mM) (Pierce) for 30 minutes at R.T. The reaction was stopped by adding 20 mM Tris, 50 mM glycine, 2 mM EDTA (pH7.4) for 15 minutes at R.T. The

samples were processed by SDS-PAGE and protein complexes, indicating protein-protein interaction, were visualized by Western blots.

**B) Using conditioned media.** Subconfluent 293 T or T47D cells expressing full length or different deletion constructs of ADAMTS1 or pCDNA (negative control) grown on 24 well plates were washed twice with serum-free DMEM and incubated in 300  $\mu$ l of serum-free media for 18 h in presence or absence of VEGF<sub>165</sub> (150 ng), VEGF<sub>121</sub> (125 ng) (provided by Dr. Gera Neufeld) with or without heparin (1.5  $\mu$ g). Conditioned media was collected and the interacting proteins were crosslinked and analyzed as described above.

**Xenograft tumor assays and tumor protein extraction.** Control and ADAMTS1 tumors were generated in 6 week old male nude mice (Charles River Laboratories) by subcutaneous flank injection of both T47D Control (empty vector) or ADAMTS1 ( $5 \times 10^6$  cells / injection). When tumors reached 1,500 mm<sup>3</sup>, mice were euthanized and dissected tumors were diced, sieved, and solubilized in lysis buffer (1% Triton X-100, 10 mM tris-HCl, pH 7.6, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 2.1 mM sodium orthovanadate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 2  $\mu$ g/ml of aprotinin) at 4°C for 1 h. Insoluble material was removed by centrifugation at 4°C for 1h at 14,000 x g.

**Expression of the carboxy-terminal (CT) region of ADAMTS1 in T47D cells.** The construct for expression of the CT region of ADAMTS1 corresponding to Pro<sup>549</sup> - Ser<sup>951</sup> was obtained by PCR of the full-length cDNA. A *KpnI* site was introduced at the 5' end by site-directed mutagenesis using the following forward oligo: 5'TTTTCATGGTACCTGGGGAATGTGGG-3'. The reverse oligo used was, 5'-ACTGCATTCTGCCTTTGTGCAAAAAGTC-3'. The resulting

## ADAMTS1 binds to VEGF<sub>165</sub>

PCR product was cloned into the pSecTag2/Hygro B vector (Invitrogen) using *KpnI/EcoRV*. Stable transfectant clones were generated using this plasmid in T47D cells by calcium phosphate transfection and selected with hygromycin-B (300 µg/ml).

## Results

### ADAMTS1 inhibits mitogenic effect of VEGF<sub>165</sub> affecting VEGFR2 phosphorylation

In a previous report we showed that ADAMTS1 antagonizes endothelial cell proliferation induced by a combination of FGF-2 and VEGF<sub>165</sub> (18). To further dissect the mechanism of action of ADAMTS1, we repeated these experiments using each growth factor independently and in combination. Addition of recombinant ADAMTS1 decreased cell proliferation induced by FGF2 (49% +/- 16%) and FGF-2 + VEGF<sub>165</sub> (78% +/- 18%). Proliferative signals mediated by VEGF<sub>165</sub> were completely blocked in the presence of ADAMTS1 (Fig. 1A).

The mitogenic action of all VEGF isoforms is mediated through binding to the 205 kD receptor tyrosine kinase VEGFR2 (31). Therefore, we tested if activation of VEGFR2 was affected by ADAMTS1. VEGFR2 phosphorylation was strongly inhibited by ADAMTS1 in several endothelial cell cultures including PAE-VEGFR2 (Fig. 1B), BAEC (Fig. 1C) and human aortic endothelial cells (data not shown).

Stimulation of BAEC with increasing concentrations of VEGF<sub>165</sub> resulted in a dose-dependent phosphorylation of VEGFR2 and ADAMTS1 reduced the levels of phospho-VEGFR2 in all cases (Fig. 1D). Together these results imply a direct link between VEGF<sub>165</sub> signaling and the anti-angiogenic effects mediated by ADAMTS1.

As a possible explanation for the ADAMTS1 inhibition of VEGFR2 phosphorylation, we tested whether binding of VEGF<sub>165</sub> to the endothelial cell surface was affected by ADAMTS1. <sup>125</sup>I-VEGF<sub>165</sub> bound to HAEC (Fig. 2A) and PAE-VEGFR2 cells (Fig. 2B) in a concentration dependent manner, however it did not bind to VEGFR2-deficient cells (PAE) (Fig. 2B). It has been documented that saturation binding occurs at 10 ng/ml with VEGF<sub>165</sub> (32). The specific binding of <sup>125</sup>I-VEGF<sub>165</sub> (16 ng/ml) to HAEC (Fig. 2A) was 14.7%, while saturation binding in

PAE-VEGFR2 was lower, 8.9% after incubation with 12 ng/ml of radioiodinated VEGF (Fig. 2B). Presence of purified ADAMTS1 reduced <sup>125</sup>I-VEGF<sub>165</sub> specific binding by 69% (Fig. 2C).

### **VEGF co-purifies with ADAMTS1**

It has been reported that TSP repeats in thrombospondin 1 and in connective tissue growth factor bind to VEGF<sub>165</sub> and modulate its activity on endothelial cells (33, 34). The CT of ADAMTS1 contains three domains that share significant homology to the TSP repeats of the TSP1 molecule (18). Therefore, we investigated the possibility of an interaction between ADAMTS1 and VEGF<sub>165</sub> as a regulatory mechanism to explain the effect of ADAMTS1 on VEGFR2 phosphorylation and the binding to the endothelial cell surface. We found that indeed VEGF (Fig. 3A, lane 1-3) was present in the samples containing ADAMTS1 (Fig. 3A, lane 5-7) after purification from heparin affinity chromatography (Fig. 3A, lane 1 and 5) and from chromatography on a Zn<sup>2+</sup>-chelate affinity column (Fig. 3A, lanes 2 and 6, 3 and 7). Although detection of VEGF in the first purification step was not surprising, since VEGF interacts avidly with heparin (34, 36); its presence after Zn<sup>2+</sup> chromatography was unexpected since this growth factor does not bind to Zn<sup>2+</sup>. To ascertain the degree of purification of VEGF from these two chromatography procedures in the absence of ADAMTS1, conditioned media from parental cell lines transfected with vector alone was subjected to the same chromatography purification. As expected, VEGF was eluted from heparin column at 1M (Fig. 3B), whereas no binding was detected on Zn<sup>2+</sup>-chelate affinity chromatography in the absence of ADAMTS1 (Fig. 3C). 293T cells have been shown previously to secrete VEGF, our results concur with those findings (37, 38). These results provide evidence that VEGF binds to ADAMTS1.

