

REPUBLIC OF SOUTH AFRICA  
PATENTS ACT, 1978**PUBLICATION PARTICULARS AND ABSTRACT**

(Section 32(3)(a) – Regulation 22(1)(g) and 31)

OFFICIAL APPLICATION NO.

LODGING DATE

ACCEPTANCE DATE

21 06 2004/7373

22 17 MAR 2003

43 14 - 9 - 05

INTERNATIONAL CLASSIFICATION

NOT FOR PUBLICATION

51

A61K

CLASSIFIED BY: WIPO

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EARLIEST PRIORITY CLAIMED

COUNTRY

33 US

NUMBER

31 60/364,047

DATE

32 15 MAR 2002

TITLE OF INVENTION

54

CELL SURFACE TROPOMYOSIN AS A TARGET OF ANGIOGENESIS INHIBITION

57

ABSTRACT (NOT MORE THAN 150 WORDS)

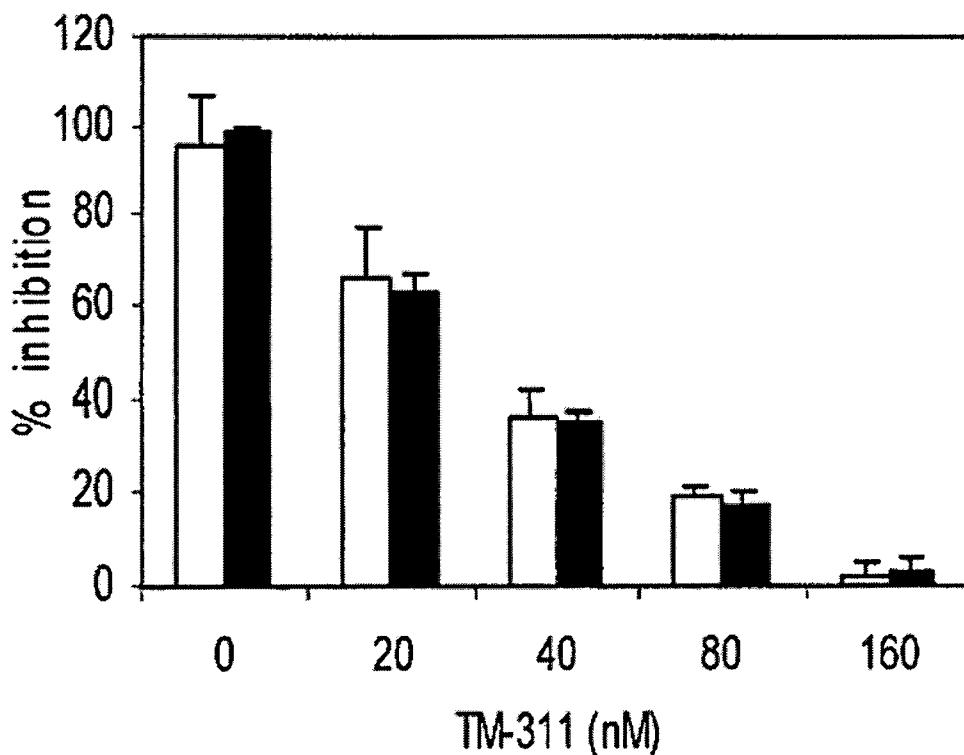
NUMBER OF SHEETS

118

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The figure of the drawing to which the abstract refers is attached.

## ABSTRACT

The present invention is directed to novel methods for inhibiting angiogenesis and treating tumors and cancer by targeting tropomyosin (Tpm) expressed on the surface of endothelial cells and/or tumor cells, to Tpm polypeptides and peptides, as well as variants and derivatives thereof that bind inhibitors of angiogenesis, and to anti-Tpm antibodies that block or stimulate angiogenesis. Cyclic peptides that bind to the D5 subunit of HK<sub>a</sub> and inhibit angiogenesis are also included. Method for screening test compounds as candidate antiangiogenic molecule that binds to Tpm are disclosed, as are affinity ligands comprising the proteins, peptides, variants and derivatives of the invention.



## CELL SURFACE TROPOMYOSIN AS A TARGET OF ANGIOGENESIS INHIBITION

### BACKGROUND OF THE INVENTION

#### Field of the Invention

The present invention in the field of biochemistry and medicine is directed to novel methods for inhibiting angiogenesis and treating tumors and cancer by targeting tropomyosin expressed on the surface of endothelial cells and/or tumor cells, to tropomyosin polypeptides and peptides that bind inhibitors of angiogenesis, and to anti-tropomyosin antibodies that block or stimulate angiogenesis.

#### Description of the Background Art

Angiogenesis, the formation of new capillaries from pre-existing ones (Folkman, J., *N. Engl. J. Med.*, 1971, 285:1182-1186; Hanahan D. *et al.*, *Cell*, 1996, 86:353-364), is a normal part of embryonic development, wound healing and female reproductive function. However, angiogenesis also plays a pathogenic role in the establishment and progression of certain diseases. Cancer, rheumatoid arthritis and diabetic retinopathy are examples of such diseases (Carmeliet P. *et al.*, *Nature*, 2000, 407:249-257). Anti-angiogenic therapy holds promise in inhibiting the progression of these diseases.

Angiogenesis can be triggered by several pro-angiogenic cytokines. In the setting of cancer, tumor cells under hypoxic conditions secrete vascular endothelial growth factor (VEGF) and/or fibroblast growth factor (bFGF). These proteins diffuse and bind to specific receptors on endothelial cells (ECs) in the local vasculature, perturbing the balance of pro- and anti-angiogenic forces in favor of angiogenesis. As a consequence of binding these proteins, ECs are activated to (a) secrete enzymes that induce remodeling of the associated tissue matrix, and (b) change the patterns and levels of expression of adhesion molecules such as integrins. Following matrix degradation, ECs proliferate and migrate toward the hypoxic tumor, resulting in the generation and maturation of new blood vessels.

Interestingly, many anti-angiogenic factors result from the degradation of matrix proteins – *i.e.*, are a result of the action of pro-angiogenic enzymes. Examples include endostatin, a fragment of collagen XIII (O'Reilly, M. S. *et al.*, *Cell* 1997, 88:277-285); kringle 5 of

plasminogen (O'Reilly, M. S. *et al.*, *Cell*, 994, 79:315-328) and PEX, the C-terminus non-catalytic subunit of MMP-2 (Brooks P.C. *et al.*, *Cell*, 1998, 92:391-400).

The concept has emerged that, due to the abundance of pro-angiogenic factors, these anti-angiogenic molecules are unable to overcome the pro-angiogenic balance in a primary tumor. However, since they are secreted into circulation, these anti-angiogenic molecules are capable of inhibiting angiogenesis at other locations where tumor cells may have begun to invade. Consequently, micro-metastases comprising these tumor cells at these new locations remain dormant. This hypothesis explains the puzzling observation made by surgeons many years ago: at various times after surgical removal of a primary tumor in a patient with no obvious metastatic disease, the patient returns with advanced metastatic disease.

Thus, clinical intervention by treatment with one or more of the anti-angiogenic factors could inhibit the angiogenic process and halt tumor growth as well as metastasis. Significant evidence in the literature (cited above) supports this notion.

Unregulated angiogenesis contributes to the pathology of not only many neoplastic diseases but also a number of non-neoplastic diseases associated with abnormal neovascularization including arthritis, various ocular disorders, and psoriasis. See, for example, Moses *et al.*, 1991, *Biotech.* 9: 630-634; Folkman *et al.*, 1995, *N. Engl. J. Med.*, 333:1757-1763; Auerbach, R *et al.*, 1985, *J. Microvasc. Res.* 29:401-411; Folkman, 1985, *Adv Canc Res* 43:175-203; Patz, A, 1982, *Am. J. Ophthalmol.* 94:715-743; Patz, A, 1982, *Am. J. Ophthalmol.* 94:552-554. Maintenance of the avascularity of the cornea, lens, and trabecular meshwork is crucial for vision as well as to normal ocular physiology. A number of ocular diseases, some of which lead to blindness, result from ocular neovascularization and include diabetic retinopathy, neovascular glaucoma, ocular inflammatory diseases and ocular tumors (*e.g.*, retinoblastoma). Other eye diseases which are associated with neovascularization, including retrobulbar fibroplasia, uveitis, retinopathy of prematurity, and macular degeneration. About twenty eye diseases are associated with choroidal neovascularization and about forty with iris neovascularization (Waltman DD *et al.*, 1978, *Am. J. Ophthal.* 85:704-710 and Gartner, S. *et al.*, 1978, *Surv. Ophthal.* 22:291-312. Current treatments of these diseases, especially once neovascularization has occurred, are frequently inadequate to stave off blindness. Studies have suggested that vasoinhibitory factors which are present in normal ocular tissue (cornea and vitreous) are lost in the diseased states.

### Tropomyosin (Tpm)

Tropomyosin (Tpm) was first discovered in skeletal muscle in 1948 (Bailey, K., *Biochem. J.* (1948) 43:271-279). Tpm binds the troponin complex, thereby playing a role in the modulation of  $\text{Ca}^{+2}$ -regulated muscle contraction (Perry SV, *J Muscle Res. Cell Motil.* (2001) 22:5-49; Squire JM *et al.*, *FASEB J.* (1998) 12:761-71). Tpm was later found to be an ubiquitous protein (Lin JJ *et al.*, *Int. Rev. Cytol.* (1997) 170:1-38) that is found in vertebrate non-muscle cells in many isoforms arising from four different genes as a consequence of alternative splicing and differential promoter regulation (Lin *et al.*, *supra*). It binds actin, a component of the actin cytoskeleton that is involved in maintaining cell integrity and in the processes of motility, cytokinesis and exocytosis. As many other coiled coil proteins, Tpm's sequence comprises repetitions of a heptapeptide motif in a  $\alpha$ -helix conformation (Squire *et al.*, *supra*; Lin *et al.*, *supra*). Each unit of the dimer is a rod-like molecule that intertwines with the other.

The first link between Tpm and angiogenesis came from a study testing the hypothesis that endostatin mediates its anti-angiogenic effect by the interaction with intracellular Tpm isoform 3 (hTm3) (MacDonald NJ *et al.*, *J. Biol. Chem.* (2001) 276:25190-196). This study showed binding of endostatin to permeabilized human umbilical vein ECs (HUVEC) by fluorescence microscopy and further characterized the binding of endostatin to hTm3 *in vitro* by Surface Plasmon Resonance (SPR). A Kd of approximately 100  $\mu\text{M}$  was disclosed. This is in apparent contradiction with a significant body of literature showing endostatin biological activity in the nanomolar range (O'Reilly MS *et al.*, *Cell* (1997) 88:277-285; Boehm T *et al.*, *Nature* (1997) 390:404-407). McDonald *et al.* concluded that endostatin is internalized and binds to intracellular Tpm, resulting in disruption of the actin cytoskeleton and leading to apoptosis. However, this explanation appeared unlikely to the present inventors. This reference provides no indication as to the identity of the cell-surface receptor for endostatin. The Kd of binding to immobilized hTm3, said to reflect intracellular binding, does not correspond to the measured biological activity which should properly reflect binding to a relevant receptor. Moreover, there was no suggestion that Tpm was expressed on the cell surface or could serve as a cell surface receptor for endostatin or any other ligand on any type of cell.

Although Tpm is generally known to be an intracellular protein, it has been reported to be the extracellular domain ("ECD") of at least two chimeric oncoproteins that were created by chromosomal translocations: the Trk oncoprotein (Nakagawara A, *Cancer Lett.* (2001) 169:107-14) and in the TPM3-ALK fusion protein (Lamant L *et al.*, *Blood* (1999) 93:3088-9510).

Extracellular dimeric Tpm seemed to permanently activate the tyrosine kinase domain of the fusion protein.

Kesari KV *et al.* (*Clin. Exp. Immunol.* (1999), 118:219-227) studied Tpm isoform 5 (hTM5), an intracellular protein as a putative target autoantigen in ulcerative colitis. hTM5 was found on the surface of colon epithelial cells and cells of the LS-180 colon cancer cell line, but not on small intestinal epithelial cells, in a form loosely associated with the membrane bound colon epithelial protein (CEP). (LS-180 cells spontaneously released hTM5 as well as CEP into the culture medium.) The authors concluded that hTM5 is externalized in colon but not in small intestinal epithelial cells and, based on the physical association with CEP, suggested a possible chaperone function of CEP in the transport of hTM5.

However, any physiological role for Tpm on the cell surface in the limited number of aforementioned instances remains obscure. Moreover, there are no reports in the literature of Tpm residing on the surface of ECs.

### **SUMMARY OF THE INVENTION**

The present invention is directed to an isolated tropomyosin (Tpm)-related anti-angiogenic receptor polypeptide or peptide which,

- (a) is a fragment of a full length native Tpm protein expressed on the surface of endothelial cells or a variant of the fragment,
- (b) has a molecular mass of about 17 kDa and corresponds in its sequence to, or is a variant of, an internal fragment of a native Tpm isoform, preferably a human isoform, which is a binding site for antiangiogenic polypeptide agents, and
- (c) binds to the antiangiogenic polypeptide agents which bind to the native Tpm internal fragment binding site;

wherein the peptide has between about 4 and about 40 amino acids; and the variant of the polypeptide or peptide is a conservative substitution variant of a native Tpm sequence; and the isolated anti-angiogenic receptor polypeptide, peptide or variant has substantially the same biochemical activity of binding to the antiangiogenic polypeptide agents as does the native Tpm internal fragment.

In a preferred embodiment of the above isolated polypeptide, peptide or variant, the native Tpm isoform has an amino acid sequence selected from the group consisting of SEQ ID

NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, and SEQ ID NO:19.

In yet another preferred isolated polypeptide, peptide or variant, the internal fragment of the native Tpm has an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, and SEQ ID NO:20.

The above isolated polypeptide, peptide or variant of claim is preferably one which binds to (a) human histidine-proline rich glycoprotein (HPRG); (b) rabbit HPRG; (c) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of human or rabbit HPRG; (d) two chain human kininogen human kininogen (HK<sub>a</sub>); (e) the D5 domain of HK<sub>a</sub>; or (f) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of the HK<sub>a</sub> or the D5 domain thereof. The above polypeptide, peptide or variant preferably binds to one or more of SEQ ID NO:21, 22, 23, 24, 25 and 26.

When the foregoing is a peptide or peptide variant, it may be capped at its N-terminus, its C-terminus, or both its N- and its C-terminus.

Also provided is a cyclic peptide which of between about 4 and about 20 amino acids which binds to the D5 domain of HK<sub>a</sub> and inhibit angiogenesis in an *in vitro* or *in vivo* assay of angiogenesis.

All the methods summarized herein that rely on a linear antiangiogenic peptide may be practiced with the antiangiogenic cyclic peptide of the present invention.

The invention is also directed to an antibody, preferably a monoclonal antibody (mAb), more preferably a human or humanized mAb, or an antigen-binding fragment (ABF) thereof which antibody is specific for an epitope of a Tpm isoform expressed on the surface an activated endothelial cell, which antibody or ABF has:

- (a) antiangiogenic activity in that it binds to the activated endothelial cell, causing the generation of an antiangiogenic signal in the cell, resulting in (i) inhibition of migration, invasion, proliferation or angiogenesis, or (ii) apoptosis; or
- (b) proangiogenic activity in that it binds, preferably by competitive binding, to Tpm on the endothelial cell and inhibits the binding to the cell of a Tpm -binding antiangiogenic agent, thereby permitting or promoting migration, invasion, proliferation or angiogenesis that would otherwise be inhibited by the antiangiogenic agent.

The above antibody or ABF preferably is specific for an epitope that is present in, or formed by, a polypeptide or peptide of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, or SEQ ID NO:20.

The antibody or ABF is preferably one wherein the Tpm -binding antiangiogenic agent is a) human histidine-proline rich glycoprotein (HPRG); (b) rabbit HPRG; (c) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of human or rabbit HPRG; (d) two chain human kininogen human kininogen (HK<sub>a</sub>); (e) the D5 domain of HK<sub>a</sub>; or (f) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of the HK<sub>a</sub> or the D5 domain thereof.

The antibody of ABF which may be used for detecting a Tpm polypeptide or peptide that serves as an anti-angiogenic receptor on endothelial cells is preferably detectably labeled. A preferred detectable label includes a radionuclide, a PET-imageable agent, an MRI-imageable agent, a fluorescer, a fluorogen, a chromophore, a chromogen, a phosphorescer, a chemiluminescer or a bioluminescer. Preferred radionuclides include of <sup>3</sup>H, <sup>14</sup>C, <sup>35</sup>S, <sup>67</sup>Ga, <sup>68</sup>Ga, <sup>72</sup>As, <sup>89</sup>Zr, <sup>97</sup>Ru, <sup>99</sup>Tc, <sup>111</sup>In, <sup>123</sup>I, <sup>125</sup>I, <sup>131</sup>I, <sup>169</sup>Yb and <sup>201</sup>Tl. Preferred fluorescers or fluorogens include fluorescein, rhodamine, dansyl, phycoerythrin, phycocyanin, allophycocyanin, *o*-phthaldehyde, fluorescamine, a fluorescein derivative, Oregon Green, Rhodamine Green, Rhodol Green and Texas Red.

Also provided is a diagnostically useful Tpm-binding antibody composition comprising the above detectably labeled antibody or ABF and a diagnostically acceptable carrier.

Another embodiment is a therapeutically useful antiangiogenic antibody or ABF that targets Tpm or an epitope thereof and inhibits angiogenesis *in vitro* or *in vivo*; such a composition comprises the above antibody or ABF to which is optionally bound, directly or indirectly, a therapeutically active moiety, such as a radionuclide, a drug or a toxin. radionuclide is selected from the group consisting of <sup>47</sup>Sc, <sup>67</sup>Cu, <sup>90</sup>Y, <sup>109</sup>Pd, <sup>125</sup>I, <sup>131</sup>I, <sup>186</sup>Re, <sup>188</sup>Re, <sup>199</sup>Au, <sup>211</sup>At, <sup>212</sup>Pb and <sup>217</sup>Bi.

A therapeutic antiangiogenic pharmaceutical composition comprises the above antibody (optionally labeled with a therapeutic label) and a pharmaceutically acceptable carrier, preferably in a form suitable for injection. The composition may be one that inhibits angiogenesis *in vitro*, *in vivo*, or both.

Also provided herein is a therapeutically useful proangiogenic antibody or ABF as above (and composition that includes a pharmaceutically acceptable carrier) which antibody targets Tpm or an epitope of Tpm and stimulates angiogenesis *in vitro* or *in vivo*. This therapeutic antibody or pharmaceutical composition is preferably in a form suitable for injection.

The invention also provides a method for inhibiting endothelial cell migration, invasion, proliferation or angiogenesis, or for inducing endothelial cell apoptosis, comprising contacting endothelial cells with an effective amount of a antiangiogenic polypeptide or peptide that binds to Tpm expressed on the surface of activated endothelial cells, and thereby causes the inhibition or the apoptosis.

In this method, the Tpm-binding polypeptide may be (a) human histidine-proline rich glycoprotein (HPRG); (b) rabbit HPRG; (c) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of human or rabbit HPRG; (d) two chain human kininogen human kininogen (HK<sub>a</sub>); (e) the D5 domain of HK<sub>a</sub>; or (f) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of the HK<sub>a</sub> or the D5 domain thereof; (g) troponin T; (h) tropomodulin; (i) caldesmon; (j) actin; (k) calponin; (l) pEL98; (m) glutamic dehydrogenase; and (n) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of any of (g)-(m).

Also provided is a method for treating a subject, preferably human, having a disease or condition associated with undesired cell migration, invasion, proliferation, or angiogenesis, comprising administering to the subject an effective angiogenesis-inhibiting amount of the above pharmaceutical composition. When the disease is one in which the subject has a tumor, the angiogenesis inhibition results in reduction in size or growth rate of the tumor or destruction of the tumor.

Another embodiment is a method for stimulating angiogenesis in a subject in need of enhanced angiogenesis, comprising administering to the subject an effective amount of the present pharmaceutical composition that comprises a proangiogenic antibody or ABF.

Another embodiment is a method for detecting in a biological sample the presence of Tpm of an isoform expressed on the surface of activated endothelial cells, comprising the steps of (a) contacting the sample with the present antibody or ABF in detectably labeled form, and (b) detecting the presence of the label associated with the sample.

An alternate method for detecting the presence of Tpm in a biological sample comprises (a) contacting the sample with the detectably labeled antiangiogenic polypeptide or peptide of the invention that binds to Tpm expressed on the surface of activated endothelial cells; and (b) detecting the presence of the label associated with the sample. Examples of such antiangiogenic polypeptides or peptides include (a) human histidine-proline rich glycoprotein (HPRG); (b) rabbit HPRG; (c) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of human or rabbit HPRG; (d) two chain human kininogen human kininogen (HK<sub>a</sub>); (e) the D5 domain of HK<sub>a</sub>; or (f) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of the HK<sub>a</sub> or the D5 domain thereof.

Samples which may be tested with the above methods include plasma, serum, cells, a tissue, an organ, and an extract of the cells, tissue or organ.

In the foregoing method, the contacting and the detecting may be performed *in vitro*; alternatively, contacting is *in vivo* and the detecting is *in vitro*. The method may also be practiced wherein the contacting is *in vitro* and the detecting is *in vivo*. Finally, both contacting and detecting may be *in vivo*.

The present invention includes a screening test to identify a test compound as a candidate antiangiogenic molecule that binds to Tpm. Such a test comprises:

- (a) adding the test compound to a mixture of a source of Tpm and a Tpm -binding antiangiogenic polypeptide or peptide agent or anti-Tpm antibody, wherein at least one of (i) the Tpm or (ii) the agent or antibody is detectably labeled
- (b) in parallel, mixing similar amounts of the Tpm and the agent or antibody in the absence of the test compound; and
- (c) measuring the binding of the agent with the Tpm in (a) and (b);

wherein, if the binding in (a) is less than the binding in (b), the test is considered positive for the test compound being an inhibitor of the binding, thereby identifying the test compound as a candidate antiangiogenic molecule.

The above screening test may further comprise testing a test compound that has been identified as a candidate antiangiogenic molecule for its activity as an inhibitor of angiogenesis in an *in vitro* or *in vivo* angiogenesis assay.

Also provided herein is an affinity ligand useful for binding to or isolating a Tpm-binding antiangiogenic molecule or cells expressing the binding molecule, comprising the isolated polypeptide or peptides described above immobilized to a solid support or carrier.

The above affinity ligand provides a basis for a method for isolating a Tpm-binding antiangiogenic molecule from a complex mixture comprising:

- (a) contacting the mixture with the affinity ligand;
- (b) allowing any material in the mixture to bind to the ligand;
- (c) removing unbound material from the ligand; and
- (d) eluting the bound Tpm -binding molecule.

The anti-angiogenic receptor polypeptide or peptide may :

- (i) have the sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, or SEQ ID NO:20;
- (ii) be a Tpm-binding peptide fragment of one of the above sequences; or
- (iii) be a Tpm-binding conservative substitution variant of one of these sequences or of the peptide fragment thereof.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

**Figure 1A and 1B** show inhibition of EC proliferation by HKa and prevention by an anti-Tpm antibody. Fig. 1A shows inhibition of bFGF (10 ng/ml) induced EC proliferation by 20 nM HKa (open bars) and 60 nM HKa domain 5 (black bars) is prevented by increasing concentrations of mAb TM-311. Fig. 1B shows the effect of mAb TM-311 and antibodies against other EC HK- or HKa-binding proteins on the inhibition of EC proliferation by HKa. Proliferation was measured after addition of (1) 10 ng/ml bFGF (stimulus), (2) bFGF + 20 nM HKa, or (3) bFGF + HKa + antibody (300 nM). The antibodies tested were polyclonal antibodies against the urokinase receptor (Anti-uPAR) or cytokeratin 1 (Anti-CK 1), or mAbs against the receptor for the globular heads of C1q (Anti-gC1qR) or against Tpm (TM-311). Control antibodies included MOPC-21 (murine IgG1) for the mAbs and nonimmune rabbit IgG (NRIgG) for the polyclonals.

**Figure 2** shows the effect of mAb TM-311 on HKa-induced EC apoptosis, assessed by endonucleolytic cleavage of DNA. EC DNA was isolated from cells cultured for 12 hours in the presence of mAb TM-311 alone (lane 1), 20 nM HKa (lane 2), 20 nM HKa + 60 nM TM-311 (lane 3), 50 nM HKa domain 5 (lane 4), 50 nM HKa domain 5 + 150 nM mAb TM-311 (lane 5), 2  $\mu$ M 2-methoxyestradiol (lane 6) or 2  $\mu$ M 2-methoxyestradiol + 6  $\mu$ M mAb TM-311 (lane 7).

**Figure 3 shows the results of immunoprecipitating endothelial Tpm by mAb TM-311.** Cell surface proteins on proliferating and confluent HUVEC were labeled with NHS-LC biotin. Detergent extracts were prepared, and equal amounts of protein from each culture were immunoprecipitated using TM-311 or MOPC-21. Immunoprecipitated proteins were separated using 10% SDS-PAGE, transferred to PVDF, and detected using streptavidin-peroxidase and chemiluminescence.

**Figures 4A-4C is a set of photomicrographs showing confocal laser scanning microscopic analysis of proliferating and confluent ECs.** Fig 1A shows proliferating ECs stained with control MOPC-21. Fig. 1B shows proliferating ECs stained with TM-311. Fig. 4C shows confluent ECs stained with TM-311. The image in (C) represents a compilation of 8 individual confocal “cuts” (necessary to visualize the cells), while that in (B) represents only a single cut. All cells were permeabilized by exposure to 0.1% Triton-X-100 prior to staining.

**Figure 5 shows the cross-linking of HKa to EC surface proteins.** Biotin-HKa was incubated with confluent (lanes 1 and 2) or proliferating (lanes 3 and 4) ECs in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of a 20-fold molar excess of unlabeled HKa prior to cross-linking using BS<sup>3</sup>. Biotin-HKa was also incubated with MDA-MB-231 breast carcinoma cells under identical conditions (lane 5). Detergent extracts were separated by SDS-PAGE, then transferred to PVDF and detected by chemiluminescence. The arrowhead denotes a prominent band of about 140-150 kDa, the expected size of an HKa-Tpm complex.

**Figures 6A and 6B** show specific binding of HKa to proliferating ECs and its inhibition by mAb TM-311. Fig. 6A shows specific binding of HKa to ECs cultured under conditions inducing proliferation. Unfixed cells were incubated with increasing concentrations of biotin-HKa in the absence or presence of 10  $\mu$ M Zn<sup>2+</sup>. Cell-bound biotin-HKa was detected using streptavidin peroxidase and the peroxidase substrate turbo-TMB. The curve was fit by nonlinear regression, yielding a K<sub>d</sub> of about 2.5 nM. Fig. 6B shows inhibition of the binding of biotin-HKa to proliferating ECs by mAb TM-311. Biotin-HKa (20 nM) was incubated with ECs in the presence of 10  $\mu$ M Zn<sup>2+</sup> and increasing concentrations of mAb TM-311.

**Figures 7A-7C** show specific binding of HKa to purified immobilized Tpm and its inhibition. Fig. 7A shows specific binding of HKa to purified chicken gizzard Tpm immobilized to polystyrene (in 96 well microplates). Plates were incubated with increasing concentrations of biotin-HKa in the absence or presence of 10  $\mu$ M Zn<sup>2+</sup>. Bound ligand was measured using streptavidin-peroxidase and turbo-TMB, and the curve fit by nonlinear regression, yielding a K<sub>d</sub>

of about 2.5 nM. Fig. 7B shows Inhibition of binding of 20 nM biotin-HKa to immobilized Tpm (as above) by mAb TM-311 in the presence of 10  $\mu$ M Zn<sup>2+</sup>. Fig. 7C shows that HKa domain 5 (D5) inhibits binding of 20 nM biotin-HKa to purified Tpm. The IC<sub>50</sub> for this inhibition was about 8.1 nM.