## ADAMTS1 binds to VEGF<sub>165</sub>

The two molecules can be dissociated and purified from one another by gel filtration chromatography (Fig. 4A). When bound to ADAMTS1, VEGF<sub>165</sub> was unable to phosphorylate VEGFR2 (Fig. 4B, sample A). However, VEGF<sub>165</sub> regained its activity when dissociated from the protease (Fig. 4B, sample 13).

## ADAMTS1 binds to VEGF<sub>165</sub>

The interaction of ADAMTS1 with VEGF<sub>165</sub> was also evaluated by co-immunoprecipitation and crosslinking assays. Conditioned media was collected from cells that were stably transfected with either vector alone (pCDNA) or ADAMTS1 and immunoprecipitated with anti-ADAMTS1 antibodies. The immunocomplexes were subsequently incubated with VEGF<sub>165</sub> and binding was evaluated by Western blots. VEGF<sub>165</sub> was detected in the ADAMTS1 immunocomplexes and addition of exogenous heparin increased binding of ADAMTS1 to VEGF<sub>165</sub> (Fig. 5A). No VEGF<sub>165</sub> was bound to immunoprecipitated complexes when media conditioned from cells expressing vector alone was used (Fig. 5A).

Crosslinking experiments were conducted with disuccinimidyl suberate to evaluate physical proximity of ADAMTS1 and VEGF<sub>165</sub>. Heterodimeric complexes of about 250kDa and 130kDa were detected with VEGF antibodies in the presence of ADAMTS1 (Fig. 5B). VEGF antibodies also recognized 130kD species in the ADAMTS1 preparations, indicating presence of VEGF in ADAMTS1 preparations as demonstrated previously (Fig. 3). We predict that the 130kD form corresponds to the sum of ADAMTS1 (87kD) and VEGF (43kD). Addition of heparin enhanced the formation of the high molecular weight complexes, however presence of heparin was not a requirement (Fig. 5B).

## ADAMTS1 binds to VEGF<sub>165</sub>

To ascertain whether ADAMTS1-VEGF<sub>165</sub> complexes were formed *in vivo*, we incubated 293T cells transfected with ADAMTS1 or empty vector with purified VEGF<sub>165</sub> and heparin. Evaluation of VEGF after crosslinking revealed a shifted band of about 130kD only in the ADAMTS1 conditioned media (Fig 5C). Under these experimental conditions, formation of the complex ADAMTS1-VEGF<sub>165</sub> required heparin. To ensure the presence of ADAMTS1 in the 130 kD band, the same blot was re-probed with anti-ADAMTS1 antibodies (Fig. 5C, blot to the right). Indeed a 130 kD band was found only in the presence of VEGF<sub>165</sub> and overlapped with the band identified by the VEGF antisera.

Crosslinking experiments were also performed with VEGF<sub>121</sub>, however no interaction was found (Fig. 5D). These results indicated that the C-terminal heparin-binding domain of VEGF participates in an interaction with ADAMTS1.

### ADAMTS1 and VEGF form complexes *in vivo*

The *in vitro* data implied that one possible mechanism for ADAMTS1 activity is binding and inactivation of VEGF<sub>165</sub> function. Therefore, we investigated whether ADAMTS1 and VEGF formed complexes *in vivo*. The experiments were performed using xenograft tumor lysates from T47D mammary carcinoma cells expressing either ADAMTS1 or vector alone (pCDNA). As previously described, we found that biochemical evaluation of VEGF from tissues/tumors reveals multiple bands immunoreactive with several independent VEGF antibodies (Fig. 6A) (39, 40), representing different VEGF forms (17, 41) as well as possible complexes formed with other proteins (42-45). Comparison between control and ADAMTS1 expressing tumors revealed a novel VEGF immunoreactive species of approximately 130 kD present exclusively in the ADAMTS1 tumor lysates (Fig. 6A). The 130 kD band co-migrated and

overlapped with a species recognized by ADAMTS1 antibodies (Fig. 6A). Furthermore, VEGF was detected in immunoprecipitates of ADAMTS1 from tumor lysates, whereas no VEGF was detected in lysates from pCDNA tumor tissue treated in an identical manner (Fig. 6B). These *in vivo* results corroborate the *in vitro* finding that ADAMTS1 and VEGF form a stable complex.

#### Defining domains of ADAMTS1 involved in VEGF<sub>165</sub> binding

To determine the domain(s) of ADAMTS1 that participate in the interaction with VEGF<sub>165</sub>, we used several deletion constructs (Fig. 7A). In these experiments, only the full length P87-ADAMTS1 was able to bind to VEGF<sub>165</sub> (Fig. 7B). Levels of each deletion protein were determined by Western blot analysis in a parallel experiment (Fig. 7C). VEGF appears as a rather diffused and wide band. It is pertinent to the interpretation of these experiments to clarify that the VEGF antibodies have a higher titer than the ADAMTS1 antibodies, with a limit of detection of approximately 10 pg (VEGF) versus 500 pg in the case of ADAMTS1 antibodies (data not shown).

#### VEGF<sub>165</sub> binds to the CT region of ADAMTS1

Previous results indicated that the interaction of VEGF<sub>165</sub> to ADAMTS1 requires the two last TSP repeats and at least part of the spacer region. To test this possibility, we generated a deletion construct consisting of the CT of ADAMTS1 from the 1<sup>st</sup> TSP repeat to the last residue (including the spacer region) (Fig. 8A). Figure 8B shows the relative expression levels of several clones which showed a truncated protein of approximately 50 kD. We found that the protein bound avidly to the cell surface of expressing cells and was only released when in the presence of heparin (Fig. 8B). As anticipated, the CT region of ADAMTS1 binds to VEGF<sub>165</sub>,

## ADAMTS1 binds to VEGF<sub>165</sub>

resulting in a 90 kD crosslinked complex (Fig. 8C). Purification of significant levels of the CT region for evaluation on angiogenesis have been hindered due to its avidity to bind matrix proteins. However we have evaluated the function of this domain in phosphorylation assays and found significant decrease of VEGFR2 phosphorylation on VEGF<sub>165</sub>-stimulated PAE-VEGFR2 in the presence of the CT truncated protein as well as full length of ADAMTS1 (Fig. 8D).

## Discussion

The results presented in this study support our previous finding that ADAMTS1 is angiogenic-inhibitory and demonstrate that this activity is due, at least in part, to its CT domain. In that region, ADAMTS1 contains three motifs that share significant homology to the antiangiogenic domain of TSP1 and a cysteine-rich region (18). It has been reported that these motifs in TSP1 and in connective tissue growth factor bind to VEGF<sub>165</sub> and negatively modulate VEGF function on endothelial cells (33, 34).

VEGF<sub>165</sub> is one of the most specific mediators of tumor angiogenesis (46-48). The importance of the growth factor and its receptor VEGFR2 to pathophysiological states has been exemplified by studies using dominant-negative VEGFR2 (48), VEGFR2 kinase inhibitors (49), and neutralizing VEGF and VEGFR2 antibodies (47, 50, 51). In all cases, suppression of VEGF signaling resulted in inhibition of angiogenesis and concomitant reduction of tumor burden. Moreover, overexpression of VEGF and VEGFR2 is associated with invasion and metastasis of human cancers (52, 53), further demonstrating the impact of this growth factor in tumor biology.

As a paracrine mediator, one of the rate-limiting steps in VEGF signaling relates to its ability to reach the target cell. The rate of diffusion of VEGF is greatly impacted by the presence of a heparin-binding domain in its CT. Splice variants of VEGF that lack this domain, such as VEGF<sub>121</sub>, have been shown to interact poorly with the extracellular environment and have a greater diffusion rate (54). Thus, the balance between the selective anchorage of VEGF by extracellular matrix proteins and its programmed release by matrix metalloproteases regulates the level of growth factor bioavailability and function. Consequently, alterations in either matrix-binding proteins or secretion of proteases have been shown to directly impact VEGF function (23, 55).

## ADAMTS1 binds to VEGF<sub>165</sub>

Our results demonstrate that ADAMTS1 binds to VEGF<sub>165</sub>, as indicated by cross-linking and co-immunoprecipitation studies, and that this binding prevents the association of this growth factor with its receptor, affecting VEGFR2 phosphorylation and endothelial cell proliferation. The interaction of ADAMTS1 with VEGF<sub>165</sub> occurs under culture conditions and is maintained through purification procedures. VEGF<sub>165</sub> was detected in purified samples of ADAMTS1 after Zn<sup>2+</sup>-chelation chromatography, but not in control samples subjected to the same purification scheme. ADAMTS1 protein purified in this manner was still quite effective in the suppression of angiogenesis, as previously demonstrated (18, 21). Our data indicates that VEGF is present in purified ADAMTS1 from 293T conditioned media. The levels of VEGF are not equimolar to ADAMTS1 and addition of exogenous VEGF results in more binding and inactivation of the growth factor. Also, we were unable to detect VEGF using silver staining of purified ADAMTS1 preparations. Consequently, ADAMTS1 is likely more potent than previously anticipated (18). Evaluation of the ADAMTS1-VEGF<sub>165</sub> complex in VEGFR2 phosphorylation assays indicated no activity, demonstrating that once bound, VEGF<sub>165</sub> is unable to activate its receptor. Using gel-filtration chromatography under denaturing conditions we have been able to separate both proteins and determine that the resulting VEGF<sub>165</sub> is active, demonstrating that the reversibility of this interaction has the potential to impact biological processes.

Interestingly, we found that ADAMTS1 did not bind to VEGF<sub>121</sub>, indicating that the interaction requires the heparin-binding domain. Kuno and colleagues have found that ADAMTS1 binds to the extracellular matrix through the spacer/cysteine rich region (20, 56). We also found that the association of ADAMTS1 with cell surfaces and matrix can be blocked/competed by heparin (21). The cross-linking results suggests that heparin might be acting as a bridge between ADAMTS1 and VEGF. The function of heparin as a chaperone has

been previously reported in similar protein-protein interactions (4, 57). *In vivo*, however, the interaction of ADAMTS1 to VEGF is not dependent on exogenous addition of heparin indicating that is either not necessary or that another heparin-like molecule, such a heparan-sulfate proteoglycan i.e. syndecan, could be the physiological mediator of this interaction.

The results presented in this study argue that the CT region of ADAMTS1 is responsible, at least in part, for the anti-angiogenic properties displayed by this molecule. Interestingly, we have previously reported that this protein is processed extracellularly resulting in the release of the CT end (21), this processing does not affect the catalytic function of the protease. Thus, ADAMTS1 processing might release two fragments with distinct/independent functions in the extracellular milieu and add to the list of extracellular matrix molecules whose functional features are multiplied by processing events. The contribution of the catalytic domain to the angio-inhibitory properties of ADAMTS1 is the subject of continuing work and is likely to be additive to the vascular inhibitory properties displayed by the C-terminal domain that we described here.

At this point, it is unclear whether the CT region in ADAMTS1 binds and modulate other growth factors. Thrombospondin1 has been shown to bind to several growth factors contributing to the regulation of many biological processes (22, 58-60). Although the murine ADAMTS1 protein has the RFK tripeptide known to activate TGF-beta in TSP1, the human ADAMTS1 homolog does not show conservation of this activating motif. However, based on extensive flanking sequence homology it is likely ADAMTS1 binds to TGF-beta, a possibility that deserves examination.

The present study has extended and provided clarification to the mechanism by which ADAMTS1 acts to suppress angiogenesis. However, the overall contribution of ADAMTS1 as a

physiological endogenous inhibitor remains to be fully clarified. Our previous results have indicated that added exogenously, ADAMTS1 is capable of blocking neovascularization *in vivo* and suppressing endothelial cell proliferation *in vitro* more effectively than recognized inhibitors, such as TSP1 and endostatin. These findings have to be reconciled, however, with the fact that loss of function mutations of ADAMTS1 in mice results in pathological states not directly linked to the vascular compartment (61). Although an extensive evaluation of the null animal with focused attention in angiogenic states is currently missing, it may not be surprising that ADAMTS1 functions in a multiplicity of biological scenarios with either partially overlapping or entirely independent functions. This has been the case for other extracellular matrix proteins and proteases, including TSP1 and TSP2, both recognized physiological inhibitors of neovascularization (62, 63). Studies that aim to determine the mechanism of action of ADAMTS1 and/or the TSP repeats are likely to enhance our understanding of the biology of angiogenesis and provide a platform for the development of drugs for pharmacological intervention in situations of unwanted neovascular growth.