**Figures 8A-8D** shows the effect of HKa and/or TM-311 on *in vivo* angiogenesis in the chick chorioallantoic membrane (CAM). Effect of bFGF (Fig. 8A), bFGF + TM-311 (Fig. 8B), bFGF + HKa (Fig. 8C) and bFGF + HKa + TM-311 (Fig. 8D). HKa inhibited basic FGF-induced angiogenesis. The antiangiogenic effects of HKa were in turn inhibited by TM-311.

**Figure 9** shows the direct binding of an anti-Tpm antibody to immobilized Tpm. Increasing amounts of a biotinylated anti-Tpm antibody were added to a 96 well plate previously coated with 200 ng of chicken gizzard Tpm. The bound anti-Tpm antibody was detected using avidin-HRP and a chromogenic substrate. The Kd was determined by non-linear regression analysis of the empirical data.

**Figure 10** shows the competition for binding of biotin-HKa to Tpm by an anti-Tpm antibody. 10 nM biotin-HKa was added to a 96 well plate previously coated with 200 ng of chicken gizzard Tpm in the presence of 10  $\mu$ M ZnCl<sub>2</sub>. Increasing amounts of anti-Tpm mAb TM311 antibody were added. Bound biotin-HKa was detected using avidin-HRP and a chromogenic substrate. Kd was determined as for Fig. 9.

**Figure 11** show the inhibition of angiogenesis by anti-Tpm mAb in the Matrigel plug model. Aliquots of Matrigel (0.5 mL) containing 400 ng/ml of bFGF, 50  $\mu$ g/ml heparin with or without 20  $\mu$ g of the anti-Tpm mAb or saline buffer were injected in the flanks of a mouse. After five days, the plugs were removed and the levels of hemoglobin determined. The level of hemoglobin in the positive control (no treatment) minus the negative control (no bFGF) was set as 100%.

**Figures 12 and 13** show inhibition of angiogenesis and tumor growth by anti-Tpm antibody in the Matrigel/MatLyLu model. Aliquots of Matrigel (0.5 mL) containing 2 x 10<sup>6</sup> MatLyLu cells with or without 30  $\mu$ g of the anti-Tpm mAb or saline buffer were injected in the flanks of a mouse. After seven days, the plugs were removed, scanned, weighed and the levels of hemoglobin determined. The level of hemoglobin in the positive control (no treatment) minus the negative control (no cells) was set as 100%.

**Figure 14** shows that HK-D5 and HPRG-H/P have approximately 1,000-fold higher affinity for immobilized Tpm than does endostatin. 10 nM biotin-HKa was added to a 96 well

plate previously coated with 200 ng of chicken gizzard Tpm in the presence of 10  $\mu$ M ZnCl<sub>2</sub>. Increasing amounts of HK-D5, HPRG-H/P domain or endostatin were added to the wells. Bound biotin-HKa was detected and Kd was calculated as for Fig. 10.

**Figures 15A and 15B** show that HPRG binds to immobilized chicken gizzard Tpm through its H/P domain. **Fig. 15A:** increasing amounts of a biotinylated HPRG, HKa or HKa-D5 were added to a 96 well Tpm-coated plate. **Fig. 15B:** 10 nM biotin-HKa was added to a 96 well Tpm- coated plate. Increasing amounts of HPRG-H/P domain or HPRG-N/C fragment were added to the wells. In both Fig. 15A and 15B, bound biotin-HKa was detected and Kd was calculated as in Fig. 10.

**Figure 16** shows that ATN-228 bound to immobilized Tpm whereas ATN-246 did not. 10 nM biotin-HKa was added to a 96 well plate Tpm- coated plate. Increasing amounts of ATN-228 or ATN-246 were added to the wells. Bound biotin-HKa was detected and Kd was calculated as for Fig. 10.

**Figure 17** shows that ATN228 but not ATN246 inhibited angiogenesis in the Matrigel plug model. Aliquots of Matrigel (0.5 mL) containing 400 ng/ml of bFGF, 50  $\mu$ g/ml heparin with or without 20  $\mu$ g ATN-228 or ATN-246 or saline buffer were injected in the flanks of a mouse. After five days, the plugs were removed and scanned.

**Figure 18** shows that ATN230 but not ATN294 inhibited MatLyLu growth in the Matrigel model/MLL. Aliquots of Matrigel (0.5 mL) containing  $2 \times 10^6$  MatLyLu cells with or without 700  $\mu$ M ATN230 or 900  $\mu$ M ATN294 or saline buffer were injected in the flanks of a mouse. After seven days, the plugs were removed and weighted.

**Figure 19** shows the effects of Tpm digestion by chymotrypsin over time. Chicken gizzard Tpm (0.4 mg/ml) was incubated with 5  $\mu$ g/ml chymotrypsin in TBS pH 7.5 at 37°C and aliquots were taken at the indicated times. SDS-PAGE sample buffer was added and the sample heated at 80°C for 4 minutes and then loaded onto and run on a 10% NuPage gel (Invitrogen).

**Figure 20** shows the results of a study identifying a fragment of Tpm that binds to HKa-D5. Chicken gizzard Tpm was partially digested with Chymotrypsin as described for Fig. 19 for 100 minutes so that a 20 kDa fragment was enriched. HK-D5 had previously immobilized in an activated Sepharose (CNBr-activated Sepharose 4 B, Amersham Biosciences). The chymotryptic fragments were incubated with the HK-D5-Sepharose resin in TBS containing 10  $\mu$ M ZnCl<sub>2</sub>, washed extensively in the same buffer and eluted in 2 M NaCl. The samples were run in a 10% NuPage gel (Invitrogen).

Figure 21 shows that ATN-310, ATN-311 and ATN-312 displace HKa that is bound to Tpm. Ten nM biotin-HKa was added to a 96 well plate previously coated with 200 ng of chicken gizzard Tpm in the presence of 10  $\mu$ M ZnCl<sub>2</sub>. Increasing amounts of ATN-310, ATN-311 or ATN-312 were added to the wells. Bound biotin-HKa was detected and Kd was calculated as in the description of Fig. 10.

### DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present inventors have discovered that Tpm is present on the surface of activated ECs (ECs) and that it is an important mediator of anti-angiogenic signals.

The induction of EC apoptosis by HKa, as well as the antiangiogenic activity of HKa in the chick chorioallantoic membrane (CAM), was completely inhibited by a monoclonal anti-Tpm antibody (mAb TM-311). TM-311 also blocked the high affinity ( $K_d \sim 2.6$  nM), Zn<sup>2+</sup>-dependent binding of HKa to both purified Tpm and to proliferating ECs. Confocal microscopic analysis of ECs stained with mAb TM-311, as well as biotin labeling of cell surface proteins on intact ECs, revealed enhanced Tpm exposure on the surface of proliferating ECs. Thus, the present inventors discovered that the antiangiogenic effects of HKa are dependent upon high affinity binding to EC cell surface Tpm.

No role for Tpm as a mediator or anti-angiogenic signals had been suggested prior to the making of the present invention.

The sequence of smooth muscle Tpm (cTPM1) from chicken gizzard (ExPASy accession # P04267) is shown below as SEQ ID NO:1

	1	11	21	31	41	51	
1	MEAIIKKKMQM	LKLDKENAID	RAEQAEADKK	QAEDRCKQLE	EEQOGLOKKL	KGTEDEVEKY	60
61	<u>SESVKEAQEK</u>	<u>LEQAEKKATD</u>	<u>AAAEVASLNR</u>	<u>RIQLVEEELD</u>	<u>RAQERLATAL</u>	<u>QKLEEAEKAA</u>	120
121	<u>DESERGMKVI</u>	<u>ENRAMKDEEK</u>	<u>MELQEMQLKE</u>	<u>AKHIAEEADR</u>	<u>KYEEVARKLV</u>	<u>VLEGELERSE</u>	180
181	<u>ERAEVAESRV</u>	<u>RQLEEEELRTM</u>	<u>DQSLKSLIAS</u>	<u>EEEYSTKEDK</u>	<u>YEEEIKLLGE</u>	<u>KLKEAETRAE</u>	240
241	FAERSVAKLE	KTIDDLEESL	ASAKEENVGI	HQVLDQTLLE	LNNL		

The Tyr (Y) residues after which chymotrypsin cleaved are underscored. A fragment corresponding to residues 61-214 of SEQ ID NO:1 (underscored above) was identified as a shorter polypeptide that binds antiangiogenic polypeptide ligands such as HKa D5 (see below). This polypeptide has the following sequence and is designated SEQ ID NO:2

1	SESVKEAQEK	LEQAEKKATD	AAAEVASLNR	RIQLVEEELD	RAQERLATAL	QKLEEAEKAA	60
61	DESERGMKVI	ENRAMKDEEK	MELQEMQLKE	AKHIAEEADR	KYEEVARKLV	VLEGELERSE	120
121	ERAEVAESRV	RQLEEEELRTM	DQSLKSLIAS	EEEYSTKEDK	YEEEIKLLGE	KLKEAETRAE	154

This polypeptide is termed “Antiangiogenic Ligand-Binding Polypeptide” or “AALBP”.

The present inventors discovered the AALBP by passing a chymotryptic digest of cTPM1 over an affinity column comprising an antiangiogenic polypeptide, specifically, the D5 domain of human kininogen, HKa. Material that bound to the column was subjected to N-terminal sequencing. The sequence was determined to be SESVKEAQE, corresponding to residues 61-69 of cTPM1 (SEQ ID NO:1) and residues 1-9 of SEQ ID NO:2. A minor contaminant had a different N terminal sequence. The size of the D5-binding polypeptide and hence its C-terminus was determined by mass spectrometry (MS), specifically, matrix-assisted laser desorption time-of-flight (MALDI-TOF) MS.

Eight human isoforms of Tpm are known. - *similar- two groups- don't know which isoform it is.* They are described below and their sequences shown (in an aligned format). Additional information about certain alignment comparisons between chicken and human (and chicken and chicken) Tpm is given. Also shown are the polypeptide fragments that correspond to the AALBP of cTPM1

TABLE 1

Abbrev	Tropomyosin species/type/isoform	ExPASY Accession #	SEQ ID NO:
cTPM1	chicken tropomyosin 1, smooth muscle (gizzard)	P04267	SEQ ID NO:1
cTMP2	chicken tropomyosin 2, smooth muscle (gizzard)	P04262	SEQ ID NO:3
hTPM1	human tropomyosin $\alpha$ chain, skeletal muscle	P09493	SEQ ID NO:5
hTPM2	human tropomyosin $\beta$ chain, fibroblast and muscle-type	P06468	SEQ ID NO:7
hTPM3	human tropomyosin $\alpha$ chain skeletal, muscle type	P06753	SEQ ID NO:9
hTPM4	human tropomyosin, fibroblast non-muscle type	P07226	SEQ ID NO:11
hTPMB	human tropomyosin $\beta$ chain, skeletal muscle.	P07951	SEQ ID NO:13
hTPMF	human tropomyosin $\alpha$ chain, fibroblast isoform TM3	P09494	SEQ ID NO:15
hTPMN	human tropomyosin, cytoskeletal type	P12324	SEQ ID NO:17
hTPMS	human tropomyosin $\alpha$ chain, smooth muscle	P10469	SEQ ID NO:19

Alignment information, including % identity between certain of the full length Tpm sequences are shown in Table 2.

TABLE 2

Alignment comparison	% Identity	# residues overlap	Score
human TPM2 and chicken gizzard TPM1	95.4	284	1315.0
human TPM3 and chicken gizzard TPM1	77.8	284	1083.0
chicken gizzard TPM1 and chicken gizzard TPM2	74.6	284	1040.0

Table 3, below shows the full length sequences of two chicken and eight human Tpm isoforms. Also indicated by underscoring is the shorter polypeptides corresponding to the AALBP as described above for cTPM1. The amino acid sequences of these shorter polypeptides appear separately in Table 4.

TABLE 3

Abbrev	SEQUENCE	Residues	SEQ ID NO:
hTPM1	MDAIKKKMQM LKLDKENALD RAEQAEADKK AAEDRSKQLE DELVSLQKKL	50	SEQ ID NO:5
ctPM1	MEAIIKKKMQM LKLDKENAID RAEQAEADKK QAEDRCKQLE EEQQGLQKKL	50	SEQ ID NO:1
hTPM2	MDAIKKKMQM LKLDKENAID RAEQAEADKK QAEDRCKQLE EEQQALQKKL	50	SEQ ID NO:7
ctPM2	MDAIKKKMQM LKLDKENALD RAEQAEADKK AAEERSKQLE DDIVQLEKQL	50	SEQ ID NO:3
hTPM3	MEAIIKKKMQM LKLDKENALD RAEQAEAEQK QAEERSKQLE DELAAMQKKL	50	SEQ ID NO:9
hTPM4	..... MAGL NSLEAVRKI	14	SEQ ID NO:11
hTPMB	MDAIKKKMQM LKLDKENAID RAEQAEADKK QAEDRCKQLE EEQQALQKKL	50	SEQ ID NO:13
hTPMF	MDAIKKKMQM LKLDKENALD RAEQAEADKK AAEDRSKQLE DELVSLQKKL	50	SEQ ID NO:15
hTPMN	..... MAGI TTIEAVRKI	14	SEQ ID NO:17
hTPM1	KGTEDELDKY <u>SEALKDAQEK</u> LELAEKKATD AEADVASLNR RIQLVEEELD	100	SEQ ID NO:5
ctPM1	KGTEDEVEKY <u>SESVKEAQEK</u> LEQAEKKATD AAEAVASLNR RIQLVEEELD	100	SEQ ID NO:1
hTPM2	KGTEDEVEKY <u>SESVKEAQEK</u> LEQAEKKATD AEADVASLNR RIQLVEEELD	100	SEQ ID NO:7
ctPM2	RVTEDSRDQV <u>LEELHKSEDS</u> LLSAEEAAK AESEVASLNR RIQLVEEELD	100	SEQ ID NO:3
hTPM3	KGTEDELDKY <u>SEALKDAQEK</u> LELAEKKAAD AAEAVASLNR RIQLVEEELD	100	SEQ ID NO:9
hTPM4	QALQQQADEA <u>EDRAQGLQRE</u> LDGERERREK AEGDVAALNR RIQLVEEELD	64	SEQ ID NO:11
hTPMB	KGTEDEVEKY <u>SESVKEAQEK</u> LEQAEKKATD AEADVASLNR RIQLVEEELD	100	SEQ ID NO:13
hTPMF	KGTEDELDKY <u>SEALKDAQEK</u> LELAEKKATD AEADVASLNR RIQLVEEELD	100	SEQ ID NO:15
hTPMN	QVLQQQADDA <u>EERAERLQRE</u> VEGERRAREQ AAEAVASLNR RIQLVEEELD	64	SEQ ID NO:17
hTPMS	..... CRL <u>RIFLRTASSE</u> HLHERKLRET AEADVASLNR RIQLVEEELD	43	SEQ ID NO:19
hTPM1	RAQERLATAL QKLEEAAKAA DESERGMKVI <u>ESRAQKDEEK</u> MEIQETQLKE	150	SEQ ID NO:5
ctPM1	RAQERLATAL QKLEEAAKAA DESERGMKVI ENRAMKDEEK MEIQEMQLKE	150	SEQ ID NO:1
hTPM2	RAQERLATAL QKLEEAAKAA DESERGMKVI ENRAMKDEEK MEIQEMQLKE	150	SEQ ID NO:7
ctPM2	RAQERLATAL QKLEEAAKAA DESERGMKVI ENRAQKDEEK MEIQETQLKE	150	SEQ ID NO:3
hTPM3	RAQERLATAL QKLEEAAKAA DESERGMKVI ENRALKDEEK MEIQETQLKE	150	SEQ ID NO:9
hTPM4	RAQERLATAL QKLEEAAKAA DESERGMKVI ENRAMKDEEK MEIQEMQLKE	114	SEQ ID NO:11
hTPMB	RAQERLATAL QKLEEAAKAA DESERGMKVI ENRAMKDEEK MEIQEMQLKE	150	SEQ ID NO:13
hTPMF	RAQERLATAL QKLEEAAKAA DESERGMKVI <u>ESRAQKDEEK</u> MEIQETQLKE	150	SEQ ID NO:15
hTPMN	RAQERLATAL QKLEEAAKAA DESERGMKVI ENRALKDEEK MEIQETQLKE	114	SEQ ID NO:17
hTPMS	RAQERLATVL QKLEEAAKAA DESERGMKVI <u>ESRAQKDEEK</u> METQETQLKE	93	SEQ ID NO:19
hTPM1	AKHIAEDADR KYEEVARKLV <u>IIIESDLERA</u> E ERAELSEGKC AELEEEELKTV	200	SEQ ID NO:5
ctPM1	AKHIAEEADR KYEEVARKLV VLEGELERSE ERAEVAESRV RQLEELRTM	200	SEQ ID NO:1
hTPM2	AKHIAEDSDR KYEEVARKLV <u>IIEGLER</u> SE ERAEVAESRA RQLEELRTM	200	SEQ ID NO:7
ctPM2	AKHIAEEADR KYEEVARKLV <u>ILEGDLERA</u> E ERAELSESKC AELEEEELKLV	200	SEQ ID NO:3
hTPM3	AKHIAEEADR KYEEVARKLV <u>IIEGLER</u> TE ERAELAESKC SELEEEELKNV	200	SEQ ID NO:9
hTPM4	AKHIAEEADR KYEEVARKLV <u>ILEGDLERA</u> E ERAEVSELKC GDLEEEELKNV	164	SEQ ID NO:11
hTPMB	AKHIAEDSDR KYEEVARKLV <u>ILEGELER</u> SE ERAEVAESKC GDLEEEELKIV	200	SEQ ID NO:13
hTPMF	AKHIAEDADR KYEEVARKLV <u>IIIESDLERA</u> E ERAELSEGQV RQLEEQQLRIM	200	SEQ ID NO:15
hTPMN	AKHIAEEADR KYEEVARKLV <u>IIEGLER</u> TE ERAELAESRC REMDEQIRLM	164	SEQ ID NO:17
hTPMS	AKHIAEDADR KYEEVARKLV <u>IIIESDLERA</u> E ERAELSEGQV RQLEEQQLRIM	143	SEQ ID NO:19
hTPM1	TNNLKSLEAQ AEK <u>YSQKEDR</u> YEEEIKVLSD KLKEAETRAE FAERSVTKLE	250	SEQ ID NO:5
ctPM1	DQSLKSLIAS EEE <u>YSTKEDK</u> YEEEIKLLGE KLKEAETRAE FAERSVAKLE	250	SEQ ID NO:1
hTPM2	DQALKSLMAS <u>EEEYSTKEDK</u> YEEEIKLLEE KLKEAETRAE FAERSVAKLE	250	SEQ ID NO:7
ctPM2	TNEAKSLEAQ AEK <u>YSQKEDK</u> YEEEIKVLTD KLKEAETRAE FAERSVTKLE	250	SEQ ID NO:3
hTPM3	TNNLKSLEAQ AEK <u>YSQKEDK</u> YEEEIKILTD KLKEAETRAE FAERSVAKLE	250	SEQ ID NO:9
hTPM4	TNNLKSLEAA SEK <u>YSQKEDK</u> YEEEIKLLSD KLKEAETRAE FAERTVAKLE	214	SEQ ID NO:11
hTPMB	TNNLKSLEAQ ADK <u>YSTKEDK</u> YEEEIKLLEE KLKEAETRAE FAERSVAKLE	250	SEQ ID NO:13
hTPMF	DQTLKALMAA <u>EDKYSQKEDR</u> YEEEIKVLSD KLKEAETRAE FAERSVTKLE	250	SEQ ID NO:15
hTPMN	DQNLKCLSAE EEK <u>YSQKEDK</u> YEEEIKILTD KLKEAETRAE FAERSVAKLE	214	SEQ ID NO:17
hTPMS	DSDLESINAA EDK <u>YSQKEDR</u> YEEEIKVLSD KLKEAETRAE FAERSVTKLE	193	SEQ ID NO:19

TABLE 3 (cont.)

Abbrev	SEQUENCE	Residues	SEQ ID NO:
hTPM1	KSIDDLEDEL YAQKLKYKAI SEELDHALND MTSI	284	SEQ ID NO:5
cTPM1	KTIDDLLESL ASAKEENVGI HQVLQDTLLE LNNL	284	SEQ ID NO:1
hTPM2	KTIDDLLETL ASAKEENVEI HQTLQDTLLE LNNL	284	SEQ ID NO:7
cTPM2	KSIDDLEEKV AHAKEEENLM HQMLQDTLLE LNNM	284	SEQ ID NO:3
hTPM3	KTIDDLLEDEL YAQKLKYKAI SEELDHALND MTSI	284	SEQ ID NO:9
hTPM4	KTIDDLLEKL AQAKEENVGL HQTLQDTLNE LNCl	248	SEQ ID NO:11
hTPMB	KTIDDLLEDEV YAQKMKYKAI SEELDNALND ITSL	284	SEQ ID NO:13
hTPMF	KSIDDLEEKV AHAKEEENLSM HQMLQDTLLE LNNM	284	SEQ ID NO:15
hTPMN	KTIDDLLEDKL KCTKEEHLCT QRMLQDTLLD LNEM	248	SEQ ID NO:17
hTPMS	KSIDDLEEKV AHAKEEENLSM HQMLQDTLLE LNNM	227	SEQ ID NO:19

TABLE 4

10	20	30	40	50	60
SESVKEAQEK	LEQAEEKKATD	AEAEVASLNR	RIQLVEEELD	RAQERLATL	QKLEEAEKAA
LEELHKSEDS	LLSAEENAAK	AESEVASLNR	RIQLVEEELD	RAQERLATL	QKLEEAEKAA
SEALKDAQEK	LELAEKKATD	AEADVASLNR	RIQLVEEELD	RAQERLATL	QKLEEAEKAA
SESVKEAQEK	LEQAEEKKATD	AEADVASLNR	RIQLVEEELD	RAQERLATL	QKLEEAEKAA
SEALKDAQEK	LELAEKKAAD	AEAEVASLNR	RIQLVEEELD	RAQERLATL	QKLEEAEKAA
EDRAQGLQRE	LDGERERREK	AEGDVAALNR	RIQLVEEELD	RAQERLATL	QKLEEAEKAA
SESVKEAQEK	LEQAEEKKATD	AEADVASLNR	RIQLVEEELD	RAQERLATL	QKLEEAEKAA
SEALKDAQEK	LELAEKKATD	AEADVASLNR	RIQLVEEELD	RAQERLATL	QKLEEAEKAA
EERAERLQRE	VEGERRAREQ	AEAEVASLNR	RIQLVEEELD	RAQERLATL	QKLEEAEKAA
RIFLRTASSE	HLHERKLRET	AEADVASLNR	RIQLVEEELD	RAQERLATL	QKLEEAEKAA
70	80	9	100	110	120
DESERGMKVI	ENRAMKDEEK	MELQEMQLKE	AKHIAEEADR	KYEEVARKLV	VLEGELERSE
DESERGMKVI	ENRAQKDEEK	MEIQEIQLKE	AKHIAEEADR	KYEEVARKLV	ILEGDLERAE
DESERGMKVI	ESRAQKDEEK	MEIQEIQLKE	AKHIAEDADR	KYEEVARKLV	IIESDLERAE
DESERGMKVI	ENRAMKDEEK	MELQEMQLKE	AKHIAEDSDR	KYEEVARKLV	ILEGELERSE
DESERGMKVI	ENRALKDEEK	MELQEIQLKE	AKHIAEEADR	KYEEVARKLV	IIEGDLERTE
DESERGMKVI	ENRAMKDEEK	MEIQEMQLKE	AKHIAEEADR	KYEEVARKLV	ILEGELERAE
DESERGMKVI	ENRAMKDEEK	MELQEMQLKE	AKHIAEDSDR	KYEEVARKLV	IILEGELERSE
DESERGMKVI	ESRAQKDEEK	MEIQEIQLKE	AKHIAEDADR	KYEEVARKLV	IIESDLERAE
DESERGMKVI	ENRALKDEEK	MELQEIQLKE	AKHIAEEADR	KYEEVARKLV	IIEGDLERTE
DESERGMKVI	ESRAQKDEEK	MEIQEIQLKE	AKHIAEDADR	KYEEVARKLV	IIESDLERAE
130	140	150	154		
ERAEEVAESRV	RQLEEEELRTM	DQSLKSLIAS	EEYY		SEQ ID NO:2
ERAELSESKC	AELEEEELKLV	TNEAKSLEAQ	AEKY		SEQ ID NO:4
ERAELSEGKC	AELEEEELKTV	TNNLKSLEAQ	AEKY		SEQ ID NO:6
ERAEEVAESRA	RQLEEEELRTM	DQALKSLMAS	EEEY		SEQ ID NO:8
ERAELAESKC	SELEEEELKNV	TNNLKSLEAQ	AEKY		SEQ ID NO:10
ERAEVSELKC	GDLEEEELKNV	TNNLKSLEAA	SEKY		SEQ ID NO:12
ERAEEVAESKC	GDLEEEELKIV	TNNLKSLEAQ	ADKY		SEQ ID NO:14
ERAELSEGQV	RQLEEQQLRIM	DQTLKALMAA	EDKY		SEQ ID NO:16
ERAELAESRC	REMDEQIRLM	DQNLKCLSA	EEKY		SEQ ID NO:18
ERAELSEGQV	RQLEEQQLRIM	DSDLESINAA	EDKY		SEQ ID NO:20

The present invention includes conservative amino acid substitution variants of any of the above polypeptides. Also included in the invention is a set of overlapping peptides having at least 4 amino acids, preferably between about 10 and about 40 amino acids, which are derived from SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18 or 20, or conservative substitution variants thereof. The polypeptides and peptides of the present invention are characterized in that they either (a) bind directly to an angiogenic polypeptide or peptide such as HKa D5 or HPRG or an

angiogenic peptide fragment of HPRG or (b) inhibit the binding of such an angiogenic peptide to a Tpm protein which is either expressed on a cell surface, immobilized to a solid support, or in solution in a competitive binding assay such as is described herein.

In another embodiment, the invention encompasses peptides which are homologous to a human Tpm, preferably SEQ ID NO:5, 7 or 9 or fragments thereof, preferably SEQ ID NO:6, 8 or 10. In one embodiment, the amino acid sequence of the peptide is at least 70% identical to the sequence of a wild type fragment of human Tpm. Preferably the identity is at least 85%, more preferably, at least 90%.

In the case of the cTPM1 polypeptide SEQ ID NO:2, the TM-311mAb does not recognize this fragment. It is noteworthy that the TM-311 mAb is not anti-angiogenic in human systems that have been tested such as HUVEC or a CAM assay, even though this mAb blocks HKa binding of Tpm. This mAb is antiangiogenic and has anti-tumor activity in murine systems

The present invention is directed to antibodies, preferably mAbs that directly bind Tpm or a Tpm polypeptide or peptide such as AALBP as described above and act as inhibitors or antagonists of angiogenesis by evoking an antiangiogenic signal in a cell to which they bind via cell surface Tpm or bind to Tpm expressed on the surface of angiogenic ECs.

Although the inventors do not wishing to be bound by any particular mechanistic explanation for the results disclosed herein, they have conceived that the compositions and methods of the present invention exert antitumor effects either by (1) antiangiogenic effects mediated via generation of signal in EC's that lead to apoptosis, (2) direct apoptotic signals to tumor cells that express a Tpm polypeptide on their surface or (3) by both mechanisms.