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**Figure Legends****Figure 1. ADAMTS1 inhibits the mitogenic effect of VEGF<sub>165</sub> and dampens VEGFR2 phosphorylation**

A) ADAMTS1 inhibits VEGF<sub>165</sub> stimulated endothelial cell proliferation. BAEC were synchronized in G<sub>0</sub> by serum starvation (48 h) post confluence. Cells were seeded and stimulated with VEGF<sub>165</sub> (25 ng/ml), FGF-2 (2 ng/ml) or a combination of both, in the presence or absence of recombinant ADAMTS1 (5 µg/ml). Treatment was performed for 24 h. During the last 8 h, a pulse of 1 µCi/well of [<sup>3</sup>H]thymidine was added. Incorporated counts were determined by liquid scintillation. All counts were normalized to maximum stimulation (VEGF<sub>165</sub> and FGF-2). B) ADAMTS1 partially blocks VEGFR2 phosphorylation in VEGF<sub>165</sub> activated PAE-VEGFR2. PAE-VEGFR2 cells were stimulated with VEGF<sub>165</sub> (25 ng/ml) for 6 minutes in the presence of conditioned media from 293T cells expressing ADAMTS1 or vector alone. Phosphorylated proteins were visualized using the anti-phosphotyrosine polyclonal Ab. The membrane was stripped and reprobed with anti-VEGFR2. C) ADAMTS1 decreases VEGFR2 phosphorylation in VEGF<sub>165</sub> stimulated BAEC. BAEC were stimulated with VEGF<sub>165</sub> (50 ng/ml) as described in (B). Anti-enolase was used as loading control. Insert shows VEGFR2 levels of the same samples resolved in a parallel Western blot. D) ADAMTS1 regulates increasing VEGFR2 phosphorylation stimulated by increasing concentrations of VEGF<sub>165</sub>. BAEC were incubated with increasing concentration of VEGF<sub>165</sub> (0 to 50 ng/ml) in the presence or absence of ADAMTS1 conditioned media. Phosphoproteins were immunoprecipitated and later analyzed by Western blot using the same antibody. In each case, arrows point to phosphorylated VEGFR2 (P-VEGFR2), VEGFR2 and enolase.

**Figure 2. ADAMTS1 impairs binding of <sup>125</sup>I-VEGF<sub>165</sub> to endothelial cells**

A) Binding of <sup>125</sup>I-VEGF<sub>165</sub> to HAEC. Indicated concentrations of <sup>125</sup>I-VEGF<sub>165</sub> (2, 6, 12 and 16 ng/ml) were added to HAEC cultures. After incubation (1h at 4°C), cells were washed, solubilized in lysis buffer and counted in a gamma counter. Non-specific binding was calculated in the presence of 20-fold excess of cold VEGF<sub>165</sub>. Specific binding (circles) was determined by subtracting nonspecific binding (triangles) from total binding (squares). B) Specific binding of <sup>125</sup>I-VEGF<sub>165</sub> to endothelial cells expressing VEGFR2. PAE or PAE-VEGFR2 were incubated with indicated concentrations of <sup>125</sup>I-VEGF<sub>165</sub> and the specific binding to the cell surface was determined as described above. C) Purified ADAMTS1 decreases the binding of <sup>125</sup>I-VEGF<sub>165</sub> to the endothelial cell surface. HAEC were incubated with <sup>125</sup>I-VEGF<sub>165</sub> (16 ng/ml) in binding buffer in presence (+) or absence (-) of ADAMTS1. The insert shows total binding (<sup>125</sup>I-VEGF) and nonspecific binding (+cold VEGF).

**Figure 3. Endogenous VEGF<sub>165</sub> co-purifies with ADAMTS1**

A) VEGF<sub>165</sub> co-purifies with ADAMTS1. ADAMTS1 was purified by heparin (H) or Zn<sup>2+</sup>-chelate affinity chromatography from conditioned media of 293T cells expressing ADAMTS1. 7 µg of purified recombinant protein was resolved by SDS-PAGE under non-reducing conditions and evaluated for the presence of VEGF<sub>165</sub> by Western blot. Arrows indicate different forms of VEGF<sub>165</sub>. After stripping of the membrane, levels of purified ADAMTS1 were assessed by Western blot (MAb 5D4E11B5). B) VEGF<sub>165</sub> was purified by heparin affinity chromatography in the absence of ADAMTS1. Conditioned media from 293T cells transfected with vector alone in presence of soluble heparin (5 µg/ml) was purified by heparin affinity chromatography. Peaks corresponding to different elution steps ([NaCl]) were resolved by SDS-PAGE and evaluated by

## ADAMTS1 binds to VEGF<sub>165</sub>

Western blot for presence of VEGF. C) VEGF<sub>165</sub> was not purified by Zn<sup>2+</sup> chromatography in the absence of ADAMTS1. Conditioned media from 293T cells transfected with vector alone was subjected to Zn<sup>2+</sup>-chelate affinity chromatography. Peaks corresponding to samples eluted with different concentration of EDTA were resolved by SDS-PAGE and analyzed by immunoblot for VEGF. In all cases purified VEGF<sub>165</sub> was used as control (C).

### Figure 4. VEGF phosphorylates VEGFR2 after dissociation from ADAMTS1

A) Dissociation of interacting ADAMTS1-VEGF<sub>165</sub>. Purified ADAMTS1 complexed to VEGF<sub>165</sub> (sample 3 or 7 from Fig. 3A) was subjected to denaturing conditions and dissociated proteins were separated by gel permeation chromatography. Eluted samples were analyzed for the presence of VEGF<sub>165</sub> under reducing conditions by Western blot techniques. After stripping the membrane, levels of ADAMTS1 were assessed using the antibody 5D4E11B5. B) Dissociated VEGF<sub>165</sub> phosphorylates VEGFR2. PAE-VEGFR2 cells were stimulated with VEGF (50 ng/ml), purified ADAMTS1 (ADAMTS1 complexed to VEGF<sub>165</sub>), samples 6 (ADAMTS1) and 13 (VEGF<sub>165</sub>) from size exclusion chromatography (A). Proteins were resolved by SDS-PAGE and phosphorylated proteins were visualized using the anti-phosphotyrosine sera. The membrane was stripped and reprobred with anti-enolase to assess protein levels. SM, starting material; C, control; V, VEGF; A, purified ADAMTS1.

### Figure 5. ADAMTS1 binds to VEGF<sub>165</sub>.

A) ADAMTS1 immunocomplexes bind to VEGF<sub>165</sub>. Immunoconjugates generated by immunoprecipitation from conditioned media of 293 T cells expressing ADAMTS1 or the pCDNA vector alone were incubated with soluble ligands: VEGF<sub>165</sub> (50ng) in presence or

## ADAMTS1 binds to VEGF<sub>165</sub>

absence of heparin (50ng). The conjugates were evaluated for the presence of bound VEGF<sub>165</sub> by immunoblotting under non-reducing conditions. Arrows indicate immunoreactive VEGF<sub>165</sub> oligomers. Presence and relative levels of P87-ADAMTS1 were assessed by Western blot (insert). B) Crosslinking analysis of ADAMTS1 and VEGF<sub>165</sub>. Purified ADAMTS1 and VEGF<sub>165</sub> were incubated in the presence or absence of heparin. Bound proteins were crosslinked and complexes were analyzed under non-reducing conditions. Arrows indicate shifted VEGF<sub>165</sub> species. Arrowheads indicate the migration of VEGF<sub>165</sub> in the absence of ADAMTS1. C) ADAMTS1 from conditioned media supernatants binds to VEGF<sub>165</sub>. Conditioned media from 293T cells expressing ADAMTS1 or pCDNA were incubated in presence or absence of VEGF<sub>165</sub> and crosslinked. Complexes were evaluated under reducing conditions and probed with anti-VEGF antibodies. Levels of ADAMTS1 were evaluated by reprobing the same membrane with MAb 5D4E11B5. Arrow indicates the species recognized by both antibodies. D) ADAMTS1 does not bind to VEGF<sub>121</sub>. Complexes were analyzed under non-reducing conditions. Arrow indicates the shifted VEGF<sub>165</sub> band. Levels of ADAMTS1 were evaluated with the MAb 5D4E11B5. A1, ADAMTS1; Ctrl, Control.

### Figure 6. ADAMTS1 and VEGF form complexes *in vivo*

A) ADAMTS1 binds to VEGF<sub>165</sub> *in vivo*. Tumor lysates (150µg) from T47D xenografts expressing either ADAMTS1 or empty vector (pCDNA) were separated by SDS-PAGE under non-reducing. VEGF<sub>165</sub> was visualized by immunoblot techniques using a C-terminal specific anti-VEGF antibody. Expression of ADAMTS1 was evaluated reprobing the same membrane with a cocktail of MAbs (3B12F6, 3C8F8, 4C2C4 and 5C6D5). Arrows indicate overlapping proteins, VEGF<sub>165</sub> and ADAMTS1, in ADAMTS1 xenografts samples not present in control

tumors. Anti-enolase sera was used to assess loading levels. B) VEGF<sub>165</sub> co-immunoprecipitates with ADAMTS1 in xenograft tumors. ADAMTS1 was immunoprecipitated from control and ADAMTS1 xenograft tumor lysates using the MAb 5C6D5. Presence of co-immunoprecipitated VEGF was tested by Western blot using a C-terminal anti-VEGF antibody. Immunoprecipitated ADAMTS1 was visualized with 5C6D5 and 3C8F8 antibodies. Ctrl, Control tumor; A1, ADAMTS1 tumor.

**Figure 7. VEGF<sub>165</sub> binding requires the CT half of ADAMTS1**

A) Schematic representation of ADAMTS1 constructs. Structural motifs include: (M) Metalloprotease domain, (D) Disintegrin domain, (T) TSP repeat and (SR) Spacer region. Numbers correspond to amino-acids considering Methionine in position #1. B) VEGF<sub>165</sub> binds to full length ADAMTS1. Subconfluent T47 D cells expressing ADAMTS1 deletion constructs (Full length, FL; Truncated, T; Met-Dis, M-D; Met, M; and 1<sup>st</sup> TSP repeat, 1<sup>st</sup>) or vector alone (P) grown on 24 well plates were incubated in serum-free media in presence or absence of VEGF<sub>165</sub> and heparin. Conditioned media was crosslinked and subjected to SDS-PAGE under non-reducing conditions and VEGF<sub>165</sub> patterns were evaluated by Western blot. C) Equivalent levels of each construct were present as assessed by immunoblot with indicated antibodies.

**Figure 8. VEGF<sub>165</sub> binds to the CT region of ADAMTS1**

A) Schematic representation of all the ADAMTS1 constructs used (Fig. 7 and 8) and binding properties to VEGF<sub>165</sub>. B) Characterization of T47D clones expressing the CT (CT) region of ADAMTS1. Western blot from 24 h conditioned media in presence or absence of heparin from T47 D clones (clone 5, 32, 39 and 41). A cocktail of MAbs (3B12F6, 3C8F8, 4C2C4 and

## ADAMTS1 binds to VEGF<sub>165</sub>

5C6D5) was used to detect the protein. T47D cells transfected with vector alone (C) were used as negative control. C) VEGF<sub>165</sub> binds to the CT region of ADAMTS1. T47 D cells expressing vector alone (C), ADAMTS1 full length (A1) or the CT construct of ADAMTS1 (5, 39 and 41) were incubated in presence or absence of VEGF<sub>165</sub> and heparin. After crosslinking, VEGF<sub>165</sub> patterns were evaluated by Western blot. Arrow indicates shifting of VEGF<sub>165</sub> produced by the binding to the CT protein and the arrowhead indicates the shifting due to binding to the full length. Relative ADAMTS1 and CT protein levels were assessed by Western blot. D) The CT protein alone was sufficient to reduce VEGFR2 phosphorylation. PAE-VEGFR2 cells were stimulated with VEGF<sub>165</sub> in the presence of conditioned media from T47D cells expressing ADAMTS1, CT or vector alone. Equal amounts of cell lysates were evaluated for phosphorylated proteins by Western blot analysis. The membrane was stripped and reprobed with anti-VEGFR2 and anti-enolase to assess protein levels.

## Tables

MAbs against ADAMTS1							
#	MAbs	Titer by ELISA	I.P.	W.B.		I.F.	
				N.R.C.	R.C.	Formaldehy.	Methanol
1	1H12G8	0.868	110/87/65	110/87/65	NEG	++	+
2	2G7F10	0.726	87/65	NEG	NEG	++	-
3	3B12F6	0.624	110/87	87	NEG	++	+
4	3C8F8	0.878	110/87	110/87	NEG	++	++
5	3E4C6B4	1.025	110/87/65	110/87/65	-65	++	++
6	3E7G10	0.861	110/87/65	110/87/65	NEG	++	+/-
7	3G3A6B11	0.947	110/87/65	NEG	NEG	++	+/-
8	4A11C7	0.726	110/87/65	110/87/65	NEG	++	++
9	5C6D5	0.899	110/87	110/87	NEG	++	++
10	5D4E11B5	0.470	NEG	110/87/65	110/87/65	+	-
11	5D3H3	0.980	110/87/65	110/87/65	NEG	ND	ND
12	4C2C4	1.358	110/87	87	NEG	ND	ND
+	Mouse serum	1.755	110/87/65	110/87/65	110/87/65	ND	ND
-	Irrelevant IgG	0.093	NEG	NEG	NEG	NEG	NEG