According to this invention, any protein that can bind to cell surface Tpm and initiate an apoptotic pathway or otherwise inhibit angiogenesis can be used to treat conditions associated with undesired angiogenesis, for example, tumor growth and metastasis. The following is a nonlimiting list of Tpm-binding proteins (see, e.g., Perry SV, *J Muscle Res. Cell Motil.* (2001), 22:5-49; Squire JM *et al.*, *FASEB J.* (1998) 12:761-771; Lin JJ *et al.*, *Int. Rev. Cytol.* (1997) 170:1-38) that can be used in accordance with this invention to inhibit angiogenesis: troponin T (Smillie, LB, *Trends Biochem Sci* (1979). 4:151-155; Zot AS *et al.*, *Annu Rev Biophys Chem* (1987) 16:535-559); tropomodulin (Fowler VM, *J Biol Chem* (1987) 262:12792-800); caldesmon (Marston SB *et al.*, *Biochem J* (1991): 279:1-16); calponin (Gimona M *et al.*, In: Barany (ed), *Biochemistry of Smooth Muscle Contraction*, pp 91-103, Academic Press, New

York. (1996); Takahashi K *et al.*, *Biochem Biophys Res Commun* (1986) **141**: 20-26); pEL98 (Takenaga K *et al.*, *J Cell Biol* (1994) **124**:757-768); glutamic dehydrogenase (Akutsu S *et al.*, *Zool Sci* (2000).**17**: 871-879) and actin.

5 Troponin T with two other molecules, Troponin I and C, form a trimer that binds to Tpm. Troponin T is the subunit responsible for Tpm binding. U.S. Patent 6,025,331 (Moses , *et al.*, February 15, 2000) disclosed that all three of these proteins could inhibit bovine capillary EC proliferation induced by bFGF. This document indicated that these proteins could be used to inhibit angiogenesis. Moses, MA *et al.*, 1999, *Proc. Natl. Acad. Sci. USA* **96**:2645–2650, disclosed that human cartilage Troponin I, purified to apparent homogeneity, is a potent and 10 specific inhibitor of angiogenesis *in vivo* and *in vitro*, as well as of tumor metastasis *in vivo* using the B16-BL6 murine melanoma model. The mechanism(s) by which Troponin I inhibited capillary EC proliferation *in vitro* and angiogenesis *in vivo* was said by these authors to be unknown although the authors suggested a connection with the known effects of modulation of 15 EC cell shape via manipulation of the cytoskeleton and its regulatory elements, including actin-associated proteins, on EC cell growth and capillary morphogenesis. The existence of an EC Troponin I receptor was suggested as a means by which this protein could induce changes in EC shape that, in turn, would suppress growth of these cells. It was further suggested that, under physiological conditions, Troponin I (pI 8.88), with its relatively rich lysine content, could, 20 through affinity for heparin, bind to and compete with bFGF and perhaps VEGF for heparin sulfate proteoglycan on the EC surface.

As noted above, Troponin I does not bind to Tpm. Rather, Troponin T is the subunit that binds tropomyosin whereas Troponin I had the antiangiogenic activity in the above study.

#### Tropomyosin-binding Angiogenesis Inhibitors

According to the present invention, Tpm is targeted by several anti-angiogenic proteins. 25 These are described below.

##### 1. Histidine Proline Rich Glycoprotein and Fragments Thereof

These polypeptides and their antiangiogenic properties are described in detail in copending, commonly assigned U.S. patent application Serial No. 10/074,225, filed 14-Feb, 2001 (published as WO 02/064621 and US 2003-0082740), which is hereby incorporated by 30 reference in its entirety.

Full length human HPRG has the amino acid sequence SEQ ID NO:21

MKALIAALLL	ITLQYSACAVS	PTDCSAVEPE	AEKALDLINK	RRRDGYLFQL	LRIADAHLDR	60
VENTVYYLV	LDVQESDCSV	LSRKYWNDCE	PPDSRRPSEI	VIGQCKVIAT	RHSHESQDLR	120
VIDFNCTTSS	VSSALANTKD	SPVLIDFFED	TERYRKQANK	ALEKYKEEND	DFASFRVDRI	180
ERVARVRGGE	GTGYFVDFSV	RNCPRHHFPR	HPNVFGFCRA	DLFYDVEALD	LESPKNLVIN	240
CEVFDPQEHE	NINGVPPHLG	HPFHGGHER	SSTTKPPFKP	HGSRDHHHPH	KPHEHGPPPP	300
PDERDHSHGP	PLPQGPPPLL	PMSCSSCQHA	TFGTNGAQRH	SHNNNSSDLH	PHKHHHSHEQH	360
PHGHHPHAH	PHEHDTHRQH	PHGHHPHGH	PHGHHPHGH	PHGHHPHCHD	FQDYGPCDPP	420
PHNOQHCCHG	HGPPPGHLRR	RGPKGKPRPF	HCRQIGSVYR	LPPLRKGEVL	PLPEANFPSE	480
PLPHHKHPLK	PDNQFPQSV	SESCPGKFKS	GFPQVSMFFT	HTFPK		525

Rabbit HPRG has the amino acid sequence SEQ ID NO:22 as follows:

ATLQCSWALT	PTDCKTTKPL	AEKALDLINK	WRRDGYLFQL	LRVADAHLG	AESATVYYLV	60
LDVKETDCSV	LSRKHWEDCD	PDLTKRPSLD	VIGQCKVIAT	RYSDEYQTLR	LNDFNCTTSS	120
VSSALANTKD	SPVLFDFFIED	TEPFRKSADK	ALEVYKSESE	AYASFRVDRV	ERVTRVKGGE	180
RTNYYVDFSV	RNCSRSHFHR	HPAFGFCRAD	LSFDVEASNL	ENPEDVIISC	EVFNFEHGN	240
ISGFRPHLGK	TPLGTDGSRD	HHHPHKPHKF	GCPPPQEGED	FSEGPPLOGG	TPPLSPPFRP	300
RCRHRRPGTN	ETHRFPHHRI	<u>SVNIIHRPPP</u>	HGHHPHGPPP	HGHHPHGPPP	HGHHPHGPPP	360
RHPPHGPPPH	<u>GHPPHGPPPH</u>	<u>GHPPHGPPPH</u>	<u>GHPPHGPPPH</u>	<u>GHPPHGPHGFH</u>	<u>DHGPCDPPSH</u>	420
KEGPQDLHQH	AMGPPPKHPG	KRGPGKGHFP	FHWRRIGSVY	QLPLQKGEV	LPLPEANFPQ	480
LLRNHTHPL	KPEIQFPQV	ASERCPEEFN	GEFAQLSKFF	PSTFPK		526

italic: signal sequence

double underscore: Pro-rich domain

single underscore: His-Pro (H/P) rich domain

The H/P domain of human HPRG has the sequence SEQ ID NO:23:

HPHKHHSHEQ HPHGHHPHAH HPHEHDTHRQ HPHGHHPHGH HPHGHHPHGH HPHGHHPHCH  
DFQDYGPCDP PPHNQGHCCH GHGPPGHLR RRGPGKGPRP FHCRCQIGSVY RLPLRKGEV  
LPLPEANFPS FPLPHHKHPL KPDNQFPQ

The H/P domain of rabbit HPRG has the sequence SEQ ID NO:24:

SVNIIHRPPP HGHHPHGPPP HGHHPHGPPP HGHHPHGPPP RHPPHGPPPH GHPPHGPPPH  
GHPPHGPPPH GHPPHGPPPH GHPPHGPHGFH DHGPCDPPSHK

## 2. Human Kininogen (HK) and Fragments

The full sequence of the mature form of HK (SEQ ID NO:25) is presented below.

QESQSEEIDC	NDKDLFKAVD	AALKYNSQN	QSNNQFVLYR	IIEATKTVGS	DTFYSFKYEI	60
KEGDCPVQSG	KTWQDCEYKD	AAKAATGECT	ATVGKRSSTK	FSVATQTCQI	TPAEGPVVTA	120
QYDCLGCVHP	ISTQSPDLEP	ILRHGIQYFN	NNTQHSSLFM	LNEVKRAQRQ	VVAGLNFRIT	180
YSIVQTNC SK	ENFLFLTPDC	KSLWNGDTGE	CTDNAYIDIQ	LRIASFSQNC	DIYPGKDFVQ	240
PPTKICVGCP	RDIPTNSPEL	EETLTHHTIK	LNAENNATFY	FKIDNVKKAR	VQVVAGKKYF	300
IDFVARETT	SKESNEELTE	SCETKKLGQS	LDCNAEVYVV	PWEKKIYPTV	NCQPLGMISL	360
MKRPPGFSPF	RSSRIGEIKE	ETTVSPPHTS	MAPAQDEERD	SGKEQGHTRR	HDWGHEKQRK	420
HNLGHGHKHE	RDQGHGHQRG	<u>HGLGHGH</u> <u>EQQ</u>	HGLGHGHKFK	LDDDL	EHQGG HVLDHGKHK	480
HGHGHGHKHN	KGKKNKGKHN	WKT	TEH	LASSSS	EDSTTPSAQT	540
TFSDFQDSL	IATMMPPISP	APIQSDDDWI	PDIQTD	DPNGL	SFNPI	600
WKS	VSEINPT	TQM	KESYYFD	LTDGLS		626

The D5 domain of HK (amino acid residues 384-508 of the mature HK sequence; underscored above) is useful as an inhibitor of angiogenesis and of various EC functions including cell proliferation as disclosed in commonly assigned application of some of the

present inventors, PCT/US01/23185, filed 7/24/01, "Human Kininogen D5 Domain Polypeptides" (A. Mazar & Jose Juarez), incorporated by reference in its entirety.

The 125 residue D5 domain has the sequence SEQ ID NO:26:

VSPPHTSMAP AQDEERDSGK EQGHTRRH DW GHEKQRKHNL GHGHKHERDQ	50
GHGHQRGHGL GHGHEQQHGL GHGHKFKLDD DLEHQGGHV L DHGHKHKHGH	100
GHGKHKNKGK KNGKHNGWKT EHLAS	125

The present invention is also directed to functional homologues of human or chicken Tpm or the AALBP fragment thereof. A functional homologue must possess the biochemical and biological activity, preferably anti-angiogenic and anti-tumor activity which can be tested using *in vitro* or *in vivo* methods described herein. In view of this functional characterization, use of homologous Tpm proteins from other species, including proteins not yet discovered, falls within the scope of the invention if these proteins have sequence similarity and the recited biochemical and biological activity.

To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred method of alignment, Cys residues are aligned.

In a preferred embodiment, the length of a sequence being compared is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence. For example, preferred alignment would be with human Tpm hTPM2 (SEQ ID NO:7) or its fragment SEQ ID NO:8., at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60% and even more preferably at least 70, 80 or 90 % of the amino acid residues are aligned. The amino acid residues (or nucleotides from the coding sequence) at corresponding amino acid (or nucleotide) positions are then compared. When a position in the first sequence is occupied by the same amino acid residue (or nucleotide) as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol.* 48:444-453 (1970) algorithm which has been incorporated into the GAP program in the GCG software package (available at <http://www.gcg.com>), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at <http://www.gcg.com>), using a NWGapDNA.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acids encoding the present polypeptide sequences and the polypeptide sequences of this invention can further be used as a "query sequence" to perform a search against public databases, for example, to identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to human or murine HPRG nucleic acid molecules. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to HPRG protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. See <http://www.ncbi.nlm.nih.gov>.

Thus, a homologue of a particular isoform of a human Tpm described above is characterized as having (a) functional activity of native Tpm or a ligand-binding fragment thereof, and (b) sequence similarity to a native Tpm when determined above, of at least about 30% (at the amino acid level), preferably at least about 50%, more preferably at least about 70%, even more preferably at least about 90%.

It is within the skill in the art to obtain and express such a protein using DNA probes based on the disclosed sequences of the Tpm's. Then, the protein's biochemical and biological activity can be tested readily using art-recognized methods such as those described herein.

### Peptide Compositions

A preferred composition is, or comprises, a biologically active peptide of Tpm characterized in that it possesses the binding and/or biological activity of Tpm. Such binding is to a ligand that is preferably an antiangiogenic protein or peptide that interacts with EC's and promotes their apoptosis or otherwise generates a signal that downregulates any EC function associated with angiogenesis.

Moreover, a biologically active peptide has Tpm-like activity in an *in vitro* or *in vivo* assay of binding or of biological activity such as those characterized herein. Preferably the peptide blocks binding of HPRG or HKa D5 or anti-Tpm mAbs to (a) EC's via cell surface Tpm or (b) isolated Tpm in a direct binding assay. A preferred peptide comprises a minimal sequence needed to bind to an antiangiogenic anti-Tpm mAb.

The peptide may be capped at its N and C termini with an acyl (abbreviated "Ac") - and an amido (abbreviated "Am") group, respectively, for example acetyl (CH<sub>3</sub>CO-) at the N terminus and amido (-NH<sub>2</sub>) at the C terminus.

A broad range of N-terminal capping functions, preferably in a linkage to the terminal amino group, is contemplated, for example:

formyl;  
alkanoyl, having from 1 to 10 carbon atoms, such as acetyl, propionyl, butyryl;  
alkenoyl, having from 1 to 10 carbon atoms, such as hex-3-enoyl;  
alkynoyl, having from 1 to 10 carbon atoms, such as hex-5-ynoyl;  
aroyl, such as benzoyl or 1-naphthoyl;  
heteroaroyl, such as 3-pyrrooyl or 4-quinoloyl;  
alkylsulfonyl, such as methanesulfonyl;  
arylsulfonyl, such as benzenesulfonyl or sulfanilyl;  
heteroarylsulfonyl, such as pyridine-4-sulfonyl;  
substituted alkanoyl, having from 1 to 10 carbon atoms, such as 4-aminobutyryl;  
substituted alkenoyl, having from 1 to 10 carbon atoms, such as 6-hydroxy-hex-3-enoyl;  
substituted alkynoyl, having from 1 to 10 carbon atoms, such as 3-hydroxy-hex-5-ynoyl;  
substituted aroyl, such as 4-chlorobenzoyl or 8-hydroxy-naphth-2-oyl;  
substituted heteroaroyl, such as 2,4-dioxo-1,2,3,4-tetrahydro-3-methyl-quinazolin-6-oyl;

substituted alkylsulfonyl, such as 2-aminoethanesulfonyl;  
substituted arylsulfonyl, such as 5-dimethylamino-1-naphthalenesulfonyl;  
substituted heteroarylsulfonyl, such as 1-methoxy-6-isoquinolinesulfonyl;  
carbamoyl or thiocarbamoyl;  
substituted carbamoyl (R'-NH-CO) or substituted thiocarbamoyl (R'-NH-CS) wherein R' is alkyl, alkenyl, alkynyl, aryl, heteroaryl, substituted alkyl, substituted alkenyl, substituted alkynyl, substituted aryl, or substituted heteroaryl;  
substituted carbamoyl (R'-NH-CO) and substituted thiocarbamoyl (R'-NH-CS) wherein R' is alkanoyl, alkenoyl, alkynoyl, aroyl, heteroaroyl, substituted alkanoyl, substituted alkenoyl, substituted alkynoyl, substituted aroyl, or substituted heteroaroyl, all as above defined.

The C-terminal capping function can either be in an amide or ester bond with the terminal carboxyl. Capping functions that provide for an amide bond are designated as NR<sup>1</sup>R<sup>2</sup> wherein R<sup>1</sup> and R<sup>2</sup> may be independently drawn from the following group:

hydrogen;

alkyl, preferably having from 1 to 10 carbon atoms, such as methyl, ethyl, isopropyl;

alkenyl, preferably having from 1 to 10 carbon atoms, such as prop-2-enyl;

alkynyl, preferably having from 1 to 10 carbon atoms, such as prop-2-ynyl;

substituted alkyl having from 1 to 10 carbon atoms, such as hydroxyalkyl, alkoxyalkyl, mercaptoalkyl, alkylthioalkyl, halogenoalkyl, cyanoalkyl, aminoalkyl, alkylaminoalkyl, dialkylaminoalkyl, alkanoylalkyl, carboxyalkyl, carbamoylalkyl;

substituted alkenyl having from 1 to 10 carbon atoms, such as hydroxyalkenyl, alkoxyalkenyl, mercaptoalkenyl, alkylthioalkenyl, halogenoalkenyl, cyanoalkenyl, aminoalkenyl, alkylaminoalkenyl, dialkylaminoalkenyl, alkanoylalkenyl, carboxyalkenyl, carbamoylalkenyl;

substituted alkynyl having from 1 to 10 carbon atoms, such as hydroxyalkynyl, alkoxyalkynyl, mercaptoalkynyl, alkylthioalkynyl, halogenoalkynyl, cyanoalkynyl, aminoalkynyl, alkylaminoalkynyl, dialkylaminoalkynyl, alkanoylalkynyl, carboxyalkynyl, carbamoylalkynyl;

arylalkyl having up to 10 carbon atoms, such as phenacyl or 2-benzoylethyl;

aryl, such as phenyl or 1-naphthyl;

heteroaryl, such as 4-quinolyl;

alkanoyl having from 1 to 10 carbon atoms, such as acetyl or butyryl;

aroyl, such as benzoyl;

heteroaroyl, such as 3-quinoloyl;

OR' or NR'R'' where R' and R'' are independently hydrogen, alkyl, aryl, heteroaryl, acyl, aroyl, sulfonyl, sulfinyl, or SO<sub>2</sub>-R''' or SO-R''' where R''' is substituted or unsubstituted alkyl, aryl, heteroaryl, alkenyl, or alkynyl.

Capping functions that provide for an ester bond are designated as OR, wherein R may be: alkoxy; aryloxy; heteroaryloxy; aralkyloxy; heteroaralkyloxy; substituted alkoxy; substituted aryloxy; substituted heteroaryloxy; substituted aralkyloxy; or substituted heteroaralkyloxy.

Either the N-terminal or the C-terminal capping function, or both, may be of such structure that the capped molecule functions as a prodrug (a pharmacologically inactive derivative of the parent drug molecule) that undergoes spontaneous or enzymatic transformation within the body in order to release the active drug and that has improved delivery properties over the parent drug molecule (Bundgaard H, Ed: *Design of Prodrugs*, Elsevier, Amsterdam, 1985).

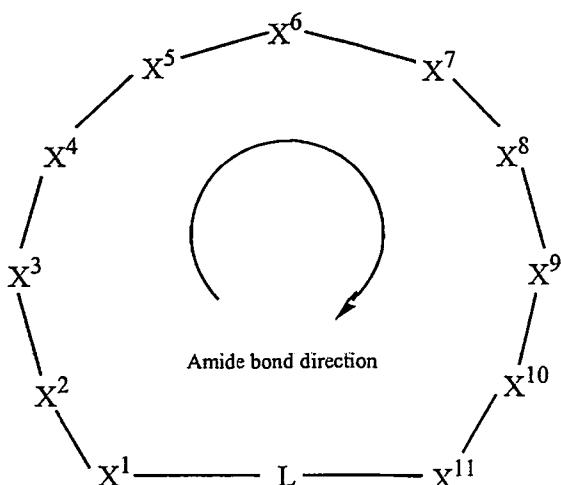
Judicious choice of capping groups allows the addition of other activities on the peptide. For example, the presence of a sulphydryl group linked to the N- or C-terminal cap will permit conjugation of the derivatized peptide to other molecules.

### Cyclic Peptides

Another class of peptides useful as angiogenesis inhibitors in accordance with the present invention are cyclic peptides (which can be considered peptide derivatives if they include additional chemical linkers). The cyclic peptides of this invention include a peptide that has between 4 and about 20 amino acids and has the property of binding to immobilized D5 domain of HK<sub>a</sub> (see above) or larger forms of HK<sub>a</sub> that include D5. The cyclic peptide must also inhibit angiogenesis in any of the known direct or surrogate assays described herein.

Methods for production of cyclic peptides, cyclization of linear peptides, *etc.*, are well-known in the art and are therefore not set forth in detail herein. See, for example, Jones *et al.*, U.S. Pat. 5,942,492; Mazar *et al.*, U.S. Pat. 6,277,818. The contents of these patents are incorporated by reference in their entirety.

A general formula of an 11-mer cyclic peptide is shown below:

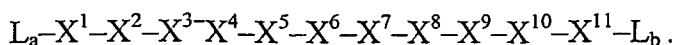


In this general formula, the amide bond (CO—NH) linking  $X^1$  to  $X^2$ , is such that the carbonyl moiety is from amino acid  $X^1$  and the amino moiety is from the amino acid  $X^2$ . The same is true for the link between  $X^2$  and  $X^3$ , and so on within this 11mer peptide. The peptide has  $X^1$  as its N-terminus and  $X^{11}$  as its C-terminus. In a preferred embodiment,  $X^1$  and  $X^{11}$  (the C-terminal residue in the linear formula of the peptide) are Cys residues joined by a disulfide bond, which cyclizes the peptide. In such a case, no additional linker (L) is necessary. Other embodiments employ linkers which are discussed below.

To prepare a compound of the above formula, L is chosen to provide, at one terminus, a functional group that can be chemically bonded to the carboxyl C atom of amino acid  $X^{11}$  and, at the other terminus, a functional group that can be chemically bonded to the  $\alpha$ -amino N atom of amino acid  $X^1$ .

It is preferred that the linker L confer water solubility to the peptide and result in an intramolecular distance of 4-12 Å between the  $C\alpha$  of the N-terminal residue  $X^1$  and the  $C\alpha$  of the C-terminal residue  $X^{11}$ .

Alternatively (again using an 11mer only as an example) the linear peptide  $X^1-X^2-X^3-X^4-X^5-X^6-X^7-X^8-X^9-X^{10}-X^{11}$  can be synthesized with an extension at  $X^{11}$  comprising a portion of the ultimate final linker group L; that extension is termed  $L_b$ . After synthesis of the peptide chain, the  $X^1$  terminus is extended with an extension that will also become part of the ultimate linker; this group is designated  $L_a$ . These steps yield a compound of the formula:



The free ends of  $L_a$  and  $L_b$  are then chemically bonded to each other. In this way, the linker L is formed during the cyclization step from pre-attached fragments  $L_a$  and  $L_b$ . In the examples

given below for L, the direction of L, reading left to right, is from to  $X^1$  to  $X^{11}$ , i.e., the C-terminus of L is bonded to  $X^1$ , and the N-terminus of L is bonded to  $X^{11}$ .

When L includes a Cys, HomoCys, Glu, Asp,  $\gamma$ -carboxyl modified Glu or a  $\beta$ -carboxyl modified Asp residue, the configuration of the enantiomeric center of such a residue can be either L- or D-.

Examples of useful linkers follow.

- L1 -CO-CH<sub>2</sub>-NH-CO-CH<sub>2</sub>-CH<sub>2</sub>-CH(CO-NH-CH<sub>2</sub>-CO-NH<sub>2</sub>)-NH-
- L2 -CO-CH<sub>2</sub>-NH-CO-CH<sub>2</sub>-CH<sub>2</sub>-CH(CO-NH-CH(CH<sub>2</sub>SH)-CO-NH<sub>2</sub>)-NH-
- L3 -CO-CH(CH<sub>2</sub>SH)-NH-CO-CH<sub>2</sub>-CH<sub>2</sub>-CH(CO-NH-CH<sub>2</sub>-CONH<sub>2</sub>)-NH-
- L4 -CO-CH<sub>2</sub>-NH-CO-CH<sub>2</sub>-CH<sub>2</sub>-CH(CO-NH-CH(CH<sub>2</sub>CH<sub>2</sub>SH)-CO-NH<sub>2</sub>)-NH-
- L5 -CO-CH(CH<sub>2</sub>CH<sub>2</sub>SH)-NH-CO-CH<sub>2</sub>-CH<sub>2</sub>-CH(CO-NH-CH<sub>2</sub>-CONH<sub>2</sub>)-NH-
- L6 -CO-CH(CH<sub>2</sub>CH<sub>2</sub>COR<sup>1</sup>)-NH-CO-CH<sub>2</sub>-CH<sub>2</sub>-CH(CO-NH-CH<sub>2</sub>-CONH<sub>2</sub>)-NH-
- L7 -CO-CH(CH<sub>2</sub>COR<sup>1</sup>)-NH-CO-CH<sub>2</sub>-CH<sub>2</sub>-CH(CO-NH-CH<sub>2</sub>-CONH<sub>2</sub>)-NH-
- L8 -CO-CH<sub>2</sub>-NH-CO-CH<sub>2</sub>-CH<sub>2</sub>-CH(CO-NH-CH(CH<sub>2</sub>CH<sub>2</sub>COR<sup>1</sup>)-CO-NH<sub>2</sub>)-NH-
- L9 -CO-CH<sub>2</sub>-NH-CO-CH<sub>2</sub>-CH<sub>2</sub>-CH(CO-NH-CH(CH<sub>2</sub>COR<sup>1</sup>)-CO-NH<sub>2</sub>)-NH-
- L10 -CO-CH<sub>2</sub>-NH-CO-CH<sub>2</sub>-CH<sub>2</sub>-CH(CO-NH-CH<sub>2</sub>-COR<sup>1</sup>)-NH-
- L11 -CO-CH(CH<sub>2</sub>CH<sub>2</sub>COOH)-NH-CO-CH<sub>2</sub>-CH<sub>2</sub>-CH(CO-NH-CH<sub>2</sub>-CONH<sub>2</sub>)-NH-
- L12 -CO-CH(CH<sub>2</sub>COOH)-NH-CO-CH<sub>2</sub>-CH<sub>2</sub>-CH(CO-NH-CH<sub>2</sub>-CONH<sub>2</sub>)-NH-
- L13 -CO-CH<sub>2</sub>-NH-CO-CH<sub>2</sub>-CH<sub>2</sub>-CH(CO-NH-CH(CH<sub>2</sub>CH<sub>2</sub>COOH)-CO-NH<sub>2</sub>)-NH-
- L14 -CO-CH<sub>2</sub>-NH-CO-CH<sub>2</sub>-CH<sub>2</sub>-CH(CO-NH-CH(CH<sub>2</sub>COOH)-CO-NH<sub>2</sub>)-NH-
- L15 -CO-CH<sub>2</sub>-NH-CO-CH<sub>2</sub>-CH<sub>2</sub>-CH(CO-NH-CH<sub>2</sub>-CO-NH-R<sup>1</sup>)-NH-

The R<sup>1</sup> group in L6-L10 is may be a weakly basic diamino group -NH-R<sup>2</sup>-NH<sub>2</sub>, where the pK<sub>a</sub> of each of the primary amino groups in the parent diamine H<sub>2</sub>N-R<sup>2</sup>-NH<sub>2</sub> is less than about 8.0 and where the pK<sub>a</sub> of the primary amino group in -NH-R<sup>2</sup>-NH<sub>2</sub>, when it is part R<sup>1</sup> is also less than about 8.0. Preferred examples of R<sup>2</sup> are *p*-phenylene, *o*-phenylene or *m*-phenylene.

For introducing the R<sup>1</sup> group into linker L15 that is part of cyclic peptide of this invention, a weakly basic amine R<sup>1</sup>NH<sub>2</sub> is preferably bonded to the glycine “spur” (which is the underscored part of L15 shown above). Amines intended for this linker are amine are not specifically limited by structure. Rather, the only requirement is that the pK<sub>a</sub> of its amino group be less than about 8.0. Aniline is a simple and prototypic example of a weakly basic amine, in fact, of the class of aromatic amines that are, in general, always weakly basic. To introduce an aromatic R<sup>1</sup> group, an aromatic amine is used. R<sup>1</sup> may be a homoaryl or a heteroaryl residue, and

may be substituted with one or more substituents drawn from a broad range. The aromatic group may be polycyclic, wherein the various rings may be fused, unfused, or even both fused and unfused. In a polycyclic aromatic group, the rings may be homocyclic or heterocyclic, or even a mixture of both. The ring may be substituted with one or more substituents drawn from a broad range. In a preferred embodiment R<sup>1</sup> in L15 is phenyl or substituted phenyl.

The R<sup>1</sup> group of L15 need not be an aromatic residue to have the requisite property of weak basicity. For example, the class of amines comprise any aromatic residue substituted with an  $\omega$ -(aminoxy)<sub>n</sub>-alkyl group of 1 to 10 carbons – this is described by the formula: H<sub>2</sub>N-O-(CH<sub>2</sub>)<sub>x</sub>- (where x=1-10). Another class of suitable amines are those having the formula H<sub>2</sub>N-CH<sub>2</sub>-CO-NH-(CH<sub>2</sub>)<sub>x</sub>-homoaryl or H<sub>2</sub>N-CH<sub>2</sub>-CO-NH-(CH<sub>2</sub>)<sub>x</sub>-heteroaryl, wherein x=2-10. The homoaryl or heteroaryl residue may be substituted with one or more substituents drawn from a broad range. As above, the homoaryl residue may be polycyclic, fused or unfused or both. The heteroaryl residue may additionally contain a homocyclic ring or more than one homocyclic rings that may be fused, unfused or even both fused and unfused. These compounds described above are non-limiting and are illustrative of the broad structural nature that can be the property of a weakly basic amine included within the scope of this invention.