**Table I. Characterization of MAbs against ADAMTS1.** ELISA data shows the reactivity of the hybridoma culture supernatant to purified ADAMTS1 immobilized on microassay plates. Numbers represent measured absorbance at 405 nm after the color reaction was developed using a secondary antibody coupled to alkaline phosphatase and p-Nitrophenyl phosphate as substrate. 110/87/65 correspond to the ADAMTS1 forms recognized by the MAbs tested on immunoprecipitation (I.P.) and Western blot (W.B.) under reducing (R.C.) and nonreducing (N.R.C.) conditions. Also evaluated was the ability of the antibodies to recognize the protein after fixation on 3% formaldehyde (Formaldehy.) or 2% methanol by immunofluorescence (I.F.). ND=Not Done; NEG=Negative

Figure 1)

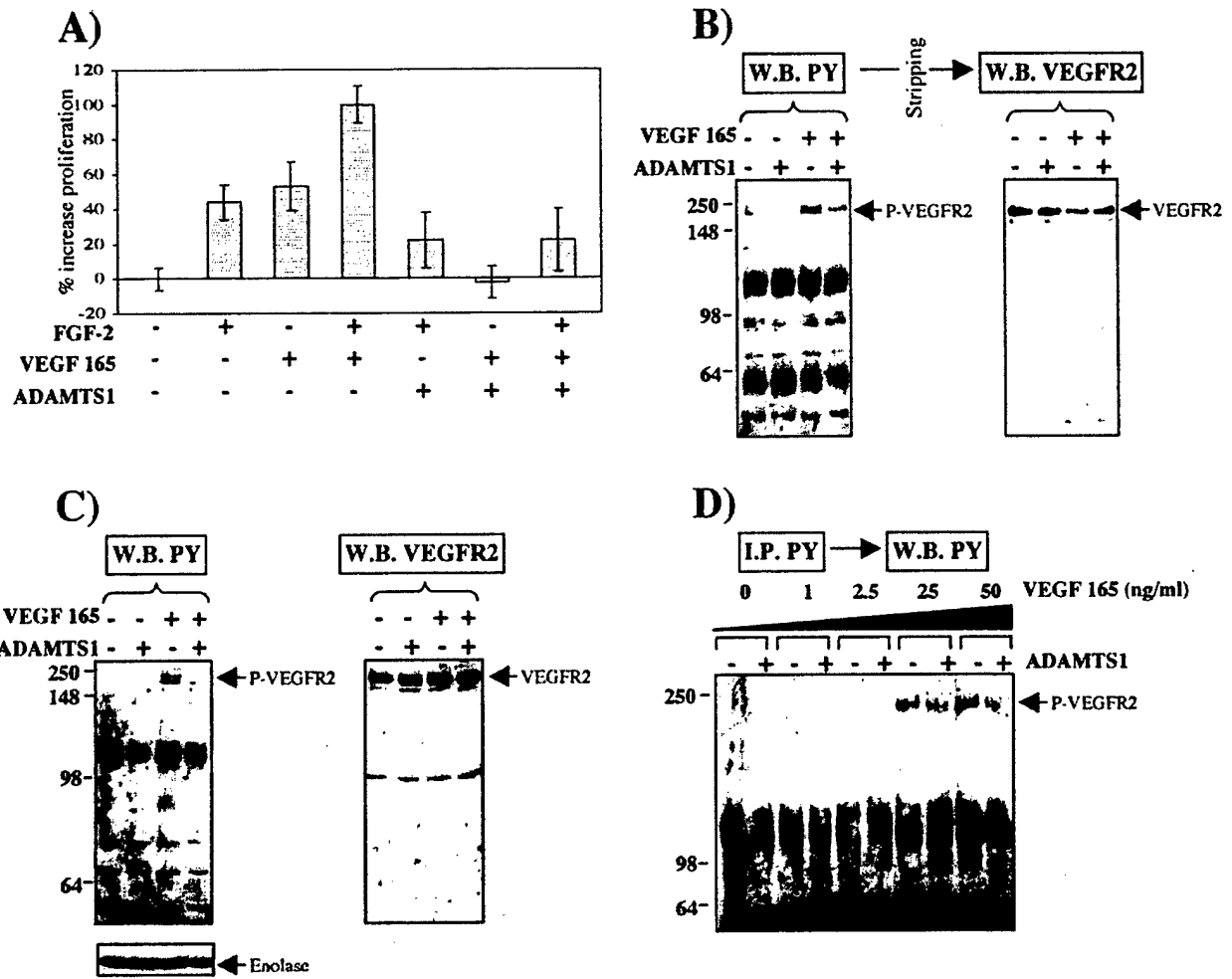


Figure 2)