Three preferred cyclic peptides and results of studies using them are described in Example VII.

#### Production of Peptides and Derivatives

##### General Chemical Synthetic Procedures

The peptides of the invention may be prepared using recombinant DNA technology. However, given their length, they are preferably prepared using solid-phase synthesis, such as that generally described by Merrifield, *J. Amer. Chem. Soc.*, 85:2149-54 (1963), although other equivalent chemical syntheses known in the art are also useful. Solid-phase peptide synthesis may be initiated from the C-terminus of the peptide by coupling a protected  $\alpha$ -amino acid to a suitable resin. Such a starting material can be prepared by attaching an  $\alpha$ -amino-protected amino acid by an ester linkage to a chloromethylated resin or to a hydroxymethyl resin, or by an amide bond to a BHA resin or MBHA resin.

Such methods, well-known in the art, are disclosed, for example, in U.S. 5,994,309 (issued 11/30/1999) which is incorporated by reference in its entirety.

Amino Acid Substitution and Addition Variants

Also included in this invention are peptides in which at least one amino acid residue and preferably, only one, has been removed and a different residue inserted in its place compared to the native sequence. For a detailed description of protein chemistry and structure, see Schulz, GE, *et al.*, *Principles of Protein Structure*, Springer-Verlag, New York, 1979, and Creighton, T.E., *Proteins: Structure and Molecular Principles*, W.H. Freeman & Co., San Francisco, 1984, which are hereby incorporated by reference. The types of substitutions which may be made in the peptide molecule of the present invention are conservative substitutions and are defined herein as exchanges within one of the following groups:

1. Small aliphatic, nonpolar or slightly polar residues: *e.g.*, Ala, Ser, Thr, Gly;
2. Polar, negatively charged residues and their amides: *e.g.*, Asp, Asn, Glu, Gln;
3. Polar, positively charged residues: *e.g.*, His, Arg, Lys;

Pro, because of its unusual geometry, tightly constrains the chain. Substantial changes in functional properties are made by selecting substitutions that are less conservative, such as between, rather than within, the above groups (or two other amino acid groups not shown above), which will differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Most substitutions according to the present invention are those that do not produce radical changes in the characteristics of the peptide molecule. Even when it is difficult to predict the exact effect of a substitution in advance of doing so, one skilled in the art will appreciate that the effect can be evaluated by routine screening assays, preferably the biological assays described below. Modifications of peptide properties including redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation or the tendency to aggregate with carriers or into multimers are assayed by methods well known to the ordinarily skilled artisan.

The present invention provides methods to inhibit or reduce angiogenesis, tumor growth, EC proliferation, EC migration or EC tube formation or to induce EC apoptosis.

The invention also provides pharmaceutical compositions comprising fragments, peptides, conformers, antibodies against, biological equivalents of or derivatives of Tpm or AALBP.

The AALBP may be obtained from Tpm isolated from any appropriate tissue source such as tissue extracts or as a product of a cell line growing in culture that produces "native" Tpm,

preferably cell surface Tpm, or the AALBP fragment of the Tpm, or a “nonnative” Tpm or AALBP that has been genetically modified, or a functional derivative thereof, such that such cells express this polypeptide or a functional derivative thereof such as a domain or shorter fragment.

Tpm fragments or derivatives are chemically synthesized, or produced by recombinant methods. Recombinant techniques known in the art include, but are not limited to DNA amplification using PCR of a cDNA library for example by reverse transcription of mRNA in cells extracts followed by PCR.

Basic texts disclosing general methods of molecular biology, all of which are incorporated by reference, include: Sambrook, J. *et al.*, *Molecular Cloning: A Laboratory Manual*, 2<sup>nd</sup> Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989; Ausubel, F.M. *et al.* *Current Protocols in Molecular Biology*, Vol. 2, Wiley-Interscience, New York, (current edition); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); Glover, D.M., ed, *DNA Cloning: A Practical Approach*, vol. I & II, IRL Press, 1985; Albers, B. *et al.*, *Molecular Biology of the Cell*, 2<sup>nd</sup> Ed., Garland Publishing, Inc., New York, NY (1989); Watson, J.D. *et al.*, *Recombinant DNA*, 2<sup>nd</sup> Ed., Scientific American Books, New York, 1992; and Old, RW *et al.*, *Principles of Gene Manipulation: An Introduction to Genetic Engineering*, 2<sup>nd</sup> Ed., University of California Press, Berkeley, CA (1981).

Fragments of Tpm are be obtained by controlled protease reaction (Borza D-B. *et al.*, *Biochemistry*, 1996, 35; 1925-1934). Chymotrypsin digestion is exemplified herein. Alternatively, Tpm can be subjected to limited plasmin digestion followed by partial reduction with dithiothreitol to create fragments of Tpm bind and block the action of Tpm-binding antiangiogenic agents. These may be useful in situations where it is desirable to promote angiogenesis by blocking endogenous homeostatic mechanisms that might otherwise limit it.

#### Chemical Derivatives of Tpm and AALBP

“Chemical derivatives” of Tpm or AALBP contain additional chemical moieties not normally a part of the protein. Covalent modifications of the polypeptide are included within the scope of this invention. Such derivatized moieties may improve the solubility, absorption, biological half life, and the like. Moieties capable of mediating such effects are disclosed, for example, in *Remington's Pharmaceutical Sciences*, 16<sup>th</sup> ed., Mack Publishing Co., Easton, PA (1980).

Such modifications may be introduced into the molecule by reacting targeted amino acid residues of the polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. Another modification is cyclization of the protein.

Cysteinyl residues most commonly are reacted with  $\alpha$ -haloacetates (and corresponding amines) to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone,  $\alpha$ -bromo- $\beta$ -(5-imidozoyl) propionic acid, chloroacetyl phosphate, N- alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4- nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylprocarbonate (pH 5.5-7.0) which agent is relatively specific for the histidyl side chain. *p*-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are derivatized with succinic or other carboxylic acid anhydrides. Derivatization with a cyclic carboxylic anhydride has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, including phenylglyoxal, 2,3- butanedione, 1,2-cyclohexanedione, and ninhydrin. Such derivatization requires that the reaction be performed in alkaline conditions because of the high  $pK_a$  of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine  $\epsilon$ -amino group.

Modification of tyrosyl residues has permits introduction of spectral labels into a peptide. This is accomplished by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizol and tetranitromethane are used to create O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side groups, aspartyl or glutamyl, may be selectively modified by reaction with carbodiimides (R-N=C=N-R') such as 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues can be converted to asparaginyl and glutaminyl residues by reaction with ammonia.

Aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions. Conversely, glutaminyl and asparaginyl residues may be deamidated to the corresponding glutamyl and aspartyl residues. Deamidation can be performed under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Derivatization with bifunctional agents is useful for cross-linking the peptide to a water-insoluble support matrix or other macromolecular carrier. Commonly used cross-linking agents include 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane.

Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patents 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Other modifications include hydroxylation of proline and lysine, phosphorylation of the hydroxyl groups of seryl or threonyl residues, methylation of the  $\alpha$ -amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, *Proteins: Structure and Molecule Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and, in some instances, amidation of the C-terminal carboxyl groups.

Also included are peptides wherein one or more D-amino acids are substituted for one or more L-amino acids.

### Diagnostic and Prognostic Compositions

The peptides of the invention can be detectably labeled and used, for example, to detect a peptide binding protein ligand or a cellular binding site/receptor (such as the binding sites on activated ECs, tumor cells, *etc.*) whether on the surface or in the interior of a cell. The fate of the peptide during and after binding can be followed *in vitro* or *in vivo* by using the appropriate method to detect the label. The labeled peptide may be utilized *in vivo* for diagnosis and prognosis, for example to image occult metastatic foci or for other types of *in situ* evaluations.

The term "diagnostically labeled" means that the polypeptide or peptide has attached to it a diagnostically detectable label. There are many different labels and methods of labeling

known to those of ordinary skill in the art, described below. General classes of labels which can be used in the present invention include radioactive isotopes, paramagnetic isotopes, and compounds which can be imaged by positron emission tomography (PET), fluorescent or colored compounds, *etc.* Suitable detectable labels include radioactive, fluorescent, fluorogenic, chromogenic, or other chemical labels. Useful radiolabels (radionuclides), which are detected simply by gamma counter, scintillation counter or autoradiography include  $^3\text{H}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{35}\text{S}$  and  $^{14}\text{C}$ .  $^{131}\text{I}$  is also a useful therapeutic isotope (see below).

A number of U.S. patents, incorporated by reference herein, disclose methods and compositions for complexing metals to larger molecules, including description of useful chelating agents. The metals are preferably detectable metal atoms, including radionuclides, and are complexed to proteins and other molecules. These documents include: US 5,627,286 (Heteroatom-bearing ligands and metal complexes thereof); US 5,618,513 (Method for preparing radiolabeled peptides); US 5,567,408; US 5,443,816 (Peptide-metal ion pharmaceutical preparation and method); US 5,561,220 (Tc- $^{99\text{m}}$  labeled peptides for imaging inflammation).

Common fluorescent labels include fluorescein, rhodamine, dansyl, phycoerythrin, phycocyanin, allophycocyanin, *o*-phthaldehyde and fluorescamine. The fluorophore, such as the dansyl group, must be excited by light of a particular wavelength to fluoresce. See, for example, Haugland, *Handbook of Fluorescent Probes and Research Chemicals*, Sixth Ed., Molecular Probes, Eugene, OR., 1996). Fluorescein, fluorescein derivatives and fluorescein-like molecules such as Oregon Green<sup>TM</sup> and its derivatives, Rhodamine Green<sup>TM</sup> and Rhodol Green<sup>TM</sup>, are coupled to amine groups using the isothiocyanate, succinimidyl ester or dichlorotriazinyl-reactive groups. Similarly, fluorophores may also be coupled to thiols using maleimide, iodoacetamide, and aziridine-reactive groups. The long wavelength rhodamines, which are basically Rhodamine Green<sup>TM</sup> derivatives with substituents on the nitrogens, are among the most photostable fluorescent labeling reagents known. Their spectra are not affected by changes in pH between 4 and 10, an important advantage over the fluoresceins for many biological applications. This group includes the tetramethylrhodamines, X-rhodamines and Texas Red<sup>TM</sup> derivatives. Other preferred fluorophores for derivatizing the peptide according to this invention are those which are excited by ultraviolet light. Examples include cascade blue, coumarin derivatives, naphthalenes (of which dansyl chloride is a member), pyrenes and pyridyloxazole derivatives. Also included as labels are two related inorganic materials that have recently been

described: semiconductor nanocrystals, comprising, for example, cadmium sulfate (Bruchez, M. *et al.*, *Science* 281:2013-2016 (1998), and quantum dots, *e.g.*, zinc-sulfide-capped Cd selenide (Chan, W.C.W. *et al.*, *Science* 281:2016-2018 (1998)).

In yet another approach, the amino group of the peptide is allowed to react with reagents that yield fluorescent products, for example, fluorescamine, dialdehydes such as *o*-phthaldialdehyde, naphthalene-2,3-dicarboxylate and anthracene-2,3-dicarboxylate. 7-nitrobenz-2-oxa-1,3-diazole (NBD) derivatives, both chloride and fluoride, are useful to modify amines to yield fluorescent products.

The peptides of the invention can also be labeled for detection using fluorescence-emitting metals such as <sup>152</sup>Eu, or others of the lanthanide series. These metals can be attached to the peptide using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA, see Example X, *infra*) or ethylenediaminetetraacetic acid (EDTA). DTPA, for example, is available as the anhydride, which can readily modify the NH<sub>2</sub>-containing peptides of this invention.

For *in vivo* diagnosis or therapy, radionuclides may be bound to the peptide either directly or indirectly using a chelating agent such as DTPA and EDTA. Examples of such radionuclides are <sup>99</sup>Tc, <sup>123</sup>I, <sup>125</sup>I, <sup>131</sup>I, <sup>111</sup>In, <sup>97</sup>Ru, <sup>67</sup>Cu, <sup>67</sup>Ga, <sup>68</sup>Ga, <sup>72</sup>As, <sup>89</sup>Zr, <sup>90</sup>Y and <sup>201</sup>Tl. Generally, the amount of labeled peptide needed for detectability in diagnostic use will vary depending on considerations such as age, condition, sex, and extent of disease in the patient, contraindications, if any, and other variables, and is to be adjusted by the individual physician or diagnostician. Dosage can vary from 0.01 mg/kg to 100 mg/kg.

The peptide can also be made detectable by coupling to a phosphorescent or a chemiluminescent compound. The presence of the chemiluminescent-tagged peptide is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescers are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester. Likewise, a bioluminescent compound may be used to label the peptides. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

In yet another embodiment, colorimetric detection is used, based on chromogenic compounds which have, or result in, chromophores with high extinction coefficients.

*In situ* detection of the labeled peptide may be accomplished by removing a histological specimen from a subject and examining it by microscopy under appropriate conditions to detect the label. Those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

For diagnostic *in vivo* radioimaging, the type of detection instrument available is a major factor in selecting a radionuclide. The radionuclide chosen must have a type of decay which is detectable by a particular instrument. In general, any conventional method for visualizing diagnostic imaging can be utilized in accordance with this invention. Another factor in selecting a radionuclide for *in vivo* diagnosis is that its half-life be long enough so that the label is still detectable at the time of maximum uptake by the target tissue, but short enough so that deleterious irradiation of the host is minimized. In one preferred embodiment, a radionuclide used for *in vivo* imaging does not emit particles, but produces a large number of photons in a 140-200 keV range, which may be readily detected by conventional gamma cameras.

*In vivo* imaging may be used to detect occult metastases which are not observable by other methods if such metastases express cell surface Tpm. Imaging could be used to stage tumors non-invasively or to detect other diseases which are associated with the presence of increased levels of surface Tpm by binding with anti-Tpm antibodies or ligands for the Tpm such as HPRG or HKa D5.

### Peptidomimetics

A preferred type of chemical derivative of the peptides described herein is a peptidomimetic compound which mimics the biological effects of Tpm, AALBP or of a biologically active peptide thereof. A peptidomimetic agent may be an unnatural peptide or a non-peptide agent that recreates the stereospatial properties of the binding elements of Tpm such that it has the binding activity or biological activity of Tpm. Similar to biologically active peptides, a peptidomimetic will have a binding face (which interacts with any ligand to which Tpm binds) and a non-binding face. Again, similar to Tpm or its peptide, the non-binding face of a peptidomimetic will contain functional groups which can be modified by various therapeutic and diagnostic moieties without modifying the binding face of the peptidomimetic. A preferred embodiment of a peptidomimetic would contain an aniline on the non-binding face of the molecule. The NH<sub>2</sub>-group of an aniline has a pKa ~ 4.5 and could therefore be modified by any NH<sub>2</sub> - selective reagent without modifying any NH<sub>2</sub> functional groups on the binding face of the peptidomimetic. Other peptidomimetics may not have any NH<sub>2</sub> functional groups on their

binding face and therefore, any  $\text{NH}_2$ , without regard for  $\text{pK}_a$  could be displayed on the non-binding face as a site for conjugation. In addition other modifiable functional groups, such as -SH and -COOH could be incorporated into the non-binding face of a peptidomimetic as a site of conjugation. A therapeutic or diagnostic moiety could also be directly incorporated during the synthesis of a peptidomimetic and preferentially be displayed on the non-binding face of the molecule.

This invention also includes compounds that retain partial peptide characteristics. For example, any proteolytically unstable bond within a peptide of the invention could be selectively replaced by a non-peptidic element such as an isostere (N-methylation; D-amino acid) or a reduced peptide bond while the rest of the molecule retains its peptide nature.

Peptidomimetic compounds, either agonists, substrates or inhibitors, have been described for a number of bioactive peptides such as opioid peptides, VIP, thrombin, HIV protease, *etc.* Methods for designing and preparing peptidomimetic compounds are known in the art (Hruby, V.J., *Biopolymers* 33:1073-1082 (1993); Wiley, R.A. *et al.*, *Med. Res. Rev.* 13:327-384 (1993); Moore *et al.*, *Adv. in Pharmacol* 33:91-141 (1995); Giannis *et al.*, *Adv. in Drug Res.* 29:1-78 (1997), which references are incorporated by reference in their entirety). These methods are used to make peptidomimetics that possess at least the binding capacity and specificity of the HPRG peptides and preferably also possess the biological activity. Knowledge of peptide chemistry and general organic chemistry available to those skilled in the art are sufficient, in view of the present disclosure, for designing and synthesizing such compounds.

For example, such peptidomimetics may be identified by inspection of the crystallographically-derived three-dimensional structure of a peptide of the invention either free or bound in complex with a ligand such as (a) heparin, plasminogen, fibrinogen, vitronectin and thrombospondin or (b) small ligands, such as heme and transition metal ions (zinc, copper and nickel). Alternatively, the structure of a peptide of the invention bound to its ligand can be gained by the techniques of nuclear magnetic resonance spectroscopy. The better knowledge of the stereochemistry of the interaction of the peptide with its ligand or receptor will permit the rational design of such peptidomimetic agents. The structure of a peptide or protein of the invention in the absence of ligand could also provide a scaffold for the design of mimetic molecules.

**Antibodies Specific for Epitopes of Tropomyosin**

The present invention provides antibodies, both polyclonal and monoclonal, reactive with an epitope of Tpm, preferably, an epitope of the AALBP fragment. These anti-Tpm antibodies may be xenogeneic, allogeneic, syngeneic, or modified forms thereof, such as humanized or chimeric antibodies. Antiidiotypic antibodies specific for the idiotype of an anti-Tpm antibody are also included.

In the following description, reference will be made to various methodologies known to those of skill in the art of immunology. Publications and other materials setting forth such known methodologies to which reference is made are incorporated herein by reference in their entireties as though set forth in full. Standard reference works setting forth the general principles of immunology include A.K. Abbas *et al.*, *Cellular and Molecular Immunology* (Fourth Ed.), W.B. Saunders Co., Philadelphia, 2000; C.A. Janeway *et al.*, *Immunobiology. The Immune System in Health and Disease*, Fourth ed., Garland Publishing Co., New York, 1999; Roitt, I. *et al.*, *Immunology*, (current ed.) C.V. Mosby Co., St. Louis, MO (1999); Klein, J., *Immunology*, Blackwell Scientific Publications, Inc., Cambridge, MA, (1990).

Monoclonal antibodies (mAbs) and methods for their production and use are described in Kohler and Milstein, *Nature* 256:495-497 (1975); U.S. Patent No. 4,376,110; Hartlow, E. *et al.*, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988); *Monoclonal Antibodies and Hybridomas: A New Dimension in Biological Analyses*, Plenum Press, New York, NY (1980); H. Zola *et al.*, in *Monoclonal Hybridoma Antibodies: Techniques and Applications*, CRC Press, 1982)).

Anti-idiotypic antibodies are described, for example, in *Idiotype in Biology and Medicine*, Academic Press, New York, 1984; *Immunological Reviews* Volume 79, 1984; *Immunological Reviews* Volume 90, 1986; *Curr. Top. Microbiol., Immunol.* Volume 119, 1985; Bona, C. *et al.*, *CRC Crit. Rev. Immunol.*, pp. 33-81 (1981); Jerne, NK, *Ann. Immunol.* 125C:373-389 (1974); Jerne, NK, In: *Idiotypes - Antigens on the Inside*, Westen-Schnurr, I., ed., Editiones Roche, Basel, 1982, Urbain, J *et al.*, *Ann. Immunol.* 133D:179- (1982); Rajewsky, K. *et al.*, *Ann. Rev. Immunol.* 1:569-607 (1983)

The term "antibody" is also meant to include both intact molecules as well as fragments thereof that include the antigen-binding site and are capable of binding to a Tpm epitope. These include, Fab and F(ab')<sub>2</sub> fragments which lack the Fc fragment of an intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact

antibody (Wahl *et al.*, *J. Nucl. Med.* 24:316-325 (1983)). Also included are Fv fragments (Hochman, J. *et al.* (1973) *Biochemistry* 12:1130-1135; Sharon, J. *et al.* (1976) *Biochemistry* 15:1591-1594.). These various fragments are to be produced using conventional techniques such as protease cleavage or chemical cleavage (see, *e.g.*, Rousseaux *et al.*, *Meth. Enzymol.*, 121:663-69 (1986))

Polyclonal antibodies are obtained as sera from immunized animals such as rabbits, goats, rodents, *etc.* and may be used directly without further treatment or may be subjected to conventional enrichment or purification methods such as ammonium sulfate precipitation, ion exchange chromatography, and affinity chromatography (see Zola *et al.*, *supra*).

The immunogen used to produce the present anti-Tpm antibodies may comprise the complete Tpm protein, or fragments or derivatives thereof. Preferred immunogens comprise all or a part of the AALBP central domain of Tpm. Immunogens comprising this domain are produced in a variety of ways known in the art, *e.g.*, expression of cloned genes using conventional recombinant methods, isolation from cells of origin, cell populations expressing high levels of Tpm, *etc.*

The mAbs may be produced using conventional hybridoma technology, such as the procedures introduced by Kohler and Milstein (*supra*) and modifications thereof (see above references). An animal, preferably a mouse is primed by immunization with an immunogen as above to elicit the desired antibody response in the primed animal.

B lymphocytes from the lymph nodes, spleens or peripheral blood of a primed, animal are fused with myeloma cells, generally in the presence of a fusion promoting agent such as polyethylene glycol (PEG). Any of a number of murine myeloma cell lines are available for such use: the P3-NS1/1-Ag4-1, P3-x63-Ag8.653, Sp2/0-Ag14, or HL1-653 myeloma lines (available from the ATCC, Rockville, MD). Subsequent steps include growth in selective medium so that unfused parental myeloma cells and donor lymphocyte cells eventually die while only the hybridoma cells survive. These are cloned and grown and their supernatants screened for the presence of antibody of the desired specificity, *e.g.*, by immunoassay techniques using the Tpm protein. Positive clones are subcloned, *e.g.*, by limiting dilution, and the mAbs are isolated.

Hybridomas produced according to these methods can be propagated *in vitro* or *in vivo* (in ascites fluid) using techniques known in the art (see generally Fink *et al.*, *Prog. Clin. Pathol.*, 9:121-33 (1984)). Generally, the individual cell line is propagated in culture and the culture

medium containing high concentrations of a single mAb can be harvested by decantation, filtration, or centrifugation.

The antibody may be produced as a single chain antibody or scFv instead of the normal multimeric structure. Single chain antibodies include the hypervariable regions from an Ig of interest and recreate the antigen binding site of the native Ig while being a fraction of the size of the intact Ig (Skerra, A. *et al.* (1988) *Science*, 240: 1038-1041; Pluckthun, A. *et al.* (1989) *Methods Enzymol.* 178: 497-515; Winter, G. *et al.* (1991) *Nature*, 349: 293-299); Bird *et al.*, (1988) *Science* 242:423; Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879; Jost CR *et al.*, *J Biol Chem.* 1994 269:26267-26273; U.S. Patents No. 4,704,692, 4,853,871, 4,94,6778, 5,260,203, 5,455,030). DNA sequences encoding the V regions of the H chain and the L chain are ligated to a linker encoding at least about 4 amino acids (typically small neutral amino acids). The protein encoded by this fusion allows assembly of a functional variable region that retains the specificity and affinity of the original antibody.

For *in vivo* use, particularly for injection into humans, it is desirable to decrease the immunogenicity of the mAb by humanizing the antibodies using methods known in the art. The humanized antibody may be the product of an animal having transgenic human Ig Constant region genes (see for example WO 90/10077 and WO 90/04036). Alternatively, the antibody of interest may be genetically engineered to substitute the CH<sub>1</sub>, CH<sub>2</sub>, CH<sub>3</sub>, hinge domains, and/or the framework domain with the corresponding human sequence (see WO 92/02190).

Antibodies can be selected for particular desired properties. In the case of an antibody to be used for therapy, antibody screening procedures can include any of the *in vitro* or *in vivo* bioassays that measure angiogenesis, cell invasion, and the like. Moreover, the antibodies may be screened in various of the tumor models described herein to see if they promote or inhibit angiogenesis (or resultant tumor growth or metastasis). In this way, antibodies that are Tpm mimics or antagonists can be selected. Thus, the present invention includes therapeutic antibodies (discussed in more detail below) that promote angiogenesis by binding to and otherwise inhibiting the action of an antiangiogenic ligand at Tpm or its AALBP "domain."

#### Use of Antibodies to Detect Cell Surface or Free Tpm or a AALBP Fragment

Antibodies specific for an epitope of Tpm are useful in immunoassays to detect molecules containing these epitopes on the surface of a cell or in a body fluid or sample, preferably serum or plasma. Such antibodies would detect Tpm, or an epitope-bearing fragment

of Tpm. Thus, if proteolysis in the tumor milieu results in release of Tpm or the AALBP in plasma or in tissue.

By measuring the levels of released Tpm from tumor cells or activated ECs, the antibodies and immunoassays of this invention are used diagnostically to monitor the progress of a disease, where Tpm levels may reflect the amount of tumor tissue present.

Any conventional immunoassay known in the art may be employed for this purpose, though Enzyme Immunoassays such as ELISA are preferred. Immunoassay methods are also described in Coligan, JE *et al.*, eds., *Current Protocols in Immunology*, Wiley-Interscience, New York 1991(or current edition); Butt, W.R. (ed.) *Practical Immunoassay: The State of the Art*, Dekker, New York, 1984; Bizollon, Ch. A., ed., *Monoclonal Antibodies and New Trends in Immunoassays*, Elsevier, New York, 1984; Butler, J.E., ELISA (Chapter 29), In: van Oss, CJ *et al.*, (eds), *IMMUNOCHEMISTRY*, Marcel Dekker, Inc., New York, 1994, pp. 759-803; Butler, J.E. (ed.), *Immunochemistry of Solid-Phase Immunoassay*, CRC Press, Boca Raton, 1991; Weintraub, B., *Principles of Radioimmunoassays*, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986; Work, TS *et al.*, *Laboratory Techniques and Biochemistry in Molecular Biology*, North Holland Publishing Company, NY, (1978) (Chapter by Chard, T., "An Introduction to Radioimmune Assay and Related Techniques").

### **In Vitro Testing of Compositions**

#### **A. Binding to immobilized Tpm in a 96 well plate.**

Binding to immobilized Tpm is carried out either by a competition assay with a known ligand, biotin-HKa, or by direct binding of the corresponding biotinylated protein. Plates are coated at room temperature with chicken gizzard Tpm (Sigma) in Tris buffer-saline (TBS) (200 ng/well). After incubation for 2 hours, wells are washed with TBS, then 1%BSA/TBS/Tween-20 is added to each well and incubate at 37°C for two hours. HKa (Enzyme Laboratories) that had been previously biotinylated with EZ-Link (Pierce) according to the manufacturers instructions, is added to the plate at a concentration of 10 nM together with ZnCl<sub>2</sub> (10 μM) and the appropriate concentration of the protein, domain or peptide competitor. The plates are incubated at room temperature and then washed with TBS/Tween-20. Avidin-HRP is added, incubated for 20 minutes at room temperature, washed with TBS/Tween-20 and the chromogenic substrate is added. The reaction is stopped with sulfuric acid and the plate read at 490 nm.

### B. Assay for EC migration

For EC migration, transwells are coated with type I collagen (50 µg/mL) by adding 200 µL of the collagen solution per transwell, then incubating overnight at 37°C. The transwells are assembled in a 24-well plate and a chemoattractant (e.g., FGF-2) is added to the bottom chamber in a total volume of 0.8 mL media. ECs, such as HUVEC, which have been detached from monolayer culture using trypsin, are diluted to a final concentration of about 10<sup>6</sup> cells/mL with serum-free media and 0.2 mL of this cell suspension is added to the upper chamber of each transwell. Inhibitors to be tested are added to both the upper and lower chambers, and the migration is allowed to proceed for 5 hrs in a humidified atmosphere at 37°C. The transwells are removed from the plate stained using DiffQuik®. Cells which did not migrate are removed from the upper chamber by scraping with a cotton swab and the membranes are detached, mounted on slides, and counted under a high-power field (400x) to determine the number of cells migrated.

### C. Biological Assay of Anti-Invasive Activity

The compositions of the invention are tested for their anti-invasive capacity. The ability of cells such as ECs or tumor cells (e.g., PC-3 human prostatic carcinoma) cells to invade through a reconstituted basement membrane (Matrigel®) in an assay known as a Matrigel® invasion assay system as described in detail by Kleinman *et al.*, *Biochemistry* 25: 312-318, 1986 and Parish *et al.*, *Int. J. Cancer* 52:378-383, 1992. Matrigel® is a reconstituted basement membrane containing type IV collagen, laminin, heparan sulfate proteoglycans such as perlecan, which bind to and localize bFGF, vitronectin as well as transforming growth factor-β (TGFβ), urokinase-type plasminogen activator (uPA), tissue plasminogen activator (tPA), and the serpin known as plasminogen activator inhibitor type 1 (PAI-1) (Chambers *et al.*, *Canc. Res.* 55:1578-1585, 1995). It is accepted in the art that results obtained in this assay for compounds which target extracellular receptors or enzymes are predictive of the efficacy of these compounds *in vivo* (Rabbani *et al.*, *Int. J. Cancer* 63: 840-845, 1995).

Such assays employ transwell tissue culture inserts. Invasive cells are defined as cells which are able to traverse through the Matrigel® and upper aspect of a polycarbonate membrane and adhere to the bottom of the membrane. Transwells (Costar) containing polycarbonate membranes (8.0 µm pore size) are coated with Matrigel® (Collaborative Research), which has been diluted in sterile PBS to a final concentration of 75 µg/mL (60 µL of diluted Matrigel® per

insert), and placed in the wells of a 24-well plate. The membranes are dried overnight in a biological safety cabinet, then rehydrated by adding 100  $\mu$ L of DMEM containing antibiotics for 1 hour on a shaker table. The DMEM is removed from each insert by aspiration and 0.8 mL of DMEM/10 % FBS/antibiotics is added to each well of the 24-well plate such that it surrounds the outside of the transwell ("lower chamber"). Fresh DMEM/ antibiotics (100 $\mu$ L), human Glu-plasminogen (5  $\mu$ g/mL), and any inhibitors to be tested are added to the top, inside of the transwell ("upper chamber"). The cells which are to be tested are trypsinized and resuspended in DMEM/antibiotics, then added to the top chamber of the transwell at a final concentration of 800,000 cells/mL. The final volume of the upper chamber is adjusted to 200  $\mu$ L. The assembled plate is then incubated in a humid 5% CO<sub>2</sub> atmosphere for 72 hours. After incubation, the cells are fixed and stained using DiffQuik® (Giemsa stain) and the upper chamber is then scraped using a cotton swab to remove the Matrigel® and any cells which did not invade through the membrane. The membranes are detached from the transwell using an X-acto® blade, mounted on slides using Permount® and cover-slips, then counted under a high-powered (400x) field. An average of the cells invaded is determined from 5-10 fields counted and plotted as a function of inhibitor concentration.

#### D. Tube-Formation Assays of Anti-Angiogenic Activity

The compounds of this invention are tested for their anti-angiogenic activity in one of two different assay systems *in vitro*.

ECs, for example, HUVEC or human microvascular ECs (HMVEC) which can be prepared or obtained commercially, are mixed at a concentration of  $2 \times 10^5$  cells/mL with fibrinogen (5mg/mL in phosphate buffered saline (PBS) in a 1:1 (v/v) ratio. Thrombin is added (5 units/ mL final concentration) and the mixture is immediately transferred to a 24-well plate (0.5 mL per well). The fibrin gel is allowed to form and then VEGF and bFGF are added to the wells (each at 5 ng/mL final concentration) along with the test compound. The cells are incubated at 37°C in 5% CO<sub>2</sub> for 4 days at which time the cells in each well are counted and classified as either rounded, elongated with no branches, elongated with one branch, or elongated with 2 or more branches. Results are expressed as the average of 5 different wells for each concentration of compound. Typically, in the presence of angiogenic inhibitors, cells remain either rounded or form undifferentiated tubes (e.g. 0 or 1 branch).

This assay is recognized in the art to be predictive of angiogenic (or anti-angiogenic) efficacy *in vivo* (Min, HY *et al.*, *Cancer Res.* 56: 2428-2433, 1996).

In an alternate assay, EC tube formation is observed when ECs are cultured on Matrigel® (Schnaper *et al.*, *J. Cell. Physiol.* 165:107-118 1995). ECs ( $1 \times 10^4$  cells/well) are transferred onto Matrigel®-coated 24-well plates, and tube formation is quantitated after 48 hrs. Inhibitors are tested by adding them either at the same time as the ECs or at various time points thereafter. Tube formation can also be stimulated by adding (a) angiogenic growth factors such as bFGF or VEGF, (b) differentiation stimulating agents (*e.g.*, PMA) or (c) a combination of these.

This assay models angiogenesis by presenting to the ECs a particular type of basement membrane, namely the layer of matrix which migrating and differentiating ECs might be expected to first encounter. In addition to bound growth factors, the matrix components found in Matrigel® (and in basement membranes *in situ*) or proteolytic products thereof may also be stimulatory for EC tube formation which makes this model complementary to the fibrin gel angiogenesis model previously described (Blood *et al.*, *Biochim. Biophys. Acta* 1032:89-118, 1990; Odedra *et al.*, *Pharmac. Ther.* 49:111-124, 1991). The compounds of this invention inhibit EC tube formation in both assays, which suggests that the compounds will also have anti-angiogenic activity.

#### E. Assays for the Inhibition of Proliferation

The ability of the compounds of the invention to inhibit the proliferation of EC's may be determined in a 96-well format. Type I collagen (gelatin) is used to coat the wells of the plate (0.1-1 mg/mL in PBS, 0.1 mL per well for 30 minutes at room temperature). After washing the plate (3x w/PBS), 3-6,000 cells are plated per well and allowed to attach for 4 hrs ( $37^{\circ}\text{C}/5\% \text{CO}_2$ ) in Endothelial Growth Medium (EGM; Clonetics) or M199 media containing 0.1-2% FBS. The media and any unattached cells are removed at the end of 4 hrs and fresh media containing bFGF (1-10 ng/mL) or VEGF (1-10 ng/mL) is added to each well. Compounds to be tested are added last and the plate is allowed to incubate ( $37^{\circ}\text{C}/5\% \text{CO}_2$ ) for 24-48 hrs. MTS (Promega) is added to each well and allowed to incubate from 1-4 hrs. The absorbance at 490nm, which is proportional to the cell number, is then measured to determine the differences in proliferation between control wells and those containing test compounds.

A similar assay system can be set up with cultured adherent tumor cells. However, collagen may be omitted in this format. Tumor cells (*e.g.*, 3,000-10,000/well) are plated and

allowed to attach overnight. Serum free medium is then added to the wells, and the cells are synchronized for 24 hrs. Medium containing 10% FBS is then added to each well to stimulate proliferation. Compounds to be tested are included in some of the wells. After 24 hrs, MTS is added to the plate and the assay developed and read as described above.

#### F. Assays of Cytotoxicity

The anti-proliferative and cytotoxic effects of the compositions may be determined for various cell types including tumor cells, ECs, fibroblasts and macrophages. This is especially useful when testing a compound of the invention which has been conjugated to a therapeutic moiety such as a radiotherapeutic or a toxin. For example, a conjugate of one of the compositions with Bolton-Hunter reagent which has been iodinated with  $^{131}\text{I}$  would be expected to inhibit the proliferation of cells expressing surface Tpm or AALBP (most likely by inducing apoptosis). Anti-proliferative effects would be expected against tumor cells and stimulated ECs but, under some circumstances not quiescent ECs or normal human dermal fibroblasts. Any anti-proliferative or cytotoxic effects observed in the normal cells would represent non-specific toxicity of the conjugate.

A typical assay would involve plating cells at a density of 5-10,000 cells per well in a 96-well plate. The compound to be tested is added at a concentration 10x the  $\text{IC}_{50}$  measured in a binding assay (this will vary depending on the conjugate) and allowed to incubate with the cells for 30 minutes. The cells are washed 3X with media, then fresh media containing [ $^3\text{H}$ ]thymidine (1  $\mu\text{Ci}/\text{mL}$ ) is added to the cells and they are allowed to incubate at 37°C in 5%  $\text{CO}_2$  for 24 and 48 hours. Cells are lysed at the various time points using 1 M NaOH and counts per well determined using a  $\beta$ -counter. Proliferation may be measured non-radioactively using MTS reagent or CyQuant<sup>®</sup> to measure total cell number. For cytotoxicity assays (measuring cell lysis), a Promega 96-well cytotoxicity kit is used. If there is evidence of anti-proliferative activity, induction of apoptosis may be measured using TumorTACS (Genzyme).

#### G. Caspase-3 activity

The ability of the compounds of the invention to promote apoptosis of EC's may be determined by measuring activation of caspase-3. Type I collagen (gelatin) is used to coat a P100 plate and  $5 \times 10^5$  ECs are seeded in EGM containing 10% FBS. After 24 hours (at 37°C in 5%  $\text{CO}_2$ ) the medium is replaced by EGM containing 2% FBS, 10 ng/ml bFGF and the desired test compound. The cells are harvested after 6 hours, cell lysates prepared in 1% Triton and

assayed using the EnzChek®Caspase-3 Assay Kit #1 (Molecular Probes) according to the manufacturers' instructions.

### **In Vivo Study of Tpm-Binding Antiangiogenic Polypeptides or Peptides**

#### **A. Corneal Angiogenesis Model**

The protocol used is essentially identical to that described by Volpert *et al.* (*J. Clin. Invest.* 98:671-679 (1996)). Briefly, female Fischer rats (120-140 gms) are anesthetized and pellets (5  $\mu$ l) comprised of Hydron®, bFGF (150 nM), and the compounds to be tested are implanted into tiny incisions made in the cornea 1.0-1.5 mm from the limbus. Neovascularization is assessed at 5 and 7 days after implantation. On day 7, animals are anesthetized and infused with a dye such as colloidal carbon to stain the vessels. The animals are then euthanized, the corneas fixed with formalin, and the corneas flattened and photographed to assess the degree of neovascularization. Neovessels may be quantitated by imaging the total vessel area or length or simply by counting vessels.

#### **B. Matrigel® Plug Assay**

This assay is performed essentially as described by Passaniti *et al.* (*Lab Invest.* 67:519-528 (1992)). Ice-cold Matrigel® (*e.g.*, 500  $\mu$ L) (Collaborative Biomedical Products, Inc., Bedford, MA) is mixed with heparin (*e.g.*, 50  $\mu$ g/ml), FGF-2 (*e.g.*, 400 ng/ml) and the compound to be tested. In some assays, bFGF may be substituted with tumor cells as the angiogenic stimulus. The Matrigel® mixture is injected subcutaneously into 4-8 week-old athymic nude mice at sites near the abdominal midline, preferably 3 injections per mouse. The injected Matrigel® forms a palpable solid gel. Injection sites are chosen such that each animal receives a positive control plug (such as FGF-2 + heparin), a negative control plug (*e.g.*, buffer + heparin) and a plug that includes the compound being tested for its effect on angiogenesis, *e.g.*, (FGF-2 + heparin + compound). All treatments are preferably run in triplicate. Animals are sacrificed by cervical dislocation at about 7 days post injection or another time that may be optimal for observing angiogenesis. The mouse skin is detached along the abdominal midline, and the Matrigel® plugs are recovered and scanned immediately at high resolution. Plugs are then dispersed in water and incubated at 37°C overnight. Hemoglobin (Hb) levels are determined using Drabkin's solution (*e.g.*, obtained from Sigma) according to the manufacturers' instructions. The amount of Hb in the plug is an indirect measure of angiogenesis as it reflects the amount of blood in the sample. In addition, or alternatively,

animals may be injected prior to sacrifice with a 0.1 ml buffer (preferably PBS) containing a high molecular weight dextran to which is conjugated a fluorophore. The amount of fluorescence in the dispersed plug, determined fluorimetrically, also serves as a measure of angiogenesis in the plug. Staining with mAb anti-CD31 (CD31 is "platelet-EC adhesion molecule or PECAM") may also be used to confirm neovessel formation and microvessel density in the plugs.

C. Chick chorioallantoic membrane (CAM) angiogenesis assay

This assay is performed essentially as described by Nguyen *et al.* (*Microvascular Res.* 47:31-40 (1994)). A mesh containing either angiogenic factors (bFGF) or tumor cells plus inhibitors is placed onto the CAM of an 8-day old chick embryo and the CAM observed for 3-9 days after implantation of the sample. Angiogenesis is quantitated by determining the percentage of squares in the mesh which contain blood vessels.

D. In Vivo Assessment Angiogenesis Inhibition and Anti-Tumor Effects Using the Matrigel® Plug Assay with Tumor Cells

In this assay, tumor cells, for example  $1-5 \times 10^6$  cells of the 3LL Lewis lung carcinoma or the rat prostate cell line MatLyLu, are mixed with Matrigel® and then injected into the flank of a mouse following the protocol described in Sec. B., above. A mass of tumor cells and a powerful angiogenic response can be observed in the plugs after about 5 to 7 days. The anti-tumor and anti-angiogenic action of a compound in an actual tumor environment can be evaluated by including it in the plug. Measurement is then made of tumor weight, Hb levels or fluorescence levels (of a dextran-fluorophore conjugate injected prior to sacrifice). To measure Hb or fluorescence, the plugs are first homogenized with a tissue homogenizer.

E. Xenograft model of subcutaneous (s.c.) tumor growth

Nude mice are inoculated with MDA-MB-231 cells (human breast carcinoma) and Matrigel® ( $1 \times 10^6$  cells in 0.2mL) s.c. in the right flank of the animals. The tumors are staged to  $200 \text{ mm}^3$  and then treatment with a test composition is initiated (100 $\mu\text{g}/\text{animal/day}$  given q.d. IP). Tumor volumes are obtained every other day and the animals are sacrificed after 2 weeks of treatment. The tumors are excised, weighed and paraffin embedded. Histological sections of the tumors are analyzed by H and E, anti-CD31, Ki-67, TUNEL, and CD68 staining.

F. Xenograft Model of Metastasis

The compounds of this invention are also tested for inhibition of late metastasis using an experimental metastasis model (Crowley, CW *et al.*, *Proc. Natl. Acad. Sci. USA* 90:5021-5025 (1993)). Late metastasis involves the steps of attachment and extravasation of tumor cells, local invasion, seeding, proliferation and angiogenesis. Human prostatic carcinoma cells (PC-3) transfected with a reporter gene, preferably the green fluorescent protein (GFP) gene, but as an alternative with a gene encoding the enzymes chloramphenicol acetyl-transferase (CAT), luciferase or LacZ, are inoculated into nude mice. This approach permits utilization of either of these markers (fluorescence detection of GFP or histochemical colorimetric detection of enzymatic activity) to follow the fate of these cells. Cells are injected, preferably iv, and metastases identified after about 14 days, particularly in the lungs but also in regional lymph nodes, femurs and brain. This mimics the organ tropism of naturally occurring metastases in human prostate cancer. For example, GFP-expressing PC-3 cells ( $1 \times 10^6$  cells per mouse) are injected iv into the tail veins of nude (*nu/nu*) mice. Animals are treated with a test composition at 100 $\mu$ g/animal/day given q.d. IP. Single metastatic cells and foci are visualized and quantitated by fluorescence microscopy or light microscopic histochemistry or by grinding the tissue and quantitative colorimetric assay of the detectable label.

G. Inhibition of Spontaneous Metastasis *In Vivo* by Antiangiogenic Agents acting at Cell Surface Tropomyosin

The rat syngeneic breast cancer system (Xing *et al.*, *Int. J. Cancer* 67:423-429 (1996) employs Mat BIII rat breast cancer cells. Tumor cells, for example about  $10^6$  suspended in 0.1 mL PBS, are inoculated into the mammary fat pads of female Fisher rats. At the time of inoculation, a 14-day Alza osmotic mini-pump is implanted intraperitoneally to dispense the test compound. The compound is dissolved in PBS (e.g., 200 mM stock), sterile filtered and placed in the minipump to achieve a release rate of about 4 mg/kg/day. Control animals receive vehicle (PBS) alone or a vehicle control peptide in the minipump. Animals are sacrificed at about day 14.

Therapeutic Outcomes

In the rats treated with the active compounds of the present invention, significant reductions in the size of the primary tumor and in the number of metastases in the spleen, lungs, liver, kidney and lymph nodes (enumerated as discrete foci) are observed. Histological and immunohistochemical analysis reveal increased necrosis and signs of apoptosis in tumors in

treated animals. Large necrotic areas are seen in tumor regions lacking neovascularization. Human or rabbit HPRG or HKa or the D5 domain and their derivatives to which <sup>131</sup>I is conjugated (either 1 or 2 I atoms per molecule of peptide) are effective radiotherapeutics and are found to be at least two-fold more potent than the unconjugated polypeptides. In contrast, treatment with control peptides fails to cause a significant change in tumor size or metastasis.

#### H. 3LL Lewis Lung Carcinoma: Primary Tumor Growth

This tumor line arose spontaneously in 1951 as carcinoma of the lung in a C57BL/6 mouse (*Cancer Res* 15:39, 1955. See, also Malave, I *et al.*, *J. Nat'l. Canc. Inst.* 62:83-88 (1979)). It is propagated by passage in C57BL/6 mice by subcutaneous (sc) inoculation and is tested in semiallogeneic C57BL/6 x DBA/2 F<sub>1</sub> mice or in allogeneic C3H mice. Typically six animals per group for subcutaneously (sc) implant, or ten for intramuscular (im) implant are used. Tumor may be implanted sc as a 2-4 mm fragment, or im or sc as an inoculum of suspended cells of about 0.5-2 x 10<sup>6</sup>-cells. Treatment begins 24 hours after implant or is delayed until a tumor of specified size (usually approximately 400 mg) can be palpated. The test compound is administered ip daily for 11 days

Animals are followed by weighing, palpation, and measurement of tumor size. Typical tumor weight in untreated control recipients on day 12 after im inoculation is 500-2500 mg. Typical median survival time is 18-28 days. A positive control compound, for example cyclophosphamide at 20 mg/kg/injection per day on days 1-11 is used. Results computed include mean animal weight, tumor size, tumor weight, survival time. For confirmed therapeutic activity, the test composition should be tested in two multi-dose assays.

#### I. 3LL Lewis Lung Carcinoma: Primary Growth and Metastasis Model

This model has been utilized by a number of investigators. See, for example, Gorelik, E *et al.*, *J. Nat'l. Canc. Inst.* 65:1257-1264 (1980); Gorelik, E. *et al.*, *Rec. Results Canc. Res.* 75:20-28 (1980); Isakov, N *et al.*, *Invasion Metas.* 2:12-32 (1982); Talmadge JE *et al.*, *J. Nat'l. Canc. Inst.* 69:975-980 (1982); Hilgard, P. *et al.*, *Br. J. Cancer* 35:78-86(1977)). Test mice are male C57BL/6 mice, 2-3 months old. Following sc, im, or intra-footpad implantation, this tumor produces metastases, preferentially in the lungs. With some lines of the tumor, the primary tumor exerts anti-metastatic effects and must first be excised before study of the metastatic phase (see also U.S. 5,639,725).

Single-cell suspensions are prepared from solid tumors by treating minced tumor tissue with a solution of 0.3% trypsin. Cells are washed 3 times with PBS (pH 7.4) and suspended in

PBS. Viability of the 3LL cells prepared in this way is generally about 95-99% (by trypan blue dye exclusion). Viable tumor cells ( $3 \times 10^4$  -  $5 \times 10^6$ ) suspended in 0.05 ml PBS are injected subcutaneously, either in the dorsal region or into one hind foot pad of C57BL/6 mice. Visible tumors appear after 3-4 days after dorsal sc injection of  $10^6$  cells. The day of tumor appearance and the diameters of established tumors are measured by caliper every two days.

The treatment is given as one or two doses of peptide or derivative, per week. In another embodiment, the peptide is delivered by osmotic minipump.

In experiments involving tumor excision of dorsal tumors, when tumors reach about 1500 mm<sup>3</sup> in size, mice are randomized into two groups: (1) primary tumor is completely excised; or (2) sham surgery is performed and the tumor is left intact. Although tumors from 500-3000 mm<sup>3</sup> inhibit growth of metastases, 1500 mm<sup>3</sup> is the largest size primary tumor that can be safely resected with high survival and without local regrowth. After 21 days, all mice are sacrificed and autopsied.

Lungs are removed and weighed. Lungs are fixed in Bouin's solution and the number of visible metastases is recorded. The diameters of the metastases are also measured using a binocular stereoscope equipped with a micrometer-containing ocular under 8X magnification. On the basis of the recorded diameters, it is possible to calculate the volume of each metastasis. To determine the total volume of metastases per lung, the mean number of visible metastases is multiplied by the mean volume of metastases. To further determine metastatic growth, it is possible to measure incorporation of <sup>125</sup>IdUrd into lung cells (Thakur, M.L. *et al.*, *J. Lab. Clin. Med.* 89:217-228 (1977)). Ten days following tumor amputation, 25 µg of fluorodeoxyuridine is inoculated into the peritoneums of tumor-bearing (and, if used, tumor-resected mice). After 30 min, mice are given 1 µCi of <sup>125</sup>IdUrd (iododeoxyuridine). One day later, lungs and spleens are removed and weighed, and a degree of <sup>125</sup>IdUrd incorporation is measured using a gamma counter.

In mice with footpad tumors, when tumors reach about 8-10 mm in diameter, mice are randomized into two groups: (1) legs with tumors are amputated after ligation above the knee joints; or (2) mice are left intact as nonamputated tumor-bearing controls. (Amputation of a tumor-free leg in a tumor-bearing mouse has no known effect on subsequent metastasis, ruling out possible effects of anesthesia, stress or surgery). Mice are killed 10-14 days after amputation. Metastases are evaluated as described above.

Statistics: Values representing the incidence of metastases and their growth in the lungs of tumor-bearing mice are not normally distributed. Therefore, non-parametric statistics such as the Mann-Whitney U-Test may be used for analysis.

Study of this model by Gorelik *et al.* (1980, *supra*) showed that the size of the tumor cell inoculum determined the extent of metastatic growth. The rate of metastasis in the lungs of operated mice was different from primary tumor-bearing mice. Thus in the lungs of mice in which the primary tumor had been induced by inoculation of larger doses of 3LL cells (1-5 x 10<sup>6</sup>) followed by surgical removal, the number of metastases was lower than that in nonoperated tumor-bearing mice, though the volume of metastases was higher than in the nonoperated controls. Using <sup>125</sup>IdUrd incorporation as a measure of lung metastasis, no significant differences were found between the lungs of tumor-excised mice and tumor-bearing mice originally inoculated with 10<sup>6</sup> 3LL cells. Amputation of tumors produced following inoculation of 10<sup>5</sup> tumor cells dramatically accelerated metastatic growth. These results were in accord with the survival of mice after excision of local tumors. The phenomenon of acceleration of metastatic growth following excision of local tumors had been repeatedly observed (for example, see U.S. 5,639,725). These observations have implications for the prognosis of patients who undergo cancer surgery.

For a compound to be useful in accordance with this invention, it should demonstrate activity in at least one of the above (*in vitro* or *in vivo*) assay systems.

#### Pharmaceutical and Therapeutic Compositions and Their Administration

The compounds that may be employed in the pharmaceutical compositions of the invention include all of the polypeptide and peptide compounds described above, as well as the pharmaceutically acceptable salts of these compounds. Pharmaceutically acceptable acid addition salts of the compounds of the invention containing a basic group are formed where appropriate with strong or moderately strong, non-toxic, organic or inorganic acids by methods known to the art. Exemplary of the acid addition salts that are included in this invention are maleate, fumarate, lactate, oxalate, methanesulfonate, ethanesulfonate, benzenesulfonate, tartrate, citrate, hydrochloride, hydrobromide, sulfate, phosphate and nitrate salts.

Pharmaceutically acceptable base addition salts of compounds of the invention containing an acidic group are prepared by known methods from organic and inorganic bases and include, for example, nontoxic alkali metal and alkaline earth bases, such as calcium,

sodium, potassium and ammonium hydroxide; and nontoxic organic bases such as triethylamine, butylamine, piperazine, and tri(hydroxymethyl)methylamine.

As stated above, the compounds of the invention possess the ability to inhibit EC proliferation, motility, or invasiveness and angiogenesis, properties that are exploited in the treatment of cancer, in particular metastatic cancer. A composition of this invention may be active *per se*, or may act as a "pro-drug" that is converted *in vivo* to the active form.

#### Therapeutically Labeled Compositions

In a preferred embodiment, the polypeptide and peptides described herein are "therapeutically conjugated" or "therapeutically labeled" (terms which are intended to be interchangeable) and used to deliver a therapeutic agent to the site to which the compounds home and bind, such as sites of tumor metastasis or foci of infection/inflammation, restenosis or fibrosis. The term "therapeutically conjugated" means that the modified peptide is conjugated to another therapeutic agent that is directed either to the underlying cause or to a "component" of tumor invasion, angiogenesis, inflammation or other pathology. A therapeutically labeled protein or peptide carries a suitable therapeutic "label" also referred to herein as a "therapeutic moiety." A therapeutic moiety is an atom, a molecule, a compound or any chemical component added to the peptide that renders it active in treating a target disease or condition, primarily one associated with undesired angiogenesis. As noted above, the peptides of the present invention are prepared by conventional means, either chemical synthesis, proteolysis of Tpm or its antiangiogenic ligands or recombinant means. The therapeutic moiety may be bound directly or indirectly to the peptide. The therapeutically labeled protein or peptide is administered as pharmaceutical composition which comprises a pharmaceutically acceptable carrier or excipient, and is preferably in a form suitable for injection.

Examples of useful therapeutic radioisotopes (ordered by atomic number) include <sup>47</sup>Sc, <sup>67</sup>Cu, <sup>90</sup>Y, <sup>109</sup>Pd, <sup>125</sup>I, <sup>131</sup>I, <sup>186</sup>Re, <sup>188</sup>Re, <sup>199</sup>Au, <sup>211</sup>At, <sup>212</sup>Pb and <sup>217</sup>Bi. These atoms can be conjugated to the peptide directly, indirectly as part of a chelate, or, in the case of iodine, indirectly as part of an iodinated Bolton-Hunter group. The radioiodine can be introduced either before or after this group is coupled to the peptide compound.

Preferred doses of the radionuclide conjugates are a function of the specific radioactivity to be delivered to the target site which varies with tumor type, tumor location and vascularization, kinetics and biodistribution of the peptide carrier, energy of radioactive emission by the nuclide, *etc.* Those skilled in the art of radiotherapy can readily adjust the dose

of the peptide in conjunction with the dose of the particular nuclide to effect the desired therapeutic benefit without undue experimentation.

Another therapeutic approach included here is the use of boron neutron capture therapy, where a boronated peptide is delivered to a desired target site, such as a tumor, most preferably an intracranial tumor (Barth, RF, *Cancer Invest.* 14:534-550 (1996); Mishima, Y. (ed.), *Cancer Neutron Capture Therapy*, New York: Plenum Publishing Corp., 1996; Soloway, A.H., *et al.*, (eds), *J. Neuro-Oncol.* 33:1-188 (1997). The stable isotope  $^{10}\text{B}$  is irradiated with low energy (<0.025eV) thermal neutrons, and the resulting nuclear capture yields  $\alpha$ -particles and  $^7\text{Li}$  nuclei which have high linear energy transfer and respective path lengths of about 9 and 5  $\mu\text{m}$ . This method is predicated on  $^{10}\text{B}$  accumulation in the tumor with lower levels in blood, ECs and normal tissue (e.g., brain). Such delivery has been accomplished using epidermal growth factor (Yang, W. *et al.*, *Cancer Res* 57:4333-4339 (1997)).