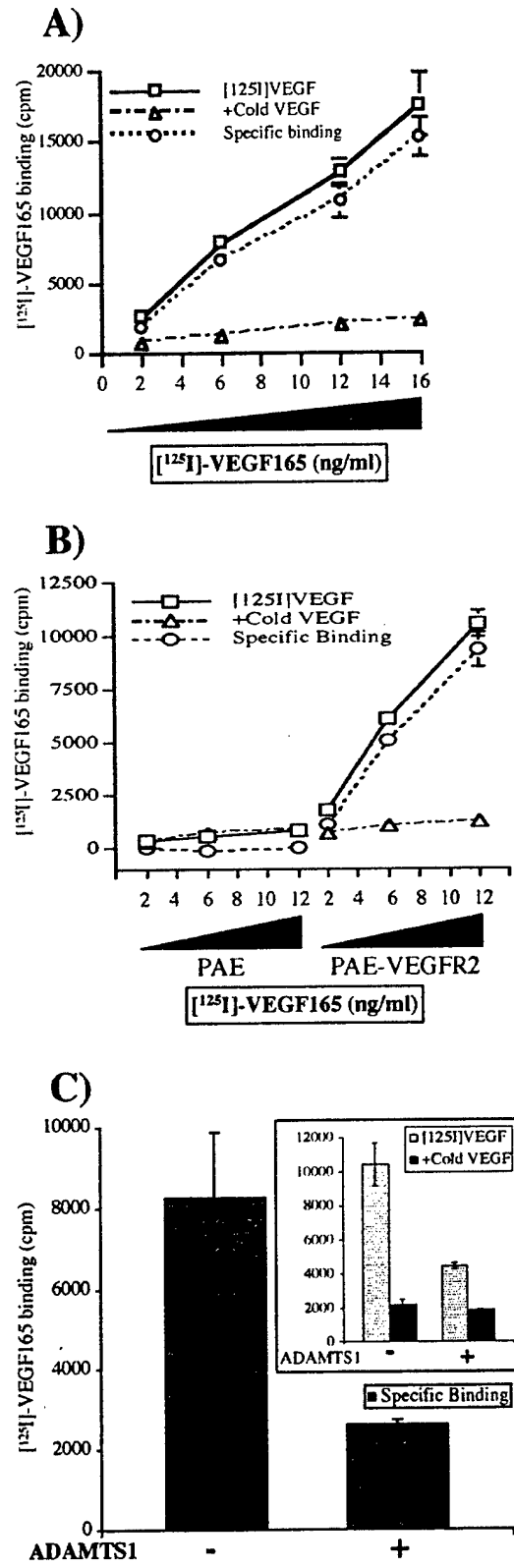


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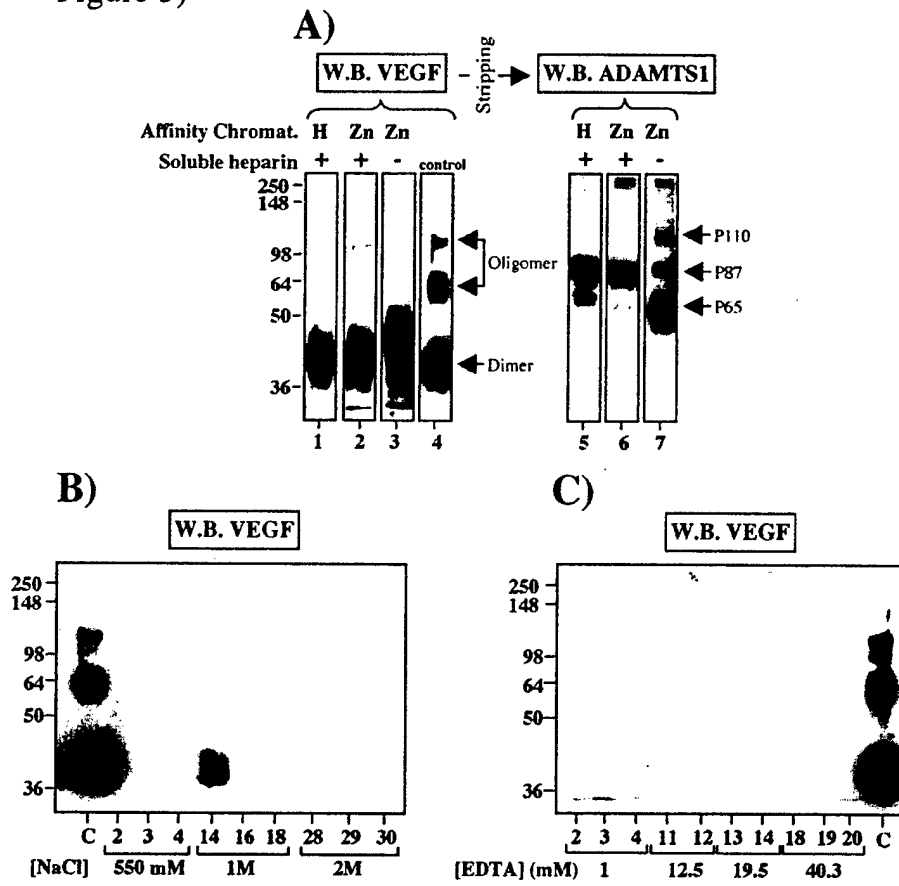


Figure 4)

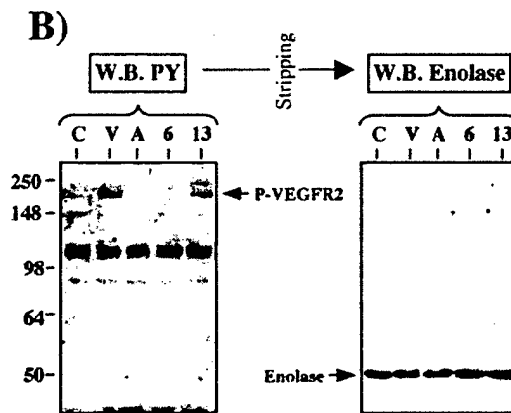
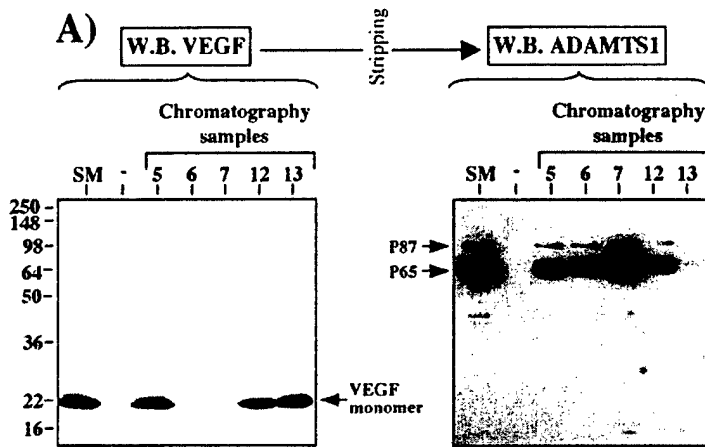


Figure 5)