Other therapeutic agents which can be coupled to the peptide compounds according to the method of the invention are drugs, prodrugs, enzymes for activating pro-drugs, photosensitizing agents, nucleic acid therapeutics, antisense vectors, viral vectors, lectins and other toxins.

Lectins are proteins, commonly derived from plants, that bind to carbohydrates. Among other activities, some lectins are toxic. Some of the most cytotoxic substances known are protein toxins of bacterial and plant origin (Frankel, AE, *et al.*, *Ann. Rev. Med.* 37:125-142 (1986)). These molecules binding the cell surface and inhibit cellular protein synthesis. The most commonly used plant toxins are ricin and abrin; the most commonly used bacterial toxins are diphtheria toxin and *Pseudomonas* exotoxin A. In ricin and abrin, the binding and toxic functions are contained in two separate protein subunits, the A and B chains. The ricin B chain binds to the cell surface carbohydrates and promotes the uptake of the A chain into the cell. Once inside the cell, the ricin A chain inhibits protein synthesis by inactivating the 60S subunit of the eukaryotic ribosome (Endo, Y. *et al.*, *J. Biol. Chem.* 262: 5908-5912 (1987)). Other plant derived toxins, which are single chain ribosomal inhibitory proteins, include pokeweed antiviral protein, wheat germ protein, gelonin, dianthins, momorcharins, trichosanthin, and many others (Strip, F. *et al.*, *FEBS Lett.* 195:1-8 (1986)). Diphtheria toxin and *Pseudomonas* exotoxin A are also single chain proteins, and their binding and toxicity functions reside in separate domains of the same protein. *Pseudomonas* exotoxin A has the same catalytic activity as diphtheria toxin. Ricin has been used therapeutically by binding its toxic  $\alpha$ -chain, to targeting molecules such as antibodies to enable

site-specific delivery of the toxic effect. Bacterial toxins have also been used as anti-tumor conjugates. As intended herein, a toxic peptide chain or domain is conjugated to a compound of this invention and delivered in a site-specific manner to a target site where the toxic activity is desired, such as a metastatic focus. Conjugation of toxins to protein such as antibodies or other ligands are known in the art (Olsnes, S. *et al.*, *Immunol. Today* 10:291-295 (1989); Vitetta, E.S. *et al.*, *Ann. Rev. Immunol.* 3:197-212 (1985)).

Cytotoxic drugs that interfere with critical cellular processes including DNA, RNA, and protein synthesis, have been conjugated to antibodies and subsequently used for *in vivo* therapy. Such drugs, including, but not limited to, daunorubicin, doxorubicin, methotrexate, and Mitomycin C are also coupled to the compounds of this invention and used therapeutically in this form.

In a preferred embodiment of this invention, a cytotoxic drug is targeted to Tpm on the surface of an EC or tumor cell by conjugating the drug to a Tpm ligand. Again, preferred ligands for cell surface Tpm include HPRG or a H/P domain peptide thereof, HKa or the D5 domain or a shorter fragment thereof, or more preferably, an antibody specific for an epitope of Tpm expressed on these cell surfaces.

The compounds of the invention, as well as the pharmaceutically acceptable salts thereof, may be incorporated into convenient dosage forms, such as capsules, impregnated wafers, tablets or injectable preparations. Solid or liquid pharmaceutically acceptable carriers may be employed.

Solid carriers include starch, lactose, calcium sulfate dihydrate, terra alba, sucrose, talc, gelatin, agar, pectin, acacia, magnesium stearate and stearic acid. Liquid carriers include syrup, peanut oil, olive oil, saline, water, dextrose, glycerol and the like. Similarly, the carrier or diluent may include any prolonged release material, such as glyceryl monostearate or glyceryl distearate, alone or with a wax. When a liquid carrier is used, the preparation may be in the form of a syrup, elixir, emulsion, soft gelatin capsule, sterile injectable liquid (e.g., a solution), such as an ampoule, or an aqueous or nonaqueous liquid suspension. A summary of such pharmaceutical compositions may be found, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton Pennsylvania (Gennaro 18th ed. 1990).

The pharmaceutical preparations are made following conventional techniques of pharmaceutical chemistry involving such steps as mixing, granulating and compressing, when necessary for tablet forms, or mixing, filling and dissolving the ingredients, as appropriate, to

give the desired products for oral, parenteral, topical, transdermal, intravaginal, intrapenile, intranasal, intrabronchial, intracranial, intraocular, intraaural and rectal administration. The pharmaceutical compositions may also contain minor amounts of nontoxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents and so forth.

The present invention may be used in the diagnosis or treatment of any of a number of animal genera and species, and are equally applicable in the practice of human or veterinary medicine. Thus, the pharmaceutical compositions can be used to treat domestic and commercial animals, including birds and more preferably mammals, as well as humans.

The term "systemic administration" refers to administration of a composition or agent such as the polypeptide, peptides or nucleic acids described herein, in a manner that results in the introduction of the composition into the subject's circulatory system or otherwise permits its spread throughout the body, such as intravenous (i.v.) injection or infusion. "Regional" administration refers to administration into a specific, and somewhat more limited, anatomical space, such as intraperitoneal, intrathecal, subdural, or to a specific organ. Examples include intravaginal, intrapenile, intranasal, intrabronchial (or lung instillation), intracranial, intra-aural or intraocular. The term "local administration" refers to administration of a composition or drug into a limited, or circumscribed, anatomic space, such as intratumoral injection into a tumor mass, subcutaneous (s.c.) injections, intramuscular (i.m.) injections. One of skill in the art would understand that local administration or regional administration often also result in entry of a composition into the circulatory system, *i.e.*, so that s.c. or i.m. are also routes for systemic administration. Injectables or infusible preparations can be prepared in conventional forms, either as solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection or infusion, or as emulsions. Though the preferred routes of administration are systemic, such as i.v., the pharmaceutical composition may be administered topically or transdermally, *e.g.*, as an ointment, cream or gel; orally; rectally; *e.g.*, as a suppository.

For topical application, the compound may be incorporated into topically applied vehicles such as a salve or ointment. The carrier for the active ingredient may be either in sprayable or nonsprayable form. Non-sprayable forms can be semi-solid or solid forms comprising a carrier indigenous to topical application and having a dynamic viscosity preferably greater than that of water. Suitable formulations include, but are not limited to, solution, suspensions, emulsions, creams, ointments, powders, liniments, salves, and the like. If desired, these may be sterilized or mixed with auxiliary agents, *e.g.*, preservatives, stabilizers, wetting

agents, buffers, or salts for influencing osmotic pressure and the like. Preferred vehicles for non-sprayable topical preparations include ointment bases, *e.g.*, polyethylene glycol-1000 (PEG-1000); conventional creams such as HEB cream; gels; as well as petroleum jelly and the like.

Also suitable for topic application as well as for lung instillation are sprayable aerosol preparations wherein the compound, preferably in combination with a solid or liquid inert carrier material, is packaged in a squeeze bottle or in admixture with a pressurized volatile, normally gaseous propellant. The aerosol preparations can contain solvents, buffers, surfactants, perfumes, and/or antioxidants in addition to the compounds of the invention.

For the preferred topical applications, especially for humans, it is preferred to administer an effective amount of the compound to an affected area, *e.g.*, skin surface, mucous membrane, eyes, *etc.* This amount will generally range from about 0.001 mg to about 1 g per application, depending upon the area to be treated, the severity of the symptoms, and the nature of the topical vehicle employed.

Antiangiogenic compositions may be administered in combination with a biodegradable, biocompatible polymeric implant which releases the troponin active agent over a controlled period of time at a selected site. Examples of preferred polymeric materials include polyanhydrides, polyorthoesters, polyglycolic acid, polylactic acid, polyethylene vinyl acetate, and copolymers and blends thereof. See, for example, *Medical Applications of Controlled Release*, Langer and Wise (eds.), 1974, CRC Press, Boca Raton, FL; *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), 1984, Wiley, NY; Ranger *et al.*, 1983, *J. Macromol. Sci. Rev. Macromol. Chem.* 23:61; Levy *et al.*, 1985, *Science* 228:190; During *et al.*, 1989, *Ann. Neurol.* 25:351; Howard *et al.*, 1989, *J. Neurosurg.* 71:105. In another embodiment, a controlled release system can be placed in proximity of the therapeutic target, *e.g.*, the brain, thus requiring only a fraction of the systemic dose (Goodson, In: *Medical Applications of Controlled Release*, *supra*, vol. 2, pp. 115-138). Other controlled release systems are discussed in a review by Langer, R, 1990, *Science* 249:1527-1533)

Other pharmaceutically acceptable carriers for polypeptide or nucleic acid compositions of the present invention are liposomes, pharmaceutical compositions in which the active protein is contained either dispersed or variously present in corpuscles consisting of aqueous concentric layers adherent to lipidic layers. The active polypeptide or peptide, or the nucleic acid is preferably present in the aqueous layer and in the lipidic layer, inside or outside, or, in any event, in the non-homogeneous system generally known as a liposomal suspension. The

hydrophobic layer, or lipidic layer, generally, but not exclusively, comprises phospholipids such as lecithin and sphingomyelin, steroids such as cholesterol, more or less ionic surface active substances such as dicetylphosphate, stearylamine or phosphatidic acid, and/or other materials of a hydrophobic nature. Those skilled in the art will appreciate other suitable embodiments of the present liposomal formulations.

Therapeutic compositions for treating tumors and cancer may comprise, in addition to the antiangiogenic polypeptide or peptide, one or more additional anti-tumor agents, such as mitotic inhibitors, *e.g.*, vinblastine; alkylating agents, *e.g.*, cyclophosphamide; folate inhibitors, *e.g.*, methotrexate, piritrexim or trimetrexate; antimetabolites, *e.g.*, 5-fluorouracil and cytosine arabinoside; intercalating antibiotics, *e.g.*, adriamycin and bleomycin; enzymes or enzyme inhibitors, *e.g.*, asparaginase, topoisomerase inhibitors such as etoposide; or biological response modifiers, *e.g.*, interferons or interleukins. In fact, pharmaceutical compositions comprising any known cancer therapeutic in combination with the peptides disclosed herein are within the scope of this invention. The pharmaceutical composition may also comprise one or more other medicaments to treat additional symptoms for which the target patients are at risk, for example, anti-infectives including antibacterial, anti-fungal, anti-parasitic, anti-viral, and anti-coccidial agents.

The therapeutic dosage administered is an amount which is therapeutically effective, as is known to or readily ascertainable by those skilled in the art. The dose is also dependent upon the age, health, and weight of the recipient, kind of concurrent treatment(s), if any, the frequency of treatment, and the nature of the effect desired, such as, for example, anti-inflammatory effects or anti-bacterial effect.

While some antibodies will bind directly to cell surface Tpm and induce antiangiogenic activity (“agonists”) other antibodies specific for epitopes of Tpm are expected to inhibit binding of and anti-angiogenic effects of HPRG via the H/P domain, D5, *etc.* Such antibodies, termed “antagonists” are useful in the induction of neovascularization and can be used to treat diseases or conditions in which increased angiogenesis is desired. Such conditions include coronary artery disease and peripheral artery disease, in which therapeutic angiogenesis is known to be beneficial (Freedman SB *et al.*, *Ann Intern Med*, 2002, 136:54-71 and *J Mol Cell Cardiol*, 2001 33:379-393; Durairaj, A. *et al.*, *Cardiol Rev*, 2000, 8:279-287; Emanueli, C *et al.*, *Br J Pharmacol*, 2001, 133:951-958; Isner, JM *et al.*, *Hum Gene Ther*, 1996, 7:959-88). In general, any form of tissue ischemia resulting from vascular occlusion, vascular disease or surgery can be treated

in this manner (Isner *et al.*, *supra*; Webster KA., *Crit Rev Eukaryot Gene Expr*, 2000, 10:113-125), for example peripheral limb ischemia or hepatic arterial occlusion in liver transplantation (Yedlicka, JW *et al.*, *J Vasc Interv Radiol*, 1991, 2:235-240) where the present antibodies will promote revascularization of ischemic tissues.

These antagonist antibodies are useful in the promotion of wound healing (including recovery from surgical wounds), which is known to be dependent upon angiogenic processes (Liekens S *et al.*, *Biochem Pharmacol*, 2001, 61:253-270; Lingen, MW, *Arch Pathol Lab Med*, 2001, 125:67-71; Raza SL *et al.*, *J Investig Dermatol Symp Proc*, 2000, 5:47-54; Tonnesen MG *et al.*, *J Investig Dermatol Symp Proc*, 2000, 5:40-46; Hunt TK, *Adv Skin Wound Care*, 2000, 13(2 Suppl):6-11; Grant DS *et al.*, *Adv Exp Med Biol*, 2000, 476:139-154; Drixler TA *et al.*, *Eur J Surg*, 2000, 166:435-446; Singer AJ *et al.*, *N Engl J Med*, 1999, 341:738-746; Martin, P, *Science*, 1997, 276:75-81) and in accelerating or enhancing fracture repair (Glowacki, J, *Clin Orthop*, 1998, 355 Suppl:S82-89).

Antagonist anti-Tpm antibodies can be used in conjunction with cellular therapy and transplantation of pancreatic islet cells in the treatment of diabetes as vascular endothelium acts to stimulate or induce pancreatic organogenesis and insulin production by pancreatic beta cells (Lammert E *et al.*, *Science*, 2001, 294:564-567; see also page 530-531). Liver organogenesis is also promoted by vasculogenic ECs and nascent vessels (Matsumoto, K. *et al.*, *Science*, 2001, 294:559-563). See also, DeFrancesco, L., *The Scientist* 15:17 (2001).

Screening of antibodies or supernatants of hybridoma cultures to detect anti-Tpm antibodies with the desired antiangiogenic or pro-angiogenic activity are performed using the *in vitro* and *in vivo* bioassays described above, such as the Matrigel® plug assay.

### Therapeutic Methods

The methods of this invention may be used to inhibit tumor growth and invasion in a subject or to suppress angiogenesis induced by tumors by inhibiting EC growth and migration. By inhibiting the growth or invasion of a tumor or angiogenesis, the methods result in inhibition of tumor metastasis. A vertebrate subject, preferably a mammal, more preferably a human, is administered an amount of the compound effective to inhibit tumor growth, invasion or angiogenesis. The compound or pharmaceutically acceptable salt thereof is preferably administered in the form of a pharmaceutical composition as described above.

Doses of the proteins (including antibodies), peptides, peptide multimers, *etc.*, preferably include pharmaceutical dosage units comprising an effective amount of the peptide. Dosage unit

form refers to physically discrete units suited as unitary dosages for a mammalian subject; each unit contains a predetermined quantity of active material (e.g., the HPRG-derived domain or peptide, or nucleic acid encoding the polypeptide) calculated to produce the desired therapeutic effect, in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of, and sensitivity of, individual subjects

By an effective amount is meant an amount sufficient to achieve a steady state concentration *in vivo* which results in a measurable reduction in any relevant parameter of disease and may include growth of primary or metastatic tumor, any accepted index of inflammatory reactivity, or a measurable prolongation of disease-free interval or of survival. For example, a reduction in tumor growth in 20 % of patients is considered efficacious (Frei III, E., *The Cancer Journal* 3:127-136 (1997)). However, an effect of this magnitude is not considered to be a minimal requirement for the dose to be effective in accordance with this invention.

In one embodiment, an effective dose is preferably 10-fold and more preferably 100-fold higher than the 50% effective dose (ED<sub>50</sub>) of the compound in an *in vivo* assay as described herein.

The amount of active compound to be administered depends on the precise peptide or derivative selected, the disease or condition, the route of administration, the health and weight of the recipient, the existence of other concurrent treatment, if any, the frequency of treatment, the nature of the effect desired, for example, inhibition of tumor metastasis, and the judgment of the skilled practitioner.

The therapeutically effective dosage for inhibition of angiogenesis *in vivo* , which include any one of more of inhibition of capillary endothelial cell proliferation, migration, and blood vessel ingrowth, may be extrapolated from *in vitro* inhibition assays described herein. The effective dosage is also dependent on the method and means of delivery. For example, in some applications, as in the treatment of psoriasis or diabetic retinopathy, the inhibitor is delivered in a topical-ophthalmic carrier. In other applications, as in the treatment of solid tumors, the inhibitor is delivered by means of a biodegradable, polymeric implant. The protein

can also be modified, for example, by polyethylene glycol treatment which would affect the effective dose.

A preferred dose of an agonist anti-Tpm for treating a subject, preferably mammalian, more preferably human, with a tumor is an amount of up to about 100 milligrams of active protein or peptide-based compound per kilogram of body weight. A typical single dosage of the peptide or peptidomimetic is between about 1 ng and about 100mg/kg body weight. For topical administration, dosages in the range of about 0.01-20% concentration (by weight) of the compound, preferably 1-5%, are suggested. A total daily dosage in the range of about 0.1 milligrams to about 7 grams is preferred for intravenous administration. The foregoing ranges are, however, suggestive, as the number of variables in an individual treatment regime is large, and considerable excursions from these preferred values are expected.

An effective amount or dose of the peptide for inhibiting EC proliferation or migration *in vitro* is in the range of about 1 picogram to about 5 nanograms per cell. Effective doses and optimal dose ranges may be determined *in vitro* using the methods described herein.

The compounds of the invention may be characterized as producing an inhibitory effect on tumor cell or EC proliferation, migration, invasion, or on angiogenesis, on tumor metastasis or on inflammatory reactions. The compounds are especially useful in producing an anti-tumor effect in a mammalian host, preferably human, harboring a tumor.

Angiogenesis inhibitors may play a role in preventing inflammatory angiogenesis and gliosis following traumatic spinal cord injury, thereby promoting the reestablishment of neuronal connectivity (Wamil, AW *et al.*, *Proc. Nat'l. Acad. Sci. USA* 95:13188-13193 (1998)). Therefore, the compositions of the present invention are administered as soon as possible after traumatic spinal cord injury and for several days up to about two weeks thereafter to inhibit the angiogenesis and gliosis that would sterically prevent reestablishment of neuronal connectivity. The treatment reduces the area of damage at the site of spinal cord injury and facilitates regeneration of neuronal function and thereby prevents paralysis. The compounds of the invention are expected also to protect axons from Wallerian degeneration, reverse aminobutyrate-mediated depolarization (occurring in traumatized neurons), and improve recovery of neuronal conductivity of isolated central nervous system cells and tissue in culture.

### **GENERAL RECOMBINANT DNA METHODS**

Basic texts disclosing general methods of molecular biology, all of which are incorporated by reference, include: Sambrook, J *et al.*, *Molecular Cloning: A Laboratory*

*Manual*, 2nd (or later) Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989; Ausubel, FM *et al.* *Current Protocols in Molecular Biology*, Vol. 2, Wiley-Interscience, New York, (current edition); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); Glover, DM, editor, *DNA Cloning: A Practical Approach*, vol. I & II, IRL Press, 1985; Albers, B. *et al.*, *Molecular Biology of the Cell*, 2nd (or later) Ed., Garland Publishing, Inc., New York, NY (1989); Watson, JD *et al.*, *Recombinant DNA*, 2nd (or later) Ed., Scientific American Books, New York, 1992; and Old, RW *et al.*, *Principles of Gene Manipulation: An Introduction to Genetic Engineering*, 2nd (or later) Ed., University of California Press, Berkeley, CA (1981).

Unless otherwise indicated, a particular nucleic acid sequence is intended to encompasses conservative substitution variants thereof (e.g., degenerate codon substitutions) and a complementary sequence. The term "nucleic acid" is synonymous with "polynucleotide" and is intended to include a gene, a cDNA molecule, an mRNA molecule, as well as a fragment of any of these such as an oligonucleotide, and further, equivalents thereof (explained more fully below). Sizes of nucleic acids are stated either as kilobases (kb) or base pairs (bp). These are estimates derived from agarose or polyacrylamide gel electrophoresis (PAGE), from nucleic acid sequences which are determined by the user or published. Protein size is stated as molecular mass in kilodaltons (kDa) or as length (number of amino acid residues). Protein size is estimated from PAGE, from sequencing, from presumptive amino acid sequences based on the coding nucleic acid sequence or from published amino acid sequences.

Specifically, cDNA molecules encoding the amino acid sequence corresponding to the Tpm polypeptide, domain or peptide fragment of the present invention, or active variants thereof, can be synthesized by the polymerase chain reaction (PCR) (see, for example, U.S. 4,683,202) using primers derived the sequence of the protein disclosed herein. These cDNA sequences can then be assembled into a eukaryotic or prokaryotic expression vector and the resulting vector can be used to direct the synthesis of the fusion polypeptide or its fragment or derivative by appropriate host cells, for example COS or CHO cells.

This invention includes isolated nucleic acids having a nucleotide sequence encoding the novel Tpm polypeptide, domain, peptide fragment, or equivalent thereof, and their use in transfecting cells *in vitro* or *in vivo* to express their polypeptide product. The term nucleic acid as used herein is intended to include such fragments or equivalents. The nucleic acid sequences of this invention can be DNA or RNA.

A cDNA nucleotide sequence encoding a Tpm polypeptide can be obtained by isolating total mRNA from an appropriate cell line. Double stranded cDNA is prepared from total mRNA. cDNA can be inserted into a suitable plasmid, bacteriophage or viral vector using any one of a number of known techniques.

In reference to a nucleotide sequence, the term "equivalent" is intended to include sequences encoding structurally homologous and/or a functionally equivalent proteins such as naturally occurring isoforms or related, immunologically cross-reactive family members of these proteins. Such isoforms or family members are defined as proteins that share function and amino acid sequence similarity to, for example, SEQ ID NO:1 or 3 or SEQ ID NO:5, 7, 9, 11, 13, 15, 17 or 19.

#### Fragments of Nucleic Acid

A fragment of the nucleic acid sequence is defined as a nucleotide sequence having fewer nucleotides than the nucleotide sequence encoding the full length Tpm protein, the AALBP fragment or smaller fragments or domains. This invention includes such nucleic acid fragments that encode polypeptides which retain (1) the ability of the Tpm polypeptide to bind an inhibitor of angiogenesis, endothelial tube formation, cell invasion or tumor growth or metastasis.

Generally, the nucleic acid sequence encoding a fragment of Tpm comprises of nucleotides from the sequence encoding the mature protein (or the active fragment thereof).

Nucleic acid sequences, particularly those that encode peptide multimers of this invention may also include linker or spacer sequences (preferably encoding Gly<sub>1-6</sub>). The nucleic acids further may include natural or modified restriction endonuclease sites and other sequences that are useful for manipulations related to cloning, expression or purification of encoded polypeptide or peptides. These and other modifications of nucleic acid sequences are described herein or are well-known in the art.

The techniques for assembling and expressing DNA coding sequences include synthesis of oligonucleotides, PCR, transforming cells, constructing vectors, expression systems, and the like; these are well-established in the art such that those of ordinary skill are familiar with standard resource materials, specific conditions and procedures.

## EXPRESSION VECTORS AND HOST CELLS

This invention includes an expression vector comprising a nucleic acid sequence encoding a Tpm polypeptide, domain, or peptide operably linked to at least one regulatory sequence.

The term "expression vector" or "expression cassette" as used herein refers to a nucleotide sequence which is capable of affecting expression of a protein coding sequence in a host compatible with such sequences. Expression cassettes include at least a promoter operably linked with the polypeptide coding sequence; and, optionally, with other sequences, *e.g.*, transcription termination signals. Additional factors necessary or helpful in effecting expression may also be included, *e.g.*, enhancers.

"Operably linked" means that the coding sequence is linked to a regulatory sequence in a manner that allows expression of the coding sequence. Known regulatory sequences are selected to direct expression of the desired protein in an appropriate host cell. Accordingly, the term "regulatory sequence" includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in, for example, Goeddel, *Gene Expression Technology. Methods in Enzymology*, vol. 185, Academic Press, San Diego, Calif. (1990)).

Thus, expression cassettes include plasmids, recombinant viruses, any form of a recombinant "naked DNA" vector, and the like. A "vector" comprises a nucleic acid which can infect, transfect, transiently or permanently transduce a cell. It will be recognized that a vector can be a naked nucleic acid, or a nucleic acid complexed with protein or lipid. The vector optionally comprises viral or bacterial nucleic acids and/or proteins, and/or membranes (*e.g.*, a cell membrane, a viral lipid envelope, *etc.*). Vectors include, but are not limited to replicons (*e.g.*, RNA replicons, bacteriophages) to which fragments of DNA may be attached and become replicated. Vectors thus include, but are not limited to RNA, autonomous self-replicating circular or linear DNA or RNA, *e.g.*, plasmids, viruses, and the like (U.S. Patent No. 5,217,879), and includes both the expression and nonexpression plasmids. Where a recombinant microorganism or cell culture is a host for an "expression vector," this includes both extrachromosomal circular and linear DNA and DNA that has been incorporated into the host chromosome(s). Where a vector is being maintained by a host cell, the vector may either be stably replicated by the cells during mitosis as an autonomous structure, or is incorporated within the host's genome.

Those skilled in the art appreciate that the particular design of an expression vector of this invention depends on considerations such as the host cell to be transfected and the nature (e.g., size) of the polypeptide to be expressed.

The present expression vectors comprise the full range of nucleic acid molecules encoding the various embodiments of the Tpm polypeptide, fragment or peptide.

Such expression vectors are used to transfect host cells (*in vitro*, *ex vivo* or *in vivo*) for expression of the DNA and production of the encoded proteins which include fusion proteins or peptides. It will be understood that a genetically modified cell expressing the Tpm polypeptide, domain, peptide fragment or multimer, may transiently express the exogenous DNA for a time sufficient for the cell to be useful for its stated purpose.

Host cells may also be transfected with one or more expression vectors that singly or in combination comprise DNA encoding at least a portion of the Tpm polypeptide or AALBP fragment or shorter peptide and DNA encoding at least a portion of a second Tpm-derived sequence (or variant), so that the host cells produce yet further Tpm polypeptide, domain or peptide fragments that include both the portions.

Methods for producing the Tpm polypeptide, domain or peptide fragments, are all conventional in the art. Cultures typically include host cells, appropriate growth media and other byproducts. Suitable culture media are well known in the art. The Tpm polypeptide, domain or peptide fragment can be isolated from medium or cell lysates using conventional techniques for purifying proteins and peptides, including ammonium sulfate precipitation, fractionation column chromatography (e.g. ion exchange, gel filtration, affinity chromatography, etc.) and/or electrophoresis (see generally, *Meth Enzymol*, 22:233-577 (1971)). Once purified, partially or to homogeneity, the recombinant polypeptides of the invention can be utilized in pharmaceutical compositions as described in more detail herein.

The term "isolated" as used herein, when referring to a molecule or composition, means that the molecule or composition is separated from at least one other compound (protein, other nucleic acid, etc.) or from other contaminants with which it is natively associated or becomes associated during processing.. An isolated composition can also be substantially pure. An isolated composition can be in a homogeneous state and can be dry or in aqueous solution. Purity and homogeneity can be determined, for example, using analytical chemical techniques such as polyacrylamide gel electrophoresis (PAGE) or high performance liquid chromatography (HPLC). It is understood that even where a protein has been isolated so as to appear as a

homogenous or dominant band in a gel pattern, there are generally trace contaminants which co-purify with it.

Prokaryotic or eukaryotic host cells transformed or transfected to express the Tpm polypeptide, domain or peptide fragment are within the scope of the invention. For example, the Tpm polypeptide, domain or peptide fragment may be expressed in bacterial cells such as *E. coli*, insect cells (baculovirus), yeast, or mammalian cells such as Chinese hamster ovary cells (CHO) or human cells (which are preferred for human therapeutic use of the transfected cells). Other suitable host cells may be found in Goeddel, (1990) *supra* or are otherwise known to those skilled in the art.

Expression in eukaryotic cells leads to partial or complete glycosylation and/or formation of relevant inter- or intra-chain disulfide bonds of the recombinant polypeptide.

Examples of vectors for expression in yeast *S. cerevisiae* include pYEpSec1 (Baldari *et al.*, (1987) *EMBO J.* 6:229-234), pMFa (Kurjan *et al.* (1982) *Cell* 30:933-943), pJRY88 (Schultz *et al.*, (1987) *Gene* 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, Calif.). Baculovirus vectors available for expression of proteins in cultured insect cells (SF 9 cells) include the pAc series (Smith *et al.*, (1983) *Mol. Cell Biol.* 3:2156-2165,) and the pVL series (Lucklow, V. A., and Summers, M. D., (1989) *Virology* 170:31-39). Generally, COS cells (Gluzman, Y., (1981) *Cell* 23:175-182) are used in conjunction with such vectors as pCDM8 (Aruffo A. and Seed, B., *supra*, for transient amplification/expression in mammalian cells, while CHO (*dhfr*-negative CHO) cells are used with vectors such as pMT2PC (Kaufman *et al.* (1987), *EMBO J.* 6:187-195) for stable amplification/expression in mammalian cells. The NS0 myeloma cell line (a glutamine synthetase expression system.) is available from Celltech Ltd.

Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the reporter group and the target protein to enable separation of the target protein from the reporter group subsequent to purification of the fusion protein. Proteolytic enzymes for such cleavage and their recognition sequences include Factor Xa, thrombin and enterokinase.

Typical fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase, maltose E binding protein, or protein A, respectively, to the target recombinant polypeptide.

Inducible non-fusion expression vectors include pTrc (Amann *et al.*, (1988) *Gene* 69:301-315) and pET 11d (Studier *et al.*, *Gene Expression Technology: Methods in Enzymology*

185, Academic Press, San Diego, Calif. (1990) 60-89). While target gene expression relies on host RNA polymerase transcription from the hybrid trp-lac fusion promoter in pTrc, expression of target genes inserted into pET 11d relies on transcription from the T7 gn10-lacO fusion promoter mediated by coexpressed viral RNA polymerase (T7gn1). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident  $\lambda$  prophage harboring a T7gn1 under the transcriptional control of the lacUV 5 promoter.

#### Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation and restriction techniques which are well understood in the art. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and re-ligated in the form desired. The DNA sequences which form the vectors are available from a number of sources. Backbone vectors and control systems are generally found on available "host" vectors which are used for the bulk of the sequences in construction. For the pertinent coding sequence, initial construction may be, and usually is, a matter of retrieving the appropriate sequences from cDNA or genomic DNA libraries. However, once the sequence is disclosed it is possible to synthesize the entire gene sequence *in vitro* starting from the individual nucleotide derivatives. The entire gene sequence for genes of sizeable length, *e.g.*, 500-1000 bp may be prepared by synthesizing individual overlapping complementary oligonucleotides and filling in single stranded nonoverlapping portions using DNA polymerase in the presence of the deoxyribonucleotide triphosphates. This approach has been used successfully in the construction of several genes of known sequence. See, for example, Edge, M. D., *Nature* (1981) 292:756; Nambair, K. P., *et al.*, *Science* (1984) 223:1299; and Jay, E., *J Biol Chem* (1984) 259:6311.

Synthetic oligonucleotides are prepared by either the phosphotriester method as described by references cited above or the phosphoramidite method as described by Beaucage, S. L., and Caruthers, M. H., *Tetrahed Lett* (1981) 22:1859; and Matteucci, M. D., and Caruthers, M. H., *J Am Chem Soc* (1981) 103:3185 and can be prepared using commercially available automated oligonucleotide synthesizers. Kinase treatment of single strands prior to annealing or for labeling is achieved using well-known methods.

Once the components of the desired vectors are thus available, they can be excised and ligated using standard restriction and ligation procedures. Site-specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which

are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, e.g., New England Biolabs, Product Catalog. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in *Meth Enzymol* (1980) 65:499-560.

Any of a number of methods are used to introduce mutations into the coding sequence to generate variants of the invention if these are to be produced recombinantly. These mutations include simple deletions or insertions, systematic deletions, insertions or substitutions of clusters of bases or substitutions of single bases. Modifications of the DNA sequence are created by site-directed mutagenesis, a well-known technique for which protocols and reagents are commercially available (Zoller, MJ *et al.*, *Nucleic Acids Res* (1982) 10:6487-6500 and Adelman, JP *et al.*, *DNA* (1983) 2:183-193)). The isolated DNA is analyzed by restriction and/or sequenced by the dideoxy nucleotide method of Sanger (*Proc Natl Acad Sci USA* (1977) 74:5463) as further described by Messing, *et al.*, *Nucleic Acids Res* (1981) 9:309, or by the method of Maxam *et al.*, *Meth. Enzymol.*, *supra*.

Vector DNA can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming host cells can be found in Sambrook *et al. supra* and other standard texts. In fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the reporter group and the target protein to enable separation of the target protein from the reporter group subsequent to purification of the fusion protein. Proteolytic enzymes for such cleavage and their recognition sequences include Factor Xa, thrombin and enterokinase.

#### Promoters and Enhancers

A promoter region of a DNA or RNA molecule binds RNA polymerase and promotes the transcription of an "operably linked" nucleic acid sequence. As used herein, a "promoter sequence" is the nucleotide sequence of the promoter which is found on that strand of the DNA or RNA which is transcribed by the RNA polymerase. The preferred promoter sequences of the present invention must be operable in mammalian cells and may be either eukaryotic or viral promoters. Although preferred promoters are described in the Examples, other useful promoters and regulatory elements are discussed below. Suitable promoters may be inducible, repressible or constitutive. A "constitutive" promoter is one which is active under most conditions

encountered in the cell's environmental and throughout development. An "inducible" promoter is one which is under environmental or developmental regulation. A "tissue specific" promoter is active in certain tissue types of an organism. An example of a constitutive promoter is the viral promoter MSV-LTR, which is efficient and active in a variety of cell types, and, in contrast to most other promoters, has the same enhancing activity in arrested and growing cells. Other preferred viral promoters include that present in the CMV-LTR (from cytomegalovirus) (Bashart, M. *et al.*, *Cell* 41:521 (1985)) or in the RSV-LTR (from Rous sarcoma virus) (Gorman, CM, *Proc. Natl. Acad. Sci. USA* 79:6777 (1982)). Also useful are the promoter of the mouse metallothionein I gene (Hamer, D., *et al.*, *J. Mol. Appl. Gen.* 1:273-288 (1982)); the TK promoter of Herpes virus (McKnight, S., *Cell* 31:355-365 (1982)); the SV40 early promoter (Benoist, C., *et al.*, *Nature* 290:304-310 (1981)); and the yeast *gal4* gene promoter (Johnston, S.A., *et al.*, *Proc. Natl. Acad. Sci. (USA)* 79:6971-6975 (1982); Silver, P.A., *et al.*, *Proc. Natl. Acad. Sci. (USA)* 81:5951-5955 (1984)). Other illustrative descriptions of transcriptional factor association with promoter regions and the separate activation and DNA binding of transcription factors include: Keegan *et al.*, *Nature* (1986) 231:699; Fields *et al.*, *Nature* (1989) 340:245; Jones, *Cell* (1990) 61:9; Lewin, *Cell* (1990) 61:1161; Ptashne *et al.*, *Nature* (1990) 346:329; Adams *et al.*, *Cell* (1993) 72:306. The relevant disclosure of all of these above-listed references is hereby incorporated by reference.

The promoter region may further include an octamer region which may also function as a tissue specific enhancer, by interacting with certain proteins found in the specific tissue. The enhancer domain of the DNA construct of the present invention is one which is specific for the target cells to be transfected, or is highly activated by cellular factors of such target cells. Examples of vectors (plasmid or retrovirus) are disclosed in (Roy-Burman *et al.*, U.S. Patent No. 5,112,767). For a general discussion of enhancers and their actions in transcription, see, Lewin, BM, *Genes IV*, Oxford University Press, Oxford, (1990), pp. 552-576. Particularly useful are retroviral enhancers (e.g., viral LTR). The enhancer is preferably placed upstream from the promoter with which it interacts to stimulate gene expression. For use with retroviral vectors, the endogenous viral LTR may be rendered enhancer-less and substituted with other desired enhancer sequences which confer tissue specificity or other desirable properties such as transcriptional efficiency.

The nucleic acid sequences of the invention can also be chemically synthesized using standard techniques. Various methods of chemically synthesizing polydeoxynucleotides are

known, including solid-phase synthesis which, like peptide synthesis, has been fully automated with commercially available DNA synthesizers (See, e.g., Itakura *et al.* U.S. Pat. No. 4,598,049; Caruthers *et al.* U.S. Pat. No. 4,458,066; and Itakura U.S. Pat. Nos. 4,401,796 and 4,373,071, incorporated by reference herein).

### Delivery of Nucleic Acid to Cells and Animals

DNA delivery involves introduction of a "foreign" DNA either (1) into a cell *ex vivo* and ultimately, into a live animal by administering the cells, or (2) directly into the animal. Several general strategies for "gene delivery" (*i.e.*, delivery of any nucleic acid vector) for purposes that include "gene therapy" have been studied and reviewed extensively (Yang, N-S., *Crit. Rev. Biotechnol.* 12:335-356 (1992); Anderson, W.F., *Science* 256:808-813 (1992); Miller, AS, *Nature* 357:455-460 (1992); Crystal, RG, *Amer. J. Med.* 92 (suppl 6A):44S-52S (1992); Zwiebel, JA *et al.*, *Ann. N.Y. Acad. Sci.* 618:394-404 (1991); McLachlin, JR *et al.*, *Prog. Nucl. Acid Res. Molec. Biol.* 38:91-135 (1990); Kohn, DB *et al.*, *Cancer Invest.* 7:179-192 (1989), which references are herein incorporated by reference in their entirety).

One approach comprises nucleic acid transfer into primary cells in culture followed by autologous transplantation of the *ex vivo* transformed cells into the host, either systemically or into a particular organ or tissue.

Preferred DNA molecules for delivery as described below encode Tpm, *e.g.*, SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17 or 19, or a AALBP fragment thereof (SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18 or 20) or shorter peptides based on these sequences..

For accomplishing the objectives of the present invention, nucleic acid therapy would be accomplished by direct transfer of a the functionally active DNA into mammalian somatic tissue or organ *in vivo*. DNA transfer can be achieved using a number of approaches described below. These systems can be tested for successful expression *in vitro* by use of a selectable marker (*e.g.*, G418 resistance) to select transfected clones expressing the DNA, followed by detection of the presence of the antigen-containing expression product (after treatment with the inducer in the case of an inducible system) using an antibody to the product in an appropriate immunoassay. Efficiency of the procedure, including DNA uptake, plasmid integration and stability of integrated plasmids, can be improved by linearizing the plasmid DNA using known methods, and co-transfection using high molecular weight mammalian DNA as a "carrier".

Examples of successful "gene transfer" reported in the art include: (a) direct injection of plasmid DNA into mouse muscle tissues, which led to expression of marker genes for an

indefinite period of time (Wolff, J.A. *et al.*, *Science* 247:1465 (1990); Acsadi, G. *et al.*, *The New Biologist* 3:71 (1991)); (b) retroviral vectors are effective for *in vivo* and *in situ* infection of blood vessel tissues; (c) portal vein injection and direct injection of retrovirus preparations into liver effected gene transfer and expression *in vivo* (Horzaglou, M. *et al.*, *J. Biol. Chem.* 265:17285 (1990); Koleko, M. *et al.*, *Human Gene Therapy* 2:27 (1991); Ferry, N. *et al.*, *Proc. Natl. Acad. Sci. USA* 88:8387 (1991)); (d) intratracheal infusion of recombinant adenovirus into lung tissues was effective for *in vivo* transfer and prolonged expression of foreign genes in lung respiratory epithelium (Rosenfeld, M.A. *et al.*, *Science* 252:431 (1991)); (e) Herpes simplex virus vectors achieved *in vivo* gene transfer into brain tissue (Ahmad, F. *et al.*, eds, *Miami Short Reports - Advances in Gene Technology: The Molecular Biology of Human Genetic Disease*, Vol 1, Boehringer Manneheim Biochemicals, USA, 1991). Gene therapy of cystic fibrosis using transfection by plasmids using any of a number of methods and by retroviral vectors has been described by Collins *et al.*, U.S. Patent 5,240,846.

Retroviral-mediated human therapy utilizes amphotrophic, replication-deficient retrovirus systems (Temin, H.M., *Human Gene Therapy* 1:111 (1990); Temin *et al.*, U.S. Patent 4,980,289; Temin *et al.*, U.S. Patent 4,650,764; Temin *et al.*, U.S. Patent No. 5,124,263; Wills, J.W. U.S. Patent 5,175,099; Miller, A.D., U.S. Patent No. 4,861,719). Such vectors have been used to introduce functional DNA into human cells or tissues, for example, the adenosine deaminase gene into lymphocytes, the NPT-II gene and the gene for tumor necrosis factor into tumor infiltrating lymphocytes. Retrovirus-mediated gene delivery generally requires target cell proliferation for gene transfer (Miller, D.G. *et al.*, *Mol. Cell. Biol.* 10:4239 (1990)). This condition is met by certain of the preferred target cells into which the present DNA molecules are to be introduced, *i.e.*, actively growing tumor cells. The DNA molecules encoding the Tpm polypeptide, domain or peptide fragments of the present invention may be packaged into retrovirus vectors using packaging cell lines that produce replication-defective retroviruses, as is well-known in the art (see, for example, Cone, R.D. *et al.*, *Proc. Natl. Acad. Sci. USA* 81:6349-6353 (1984); Mann, RF *et al.*, *Cell* 33:153-159 (1983); Miller, AD *et al.*, *Molec. Cell. Biol.* 5:431-437 (1985); Sorge, J. *et al.*, *Molec. Cell. Biol.* 4:1730-1737 (1984); Hock, RA *et al.*, *Nature* 320:257 (1986); Miller, AD *et al.*, *Molec. Cell. Biol.* 6:2895-2902 (1986). Newer packaging cell lines which are efficient and safe for gene transfer have also been described (Bank *et al.*, U.S. 5,278,056).

This approach can be utilized in a site specific manner to deliver the retroviral vector to the tissue or organ of choice. Thus, for example, a catheter delivery system can be used (Nabel, EG *et al.*, *Science* 244:1342 (1989)). Such methods, using either a retroviral vector or a liposome vector, are particularly useful to deliver the nucleic acid to be expressed to a blood vessel wall, or into the blood circulation of a tumor.

Other virus vectors may also be used, including recombinant adenoviruses (Horowitz, M.S., In: *Virology*, Fields, BN *et al.*, eds, Raven Press, New York, 1990, p. 1679; Berkner, KL, *Biotechniques* 6:616 9191988), Strauss, SE, In: *The Adenoviruses*, Ginsberg, HS, ed., Plenum Press, New York, 1984, chapter 11), herpes simplex virus (HSV) for neuron-specific delivery and persistence. Advantages of adenovirus vectors for human gene delivery include the fact that recombination is rare, no human malignancies are known to be associated with such viruses, the adenovirus genome is double stranded DNA which can be manipulated to accept foreign genes of up to 7.5 kb in size, and live adenovirus is a safe human vaccine organisms. Adeno-associated virus is also useful for human therapy (Samulski, RJ *et al.*, *EMBO J.* 10:3941 (1991) in the present invention.

Another useful vector, particularly in humans, is vaccinia virus, which can be rendered non-replicating (U.S. Patents 5,225,336; 5,204,243; 5,155,020; 4,769,330; Sutter, G *et al.*, *Proc. Natl. Acad. Sci. USA* (1992) 89:10847-10851; Fuerst, TR *et al.*, *Proc. Natl. Acad. Sci. USA* (1989) 86:2549-2553; Falkner FG *et al.*; *Nucl. Acids Res* (1987) 15:7192; Chakrabarti, S *et al.*, *Molec. Cell. Biol.* (1985) 5:3403-3409). Descriptions of recombinant vaccinia viruses and other viruses containing heterologous DNA and their uses in immunization and DNA therapy are reviewed in: Moss, B, *Curr. Opin. Genet. Dev.* (1993) 3:86-90; Moss, B, *Biotechnology* (1992) 20:345-362; Moss, B, *Curr Top Microbiol Immunol* (1992) 158:25-38; Moss, B, *Science* (1991) 252:1662-1667; Piccini, A *et al.*, *Adv. Virus Res.* (1988) 34:43-64; Moss, B *et al.*, *Gene Amplif Anal* (1983) 3:201-213.

In addition to naked DNA or RNA, or viral vectors, engineered bacteria may be used as vectors. A number of bacterial strains including *Salmonella*, BCG and *Listeria monocytogenes*(LM) (Hoiseth & Stocker, *Nature* 291, 238-239 (1981); Poirier, TP *et al.* *J. Exp. Med.* 168, 25-32 (1988); (Sadoff, J.C., *et al.*, *Science* 240, 336-338 (1988); Stover, C.K., *et al.*, *Nature* 351, 456-460 (1991); Aldovini, A. *et al.*, *Nature* 351, 479-482 (1991); Schafer, R., *et al.*, *J. Immunol.* 149, 53-59 (1992); Ikonomidis, G. *et al.*, *J. Exp. Med.* 180, 2209-2218 (1994)).

These organisms permit enteric routes of infection, providing the possibility of oral nucleic acid delivery.

In addition to virus-mediated gene transfer *in vivo*, physical means well-known in the art can be used for direct transfer of DNA, including administration of plasmid DNA (Wolff *et al.*, 1990, *supra*) and particle-bombardment mediated gene transfer (Yang, N.-S., *et al.*, *Proc. Natl. Acad. Sci. USA* 87:9568 (1990); Williams, RS *et al.*, *Proc. Natl. Acad. Sci. USA* 88:2726 (1991); Zelenin, AV *et al.*, *FEBS Lett.* 280:94 (1991); Zelenin, AV *et al.*, *FEBS Lett.* 244:65 (1989); Johnston, SA *et al.*, *In Vitro Cell. Dev. Biol.* 27:11 (1991)). Furthermore, electroporation, a well-known means to transfer genes into cell *in vitro*, can be used to transfer DNA molecules of the present invention to tissues *in vivo* (Titomirov, AV *et al.*, *Biochim. Biophys. Acta* 1088:131 ((1991)).

“Carrier mediated gene transfer” has also been described (Wu, C.H. *et al.*, *J. Biol. Chem.* 264:16985 (1989); Wu, GY *et al.*, *J. Biol. Chem.* 263:14621 (1988); Soriano, P. *et al.*, *Proc. Natl. Acad. Sci. USA* 80:7128 (1983); Wang, C-Y, *et al.*, *Proc. Natl. Acad. Sci. USA* 84:7851 (1982); Wilson, JM *et al.*, *J. Biol. Chem.* 267:963 (1992)). Preferred carriers are targeted liposomes (Nicolau, C *et al.*, *Proc. Natl. Acad. Sci. USA* 80:1068 (1983); Soriano *et al.*, *supra*) such as immunoliposomes, which can incorporate acylated mAbs into the lipid bilayer (Wang *et al.*, *supra*). Polycations such as asialoglycoprotein/polylysine (Wu *et al.*, 1989, *supra*) may be used, where the conjugate includes a molecule which recognizes the target tissue (e.g., asialoorosomucoid for liver) and a DNA binding compound to bind to the DNA to be transfected. Polylysine is an example of a DNA binding molecule which binds DNA without damaging it. This conjugate is then complexed with plasmid DNA of the present invention for transfer.

Plasmid DNA used for transfection or microinjection may be prepared using methods well-known in the art, for example using the Quiagen procedure (Quiagen), followed by DNA purification using known methods, such as the methods exemplified herein.

#### **Diseases and Disorders to be Treated**

Malignant and metastatic diseases and conditions (tumors and cancer) which can be treated in accordance with the present invention include, but are not limited to, solid tumors, e.g., carcinomas, sarcomas, lymphomas and other malignant or nonmalignant tumors such as those listed in the table below (for a review of such disorders, see any textbook of clinical

oncology, most recent edition, e.g., *Cancer: Principles & Practice of Oncology*, 5<sup>th</sup> Ed. (DeVita, V. et al., eds), Philadelphia: Lippincott-Raven Publishers, 1997)

### CANCERS/TUMORS

Acoustic neuroma	Ewing's tumor	oligodendrogloma
Adenocarcinoma	Fibrosarcoma	osteogenic sarcoma
Angiosarcoma	glioma	ovarian cancer
Astrocytoma	hemangioblastoma	pancreatic cancer
Basal cell carcinoma	hepatoma	papillary adenocarcinomas
Bile duct carcinoma	Kaposi's sarcoma	pinealoma
Bladder carcinoma	leiomyosarcoma	prostate cancer
Breast cancer	liposarcoma	renal cell carcinoma
Bronchogenic carcinoma	lung carcinoma	retinoblastoma
Cervical cancer	lymphangiosarcoma	rhabdomyosarcoma
Chondrosarcoma	lymphangioendothelioma	sebaceous gland carcinoma
Choriocarcinoma	medullary carcinoma	seminoma
Colon carcinoma	medulloblastoma	small cell lung carcinoma
Craniopharyngioma	melanoma	squamous cell carcinoma
Cystadenocarcinoma	meningioma	sweat gland carcinoma
Embryonal carcinoma	mesothelioma	synovioma
Endothelioma	myxosarcoma	testicular tumor
Ependymoma	neuroblastoma	Wilms' tumor

The present invention is directed to the treatment of ocular disorders that involve pathogenic ocular neovascularization such as that associated with, or a cause of, proliferative diabetic retinopathy, neovascular age-related macular degeneration, neovascular glaucoma, retinopathy of prematurity, sickle cell retinopathy, retinal vein occlusion, retrobulbar fibroplasia, uveitis, corneal graft neovascularization, as well as other eye inflammatory diseases, ocular tumors and diseases associated with choroidal or iris neovascularization.

Other disorders which can be treated in accordance with the present invention include, but are not limited to, uterine disease such as endometriosis, hemangioma, arthritis, psoriasis, angiofibroma, atherosclerotic plaques, delayed wound healing, granulations, hemophilic joints, hypertrophic scars, nonunion fractures, Osler-Weber syndrome, pyogenic granuloma, scleroderma, trachoma, and vascular adhesions.

Therapeutic or prophylactic utility of the present invention and the determination of therapeutically effective dosages can be determined or demonstrated *in vivo* in a suitable animal model system prior to testing in humans. Such model systems may be based on the use of rats, mice, chicken, cows, monkeys, rabbits, etc. For *in vivo* testing, prior to administration to humans, any animal model system known in the art may be used. Some preferred model systems have been set forth above.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

## EXAMPLES

### Materials and Methods for Examples

Two-chain high molecular weight kininogen (HKa) was purchased from Enzyme Research Laboratories (Bloomington, IN). Recombinant bFGF and VEGF were from Becton-Dickinson Biosciences (Franklin Lakes, NJ). NHS (sulfo)-LC-biotin and *Bis*(sulfocuccinimidyl) suberate (BS<sup>3</sup>) were from Pierce (Rockford, IL).

The anti-Tpm mAbTM-311, raised against chicken gizzard Tpm, was obtained as ascites from Sigma (St. Louis, MO), and purified with Protein A-Sepharose®. Affinity-purified rabbit antibodies that block the binding of HKa to domains 2+3 of the urokinase receptor (uPAR) have been described (Colman, RW *et al.* (1997) *J. Clin. Invest.* **100**, 1481-1487. A rabbit antibody that blocks HK binding to cytokeratin 1 was from Dr. Alvin Schmaier (Hasan, AA *et al.* (1998) *Proc. Natl. Acad. Sci., U.S.A.* **95**:3615-3620), and a mAb that blocks binding of HK to the EC gC1qR was from Dr. Berhane Geebrehewet (Joseph, K *et al.* (1996) *Proc. Natl. Acad. Sci. USA* **93**:8552-8557).

Cloning, expression, refolding and purification of kininogen domain 5. Recombinant HKa domain 5 (HKa D5) was produced as a calmodulin binding protein (CBP) conjugate in *E. coli*. Briefly, domain 5 cDNA was PCR amplified from a full-length HK cDNA using primers 5'-CGGGATCCGTAAAGTCCACCCACACTTC-3' (SEQ ID NO:27) and 5'-CGAATTCTCAGCTTGCAAATGCTC-3' (SEQ ID NO:28).

The purified PCR product was digested with *Bam*HI and *Eco*RI and ligated into the expression vector pCAL-n (Stratagene, La Jolla, CA). The vector was transformed into BL21(DE3) cells and subclones were grown and induced with 1 mM IPTG. SDS-PAGE revealed that the majority of expressed CBP-HKa D5 was in inclusion bodies. To purify these, the pellet from a 500 ml bacterial culture was lysed, homogenized in 4% Tergitol and centrifuged at 10,000 x g for 45 minutes. The purified inclusion bodies were sonicated in 7 M guanidine HCl, and the denatured protein clarified by centrifugation and then added to 1000 ml of 50 mM bicine, pH 8.8, containing 150 mM NaCl. The refolded CBP-HKa D5 was purified by affinity chromatography

on HiTrap SP (Amersham-Pharmacia, Piscataway NJ), then digested with  $\alpha$ -thrombin (2.5  $\mu$ g/mg of CBP-D5). Free HKa D5 was purified using Mono S.

Cell culture. HUVEC were isolated and cultured as previously described (Zhang, J.-C. *et al.* (2000) *FASEB J* 14:2589-2600). MDA-MB-231 breast carcinoma cells were obtained from the American Type Culture Collection.

EC proliferation assays. The effect of HKa on ECs in the absence or presence of mAb TM-311 was initially assessed using a proliferation assay, as described in Zhang *et al.*, *supra*. Relative numbers of cells remaining in each well of a 96 well microplate after incubation for 48 hours in the absence or presence of HKa were determined using the Aqueous® cell proliferation assay (Promega, Madison, WI). Results are presented as the percent inhibition of bFGF-induced EC proliferation, which essentially reflects the extent of HKa-induced EC apoptosis. Though bFGF was used as the EC mitogen in most studies of the studies described herein, identical results were obtained using VEGF.

Assessment of EC apoptosis. The effect of TM-311 on HKa- or HKa D5-induced EC apoptosis was determined using several methods. First, staining of control or HKa exposed ECs using 4', 6'-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes, Eugene OR) was employed to highlight apoptosis-associated changes in nuclear morphology. Second, apoptosis was assessed by TUNEL staining, using the Apo-Direct kit (Pharmingen, San Diego, CA). Cells were counterstained with propidium iodide to define all nuclei, and the percentage of apoptotic (TUNEL positive) nuclei determined by manual counting. Finally, to assess endothelial apoptosis by endonucleolytic cleavage of DNA, EC DNA was isolated and separated using 0.8% agarose gel electrophoresis. Gels were stained with ethidium bromide, and DNA visualized using UV light.

Exposure of Tpm on the cell surface. Two approaches were used to detect exposure of Tpm on ECs. First, confluent or proliferating ECs cultured in Lab-Tek® chambers (Nunc, Naperville IL) were fixed by exposure to 3.7% paraformaldehyde, blocked using 10% donkey serum and then incubated with either mAb TM-311 or non-immune murine IgG<sub>1</sub>. Bound antibody was detected using rhodamine-conjugated donkey anti-mouse IgG, and stained cells were examined using a Bio-Rad MRC 600 laser scanning confocal microscope. For confocal imaging, control stains were set to a black background and positive samples viewed at the same laser intensity, aperture, gain and black level settings. One-micron optical slices were taken for each sample, beginning at the coverslip surface and ending at the apical surface. Projections were acquired

using Confocal Assistant Imaging Software, v 4.02. In some experiments, cells were exposed to 0.1% Triton-X-100 in PBS for 15 minutes prior to staining.