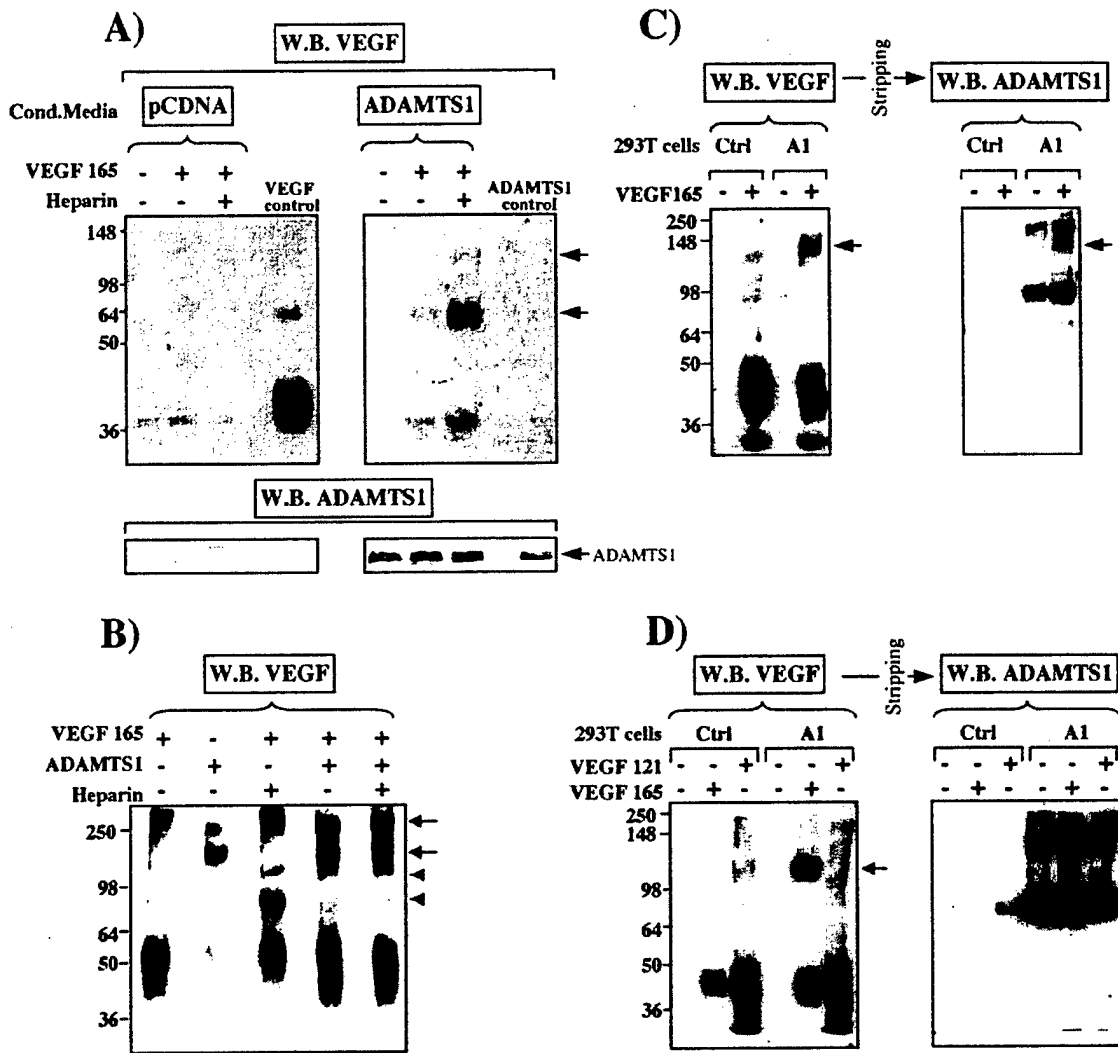


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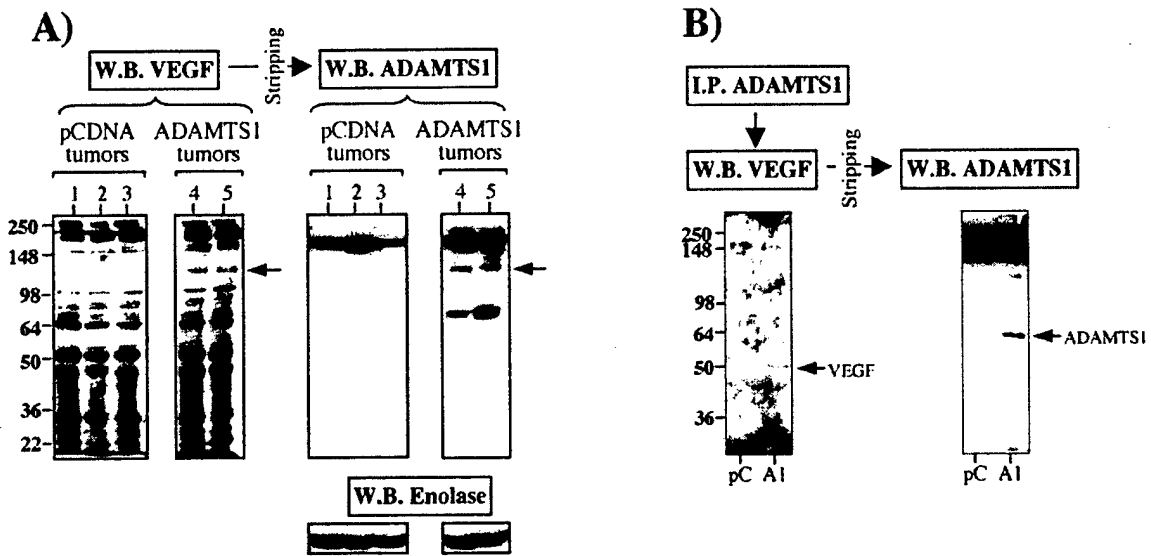
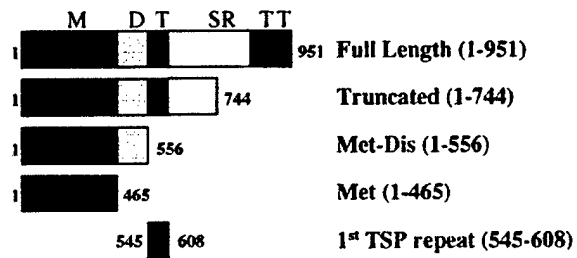
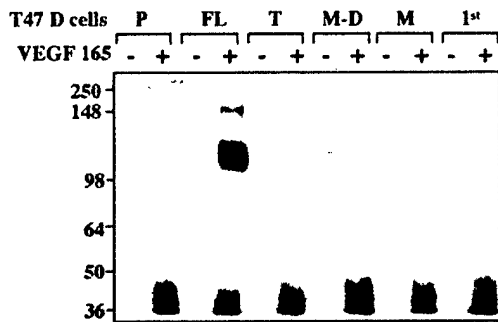


Figure 7

A)



B)



C)

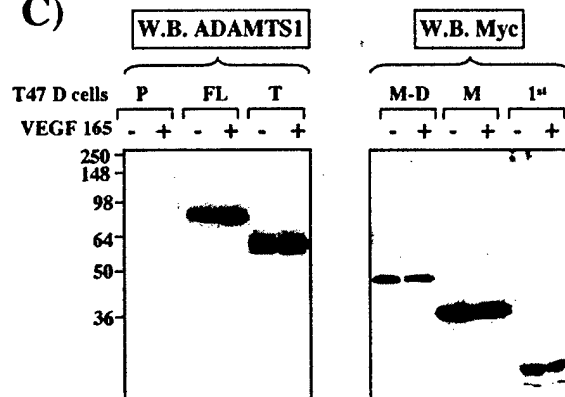


Figure 8