To further assess the availability of Tpm on the surface of confluent or proliferating ECs, intact, unfixed cells were labeled using NHS (sulfo)-LC-biotin, as described by (Ma, K *et al.*, (2000) *J. Biol. Chem.* **275**:15541-15548). After labeling, cell extracts were prepared in a buffer containing 0.1 M Tris-HCl, pH 7.4, 1% Triton X-100 and protease inhibitors, and equal amounts of protein from confluent and subconfluent cultures were immunoprecipitated using mAb TM-311. Precipitated proteins were separated by 10% SDS-PAGE, and transferred to a polyvinylidene fluoride (PVDF) membrane. Biotinylated proteins were detected by incubating the membrane with streptavidin-peroxidase and chemiluminescence reagent (Super Signal, Pierce) before exposure to Kodak XL-blue autoradiographic film.

Cross-linking of HKa to ECs. To determine whether HKa interacted with a specific protein on proliferating ECs, the present inventors determined whether it could be cross-linked to such a protein. Biotin-labeled HKa (Ma *et al.*, *supra*) was incubated with proliferating or confluent ECs for 30 minutes, at 37° C. Cells were then washed and exposed to the bifunctional, membrane-impermeable cross-linker, BS<sup>3</sup>, for 15 minutes at room temperature. Detergent extracts were prepared, and 40 µg of cell protein from proliferating and confluent cultures separated by 7.5% SDS-PAGE. Proteins were transferred to PVDF, and biotinylated proteins detected using streptavidin-peroxidase and chemiluminescence. To assess the specificity of the cross-linking procedure, studies were also performed in the presence of a 50-fold molar excess of unlabeled HKa, and with MDA-MB-231 breast carcinoma cells.

Binding of HKa to ECs and purified Tpm. Binding of HKa to ECs was measured as described by Hasan, AA *et al.* (1995) *J. Biol. Chem.* **270**:19256-19261. Briefly, 3 x 10<sup>4</sup> HUVEC/ml were cultured in 96-well microplates, then washed and incubated with increasing concentrations of biotin-HKa for two hours, at 4°C. After brief washing, cells were incubated sequentially with a 1:750 dilution of streptavidin peroxidase and the peroxidase substrate, turbo-TMB (Pierce), prior to measurement of A<sub>490</sub>. Absolute amounts of bound HKa were determined by comparison of A<sub>490</sub> values with a standard curve prepared using known amounts of biotin-HKa. Binding was measured in the presence (to determine total binding) and absence (to determine non-specific binding) of 10 µM Zn<sup>2+</sup>, and specific binding defined as the difference between total and non-specific binding (van Iwaarden, F *et al.* (1988) *J. Biol. Chem.* **263**:4698-

4703). The dissociation constant ( $K_d$ ) was determined by fitting the saturation isotherm by nonlinear regression using the Prism software program (GraphPad, San Diego, CA).

In selected experiments, the ability of mAb TM-311 to inhibit the binding of HKa to cells was assessed by incubating biotin-HKa with ECs in the presence of increasing concentrations of antibody. The concentration of TM-311 that inhibited HKa binding by 50% ( $IC_{50}$ ) was determined from plots of bound HKa versus the log of the TM-311 concentration.

To measure the binding of HKa to purified Tpm, 96 well microplates were coated with 20  $\mu$ g/ml of chicken gizzard Tpm (Sigma), or bovine serum albumin (BSA), as a control. Wells were then blocked by incubation with phosphate buffered saline containing 5% nonfat milk, and incubated with increasing concentrations of HKa (0.01-20 nM) for 2 hours. Specifically-bound HKa was quantitated as described for cell binding assays (van Iwaarden *et al.*, *supra*, and by assessing the ability of a 100-fold molar excess of unlabeled HKa to compete with biotin-HKa for binding.

In selected experiments, the ability of mAb TM-311 and recombinant HKa D5 to inhibit the binding of biotin-HKa to Tpm was assessed. These studies allowed determination of the  $K_d$  for binding of HKa D5 to Tpm using the equation:

$$K_d(D5) = IC_{50} / (1 + [HKa]/K_d(HKa))$$

where  $IC_{50}$  is the concentration of HKa D5 that inhibited HKa binding by 50%, and  $K_d(HKa)$  is the  $K_d$  for binding of HKa to Tpm.

Chick chorioallantoic membrane assay. The chick chorioallantoic membrane (CAM) assay was used to assess the role of Tpm in the antiangiogenic activity of HKa *in vivo* (Nguyen, M *et al.* (1993) *Microvasc Res* 47:40). Three day-old fertilized White Leghorn chicken eggs were cracked in sterile Petri dishes. Embryos were cultured at 37°C, under 4% CO<sub>2</sub>, until day 7, at which time a 3.0 mm filter disc containing either 30 ng bFGF (positive control), 30 ng bFGF and 10  $\mu$ g HKa or 30 ng bFGF, 10  $\mu$ g HKa and 20  $\mu$ g of mAb TM-311 was placed on the CAM. Each experimental condition was tested in at least 6 eggs. On day 10, embryos were photographed using a SPOT digital camera. Angiogenesis was quantitated by counting the number of neovessels in direct contact with the filter disc.

## EXAMPLE I

### Antibody Specific for Tpm Blocks the Action of HKa on ECs

Preliminary studies aimed at defining the structure of Zn<sup>2+</sup>-bound HKa D5 (Kumar, GA *et al.*, 2001, *Abst. Am. Chem. Soc.* 222:134) suggested structural homology between HKa D5

and endostatin, a  $Zn^{2+}$ -binding, antiangiogenic polypeptide comprised of the NC domain of collagen XVIII (O'Reilly, MS *et al.* (1997) *Cell* **88**:277-285; Ding, Y-H *et al.* (1998) *Proc. Natl. Acad. Sci., U.S.A.* **95**:10443-10446).

Prior studies by one of the present inventors and colleagues could not demonstrate involvement of previously-reported EC binding sites for HK or HKa in the antiangiogenic effects of this polypeptide (Zhang *et al.*, *supra*). A recent report suggested that the anti-angiogenic activity of endostatin was mediated through binding to Tpm (MacDonald, NJ *et al.* (2001) *J. Biol. Chem.* **276**:25190-25196). The present inventors therefore sought to determine if Tpm functioned in a similar way with HKa.

The initial study was designed to determine whether mAb TM-311 affected the ability of HKa to block growth factor-induced EC proliferation. TM-311 indeed blocked, in a concentration-dependent manner, the HKa and D5-mediated inhibition of bFGF- and VEGF-induced EC proliferation (Fig. 1A). Antibodies that block the binding of HKa to different receptors, the urokinase receptor (Colman, RW *et al.* (1997) *J. Clin. Invest.* **100**:1481-1487), gC1qR (Joseph *et al.*, *supra*; Herwald, H *et al.* (1996) *J. Biol. Chem.* **271**:13040-13047) and cytokeratin 1 (Hasan *et al.*, *supra*) did not have such an effect (Fig. 1B).

These effects reflected inhibition of HKa-induced EC apoptosis by TM-311. TM-311 prevented the endonucleolytic fragmentation of DNA (Figure 2) as well as the characteristic apoptotic changes in nuclear morphology and the increase in TUNEL-positive cells following exposure of proliferating ECs to HKa or HKa D5 (Zhang *et al.*, *supra*). Non-immune murine IgG (MOPC-21 myeloma protein) also lacked any such activity.

The specificity of these effects was addressed by evaluating the ability of mAb TM-311 to inhibit EC induced by 2-methoxyestradiol, another anti-angiogenic agent with such activity (Yue, TL *et al.* (1997) *Mol Pharm* **51**:951-962). However, even at a very high concentration of 6  $\mu$ M, mAb TM-311 did not inhibit EC apoptosis induced by 2  $\mu$ M 2-methoxyestradiol (Figure 2, lanes 6 versus 7).

## EXAMPLE II

### Tpm is Present on the Surface of Activated EC's

The results of Example I suggested an essential role for Tpm in mediating HKa-induced EC apoptosis. However, Tpm is a cytoskeletal protein, and there have been no reports of it being exposed on the endothelial surface. Indeed, in only one prior study was a single isoform of Tpm, hTM5, observed on the surface of any cell type - colon epithelial cells and cells of a colon

carcinoma line (Kesari KV *et al.*, *Clin. Exp. Immunol.* (1999). **118**:219-27). Although a recent report suggested that the antiangiogenic activity of endostatin requires interaction with Tpm, it was hypothesized that internalization of endostatin was necessary for this to occur (McDonald *et al.*, *supra*).

To assess whether Tpm is expressed on the EC surface, and to address the selectivity of HKa for proliferating ECs, the present inventors used confocal scanning laser microscopy to assess proliferating and confluent cultures of ECs stained with mAb TM-311. Proliferating cells stained specifically with TM-311 (Figure 3A versus 3B), and the surface staining of these cells was more prominent than with confluent cells (Figure 3B versus 3C). The surface staining pattern of proliferating cells was unchanged when cells were permeabilized by exposure to 0.2% Triton X-100 prior to staining, though an intracellular pool of Tpm, particularly evident in the confluent cells (Figure 3C), was more prominent.

To confirm these results of the existence of Tpm on the EC surface, surface proteins on proliferating or confluent ECs were labeled with biotin, and detergent extracts of the labeled cells were immunoprecipitated with mAb TM-311. TM-311 precipitated proteins of ~36 kDa and ~40 kDa, consistent with the expected molecular weight of Tpm (Lees-Miller, JP *et al.* (1991) *Bioessays* **13**:429-437; Lin, JJC *et al.*, (1997) *Int. Rev. Cytology* **170**:1-38), from both EC cultures (Figure 4). However, these proteins were precipitated in markedly greater amounts from the proliferating cells (Figure 4).

#### Characterization of the binding of HKa to ECs and Tpm

Several approaches were used for further evaluation of the possibility that HKa binds to Tpm on ECs.

First, the ability of mAb TM-311 to block the binding of HKa to subconfluent ECs was tested. Biotin-HKa bound specifically to these cells in a  $Zn^{2+}$ -dependent manner, with a  $K_d$  of ~2.5 nM (Figure 5A). TM-311, but not a control IgG (MOPC-21), blocked the specific binding of HKa to cells by about 90% (Figure 5B)

Next, the binding of HKa to purified Tpm immobilized in 96-well microplates was studied. The results are shown in Figs 6A-6C/PNAS. HKa bound with similar affinity ( $K_d$  of about 2.6 nM), and in a  $Zn^{2+}$ -dependent manner, to purified Tpm (Figure 6A). mAb TM-311 (Figure 6B), as well as HKa D5 (Figure 6C) blocked binding, confirming that HKa D5 binds with high affinity to Tpm (estimated  $K_d$  about 2.1 nM)

New Fig 7 shows direct binding of mAb TM-311 to immobilized Tpm by ELISA. Fig. 8 provides additional proof that this antibody competes with HKa for binding to such immobilized Tpm.

Finally, a study was done to determine whether biotinylated HKa could be cross-linked to an EC surface protein of the size of Tpm. When incubated with confluent ECs prior to exposure to the membrane-impermeable cross-linker, BS<sup>3</sup>, biotin-HKa was barely detectable in cell extracts (Figure 5, lane 1). However, HKa was detected in both an uncomplexed form ( $M_r$  ~110 kDa) and within a broad, high molecular weight complex (Figure 5, lane 3) in extracts of proliferating cells. A complex between HKa and Tpm would be expected to exhibit a  $M_r$  of ~140-150 kDa. Indeed, a discrete band of this size was observed within the broad band (Figure 5, arrow).

The specificity of this interaction is supported by the observations that complex formation was prevented by excess unlabeled HKa (Figure 5, lane 4), and that high molecular weight complexes were not observed when the cells were MDA-MB-231 breast carcinoma cells rather than ECs (Figure 5, lane 5).

The presence of HKa within complexes having a molecular size >150 kDa may result from association of the HKa-Tpm complex with actin or other Tpm binding proteins, or complexes between HKa and other EC binding proteins.

### EXAMPLE III

#### MAb Tm 311 Blocks the Anti-angiogenic Effects of HKa *In Vivo*

The CAM assay was employed to assess the functional role of Tpm in an *in vivo* angiogenesis model. The results are shown in Figure 8A-8D. HKa inhibited angiogenesis induced by bFGF-containing filter discs by about 85%, as determined by vessel counts (Fig. 8A vs. Fig. 8C). Similar results were obtained using HKa D5 (Colman, RW *et al.* (2000) *Blood* 95:543-550). However, the anti-angiogenic effect of HKa was blocked by TM-311 (Fig. 8C vs Fig. 8D), whereas non-immunie murine IgG was without effect. These results demonstrate that Tpm mediates the anti-angiogenic activity of HKa *in vivo* as well as *in vitro*, and hence may function as an “anti-angiogenic” binding site for HKa.

#### Discussion of Examples I-III

The result presented herein prove that that HKa binds with high affinity to Tpm through interactions involving the HKa D5 domain, and that inhibition of this interaction by an anti-Tpm

antibody blocks (a) the induction of EC apoptosis and (b) the inhibition of angiogenesis by HKa. These observations provide compelling evidence that the effects of HKa on proliferating ECs are mediated through direct binding to Tpm.

In addition to identifying a new EC binding site for HKa, the present invention provides insight into the biology of ECs during angiogenesis by demonstrating that Tpm, a cytoskeletal protein, is preferentially exposed on the surface of proliferating cells. Exposure of Tpm on the endothelial surface was documented by (a) direct visualization and (2) the fact that Tpm could be biotinylated NHS (sulfo) LC-biotin.

Earlier studies examining the binding of HK or HKa to ECs used confluent, static EC monolayers; however, the present findings demonstrate that under these conditions Tpm is minimally available on the cell surface, leaving other binding sites to the more prominent role in binding single chain (Hasan *et al.*, *supra*; Herwald *et al.*, *supra*; Hasan, AA *et al.*, (1994) *J. Biol. Chem.* **269**, 31822-31830; Herwald, H *et al.* (1995) *J. Biol. Chem.* **270**:14634-14642; Dedio, J *et al.* (1996) *FEBS Lett.* **399**:255-258; Dedio, J *et al.*, (1998) *J. Immunol.* **160**:3534-3542) or two-chain (Colman *et al.*, 1997, *supra*) high molecular weight kininogen.

These findings are consistent with studies demonstrating that the EC cytoskeleton undergoes dramatic structural rearrangement during the transition between a quiescent and proliferative state (Ingber, DE (1997) *Annu Rev Physiol* **59**:575-599; Ingber, DE *et al.* (1995) *J. Biomechanics* **28**:1471-1484; Huang, S *et al.* (2000) *Exp. Cell Res.* **261**:91-103)

However, other than one report in which actin was demonstrated on the surface of cultured ECs (Dudani, AK *et al.* (1996) *Br. J Haematol* **95**:168-178), there has been little evidence for exposure of cytoskeletal components on the EC surface. Hence, the present results challenge the paradigm that the cytoskeleton exists solely within the confines of the EC plasma membrane under all conditions. Moreover, the observation that mAb TM-311 inhibits the anti-angiogenic activity of HKa suggests that Tpm is available on the surface of angiogenic ECs *in vivo*.

Vertebrate cells express a number of Tpm isoforms in a cell-specific manner. The expression of Tpm in nonmuscle cells has been most intensively studied in human fibroblasts, which express at least eight isoforms (Lin *et al.*, *supra*). The present inventors have obtained results suggesting that ECs also express multiple Tpm isoforms.

The major role of Tpm is the binding and stabilizing actin filaments, protecting them from separation or depolymerization by factors such as gelsolin or actin depolymerizing factor

(Lin *et al.*, *supra*; Ishikawa, R *et al.* (1989) *J. Biol. Chem.* **264**:7490-7497). Alterations in Tpm isoform expression has been reported to play a role in cellular transformation (Lin *et al.*, *supra*; Takenaga, K *et al.*, (1988) *Molec. Cell Biol.* **8**:3934-3937; Prasad, GL *et al.*, (1993) *Proc. Natl. Acad. Sci., USA* **90**:7039-7043), suggesting that Tpm may influence the state of cytoskeletal organization. According to the present invention, alterations in the expression and cellular localization of Tpm contributes to the transition of ECs from a quiescent to an "angiogenic" phenotype.

The identification of Tpm as an endothelial binding site required for the anti-angiogenic activity of HKa raises several questions concerning the mechanism(s) by which HKa induces EC apoptosis. Though HK and HKa have anti-adhesive properties (Colman, RW *et al.* (1997) *Blood* **90**:3819-3843; Chavakis, T *et al.*, (2000) *Blood* **96**:514-522), the ability of HKa to induce apoptosis of proliferating ECs at concentrations below those generally associated with the anti-adhesive activity argues in favor of additional mechanisms to account for HKa's anti-angiogenic activity (Zhang *et al.*, *supra*). The results disclosed above suggest that HKa may affect the EC cytoskeleton directly, perhaps causing secondary alterations in downstream signaling pathways dependent upon reciprocal interactions between the cytoskeleton and integrin adhesion receptors. Indeed, biomechanical influences mediated through the cytoskeleton play a critical role in vital processes such as cell cycle entry, cellular growth, and apoptosis (Ingber *et al.*, *supra*; Huang *et al.*, *supra*).

A recent report by MacDonald *et al.*, *supra*, disclosed that an antibody reactive with an endostatin-binding cyclic peptide cross-reacted with human Tpm 3 (hTM3) and that hTM3 may be involved in the anti-angiogenic effects of endostatin *in vivo*. Both this antibody and mAb TM-311, employed in the above studies, recognize a EC protein having a molecular weight of about 38 kDa, suggesting that mAb TM-311, the Tpm isoform specificity which has not been well characterized, also recognizes hTM3.

Since TM-311 blocked HKa-induced EC apoptosis, it is possible that hTM3 may mediate the antiangiogenic activity of HKa as well. However, in the present studies, TM-311 recognized two EC proteins of ~36 and ~40 kDa (Figure 3), so that any specific role for hTM3 in mediating the activity of HKa is not conclusive. If indeed HKa binds hTM3, then this interaction may differ from that of endostatin, since the affinity of HKa for Tpm ( $K_d \sim 2.6$  nM) is orders of magnitude higher than that of endostatin ( $K_d \sim 100$   $\mu$ M). Moreover, MacDonald *et al.* *supra*, suggested that endostatin may require internalization prior to interacting with hTM3, while the

high molecular weight kininogen is not internalized by ECs (Hasan, AA *et al.*, (1995) *Blood* 85:3134-3143).

Because the present results support the conception that HKa binds directly to cell surface-exposed Tpm, it may be useful to reconsider the requirement that endostatin be internalized prior to its interaction with Tpm. Nevertheless, the observation that at least two antiangiogenic polypeptides bind to ECs through interactions with Tpm favors a broader role for Tpm, and perhaps other cytoskeletal proteins, in mediating the activity of naturally-occurring angiogenesis inhibitors.

In summary, the present invention is based on the discovery of a novel interaction between HKa and EC Tpm which underlies the antiangiogenic activity of HKa. This also serves as a basis for the development of novel agents targeted toward arrest of pathologic angiogenic processes.

#### EXAMPLE IV

##### Binding of HKa and other Antiangiogenic Agents to Tpm

The binding affinity of HKa and its domain D5 (which possesses most, if not all, of the anti-angiogenic activity) to Tpm were measured in a plate binding assay such as that described above in which chicken gizzard Tpm was immobilized to the plate. As shown in Figure 15, Kd values obtained for both proteins were approximately 1 nM. This value is identical to that obtain *in vivo* with HUVECs.

Other human Tpm human isoforms were tested in this same assay with similar results. Several other anti-angiogenic proteins also bind to Tpm. Endostatin binds to immobilized Tpm with a Kd of about 2  $\mu$ M which is 50-fold better than that reported previously (measured by SPR), but is approximately 1,000-fold lower than the affinity of HKa, or HKa-D5 binding (Figs. 14 and 15).

Troponin I is a component of the Troponin complex that binds and regulates actin contractility in muscle cells, which has also being found to be an anti-angiogenic molecule. Troponin I appears to bind to immobilized Tpm with a Kd in the nanomolar range

#### EXAMPLE V

##### Tpm Binding and Inhibition of Angiogenesis by Short Peptides derived from HPRG

The abundant multi-domain plasma protein Histidine-Proline-Rich Glycoprotein (HPRG) has been shown by some of the present inventors and their colleagues to have robust anti-

angiogenic properties (USSN 60/268370 and USSN 10/074,225, filed 2-14-02, all of which are incorporated by reference in their entirety).

Interestingly, rabbit HPRG and the Histidine-Proline rich ("H/P") domain of HPRG, which is responsible for anti-angiogenic activity, bind to Tpm with high affinity and specificity (Figs. 15A and 15B). The calculated Kd, ~ 2 nM, is similar to the Kd of HKa or HKa-D5 binding to Tpm. As a control, the rabbit N/C fragment, which corresponds to all of HPRG minus the H/P domain, shows no binding at the concentration tested (Fig 15A and B).

The H/P domain of rabbit HPRG is composed of 2 repeats of HHPHG [SEQ ID NO:29], 7 repeats of PPPHG [SEQ ID NO:30], 6 repeats of HPPHG [SEQ ID NO:31]. The human H/P domain contains 10 tandem repeats of the sequence HHPHG. A combined consensus sequence of human and rabbit is designated [H/P][H/P]PHG [SEQ ID NO:32].

In an effort to identify the minimal sequence within the H/P domain with significant anti-angiogenic activity, the present inventors synthesized and evaluated three peptides from the above mentioned consensus sequence (HHPHG, HPPHG and PPPHG). The binding affinity to immobilized Tpm of those three peptides together with activity in the Matrigel Plug assay was determined and is summarized in Table 5.

The IC<sub>50</sub> for the displacement of biotinylated-HKa for the peptides shown in Table 5 was determined as follows: 10 nM biotin-HKa is added to a 96 well plate previously coated with 200 ng of chicken gizzard Tpm in the presence of 10  $\mu$ M ZnCl<sub>2</sub>. Increasing amounts of the peptides are added to the wells. The remaining biotin-HKa bound to Tpm was detected using avidin-HRP and a chromogenic substrate. A Kd was determined by non-linear regression analysis of the empirical data. The effect on the Matrigel Plug assay was determined as follows: Matrigel (0.5 mL) containing 400 ng/ml of bFGF, 50  $\mu$ g/ml heparin with or without the peptide (300  $\mu$ M) or saline buffer are injected in the flanks of a mouse. After five days, the plugs are removed and the plugs scanned.

The peptide HHPHG binds Tpm with ~100  $\mu$ M affinity and has substantial activity in the Matrigel Plug assay. Alanine substitution of the first His in HPPHG (to yield APPHG) results in complete loss of Tpm binding and anti-angiogenic activities (Fig 16 and 17). Thus, loss of Tpm binding activity went hand-in-hand with a loss of anti-angiogenic activity in the Matrigel plug.

**Table 5**

Activity of consensus sequence peptides from HK-D5 (★) or HPRG-H/P domain

ATN#	Sequence	SEQ. ID NO:	IC <sub>50</sub> (μM)	Inhibits Angiogenesis (Matrigel® assay)
ATN16 ★	HKNKGKKN	34	99.5	Yes
ATN232 ★	TRRHDWGH	35	140.5	Yes
ATN230	HHPHG	29	121	Yes
ATN239	c-HHPHG-c	29	NB*	
ATN269	c-HHPHG	29	NB	
ATN228	HPPHG	31	150	Yes
ATN246	APPHG	33	NB	No
ATN231	c-HPPHG-c	31	NB	
ATN227	PPPHG	30	NB	
ATN229	c-PPPHG-c	30	NB	

ATN#- Attenuon code number

The IC<sub>50</sub> is determined in the Tpm binding assay; \* NB = no binding (i.e., IC<sub>50</sub>>10mM)

C= acetyl or amide cap

HHPHG also showed anti-tumor activity against MatLyLu prostate cancer cells, whereas the Ala substituted mutant, AHPHG (SEQ ID NO:36), in which binding to Tpm *in vitro* was abolished, also has significantly less anti-tumor activity (Fig 18).

The *N*-terminus of the pentapeptide HHPHG has been derivatized (capped) in an attempt to increase the pentapeptides' affinity to Tpm by increasing productive molecular contacts (or interactions). Table 6 shows a few of these derivatives including the capping groups that have already better affinities for Tpm than the parent compound. The IC<sub>50</sub> for the displacement of biotinylated-HKa for the capped peptides shown in the table was determined as follows: 10 nM biotin-HKa was added to a 96 well plate previously coated with 200 ng of chicken gizzard Tpm in the presence of 10 μM ZnCl<sub>2</sub>. Increasing amounts of the peptides were added to the wells. The remaining biotin-HKa bound to Tpm was detected using avidin-HRP and a chromogenic substrate. A Kd was determined by non-linear regression analysis of the empirical data.

**Table 6: Activity of N-capped peptides from HPRG-H/P domain**

ATN#	Common Name	IC <sub>50</sub> (μM)
ATN-278.000.01	Fmoc	55
ATN-276.000.01	4-Chlorobenzyl	23
ATN-281.000.01	3-(1-adamantyl)propanoyl	59
ATN-275.000.01	(1S)-(+)-camphorsulphonyl	30

**EXAMPLE VI****HPRG and the H/P Domain Inhibit Angiogenesis Stimulated by FGF-2 in Matrigel® Plug Model *in vivo***

The present inventors identified the region of Tpm that binds to HKa-D5. Chicken gizzard Tpm, because of its high degree of homology to different human Tpm isoforms, has been used as a model.

This Tpm was partially proteolyzed with chymotrypsin. A fragment of approximately 20-25 kDa was identified as being enriched during proteolysis by SDS-PAGE (Fig. 19). This fragment bound to the HKa-D5 domain immobilized in the Sepharose resin suggesting that it contains the region that binds to HKa-D5.

The fragment was partially purified by passing the chymotryptic digest through an affinity column of Sepharose-HKa-D5. The column was extensively washed and eluted with high salt buffer. The resulting material was run on an SDS gel which appears in (Fig. 20). The isolated chymotryptic fragment of Tpm, isolated as above was dialyzed against water and submitted to N-terminal sequencing (University of California, San Diego: Protein Sequencer Facility) and mass spectroscopy (MALDI-TOF) at the Scripps Research Institute Mass Spectrometry facility). The N terminal sequence of this fragment is shown above as residues 61-69 of SEQ ID NO:1 (or residues 1-9 or SEQ ID NO:2).

The estimated molecular mass of this fragment from MALDI-TOF analysis was ~17 kDa. The actual molecular weight of the polypeptide based on its sequence is 17,684.6 Da.

**EXAMPLE VII****Inhibition of Angiogenesis by Cyclic Peptides that Bind to HKa D5**

The following three cyclic peptides were discovered to bind HKa-D5 and were tested here.

ATN-310 CGPNWAGDGTYLGGGGPC (SEQ ID NO:37)

ATN-311 CGPNTPDGFWWVDGPC (SEQ ID NO:38)

ATN-312 CGPTIYKTDGGGETTGPC (SEQ ID NO:39)

These cyclic peptides were stabilized by a disulfide link between the two terminal Cys residues, indicated above as a "line" or "bond" between the terminal C (cysteine) residues.

A study was performed to examined whether these peptides displace HKa bound to immobilized Tpm. The results shown in Figure 21.

The binding results presented in Figure 21 show that the cyclic peptides ATN-310, ATN-311 and ATN-312 (referred to in the Figure insert as 310.000.01, 311.000.01 and 312.000.01, respectively) displaced HKa that is bound to Tpm whereas a control ATN-246 did not (not shown). In this experiment, 10 nM biotin-HKa was added to a 96 well plate previously coated with 200 ng chicken gizzard Tpm in the presence of 10  $\mu$ M ZnCl<sub>2</sub>. Increasing amounts of the three cyclic peptides were added to wells as indicated. Bound biotin-HKa was detected and Kd was calculated as in the Figure 10 description.

These cyclic peptides were also tested for their ability to inhibit bFGF-induced angiogenesis *in vivo* in a Matrigel plug assay. Matrigel (0.5 mL) containing 400 ng/ml of bFGF, 50  $\mu$ g/ml heparin with or without 10  $\mu$ M peptide (ATN-310, ATN-311 or ATN-312 or saline buffer for the controls, was injected subcutaneously in the hind flanks of a mouse as described above. All the treatments were done in triplicate. After five days, the vascularization of the Matrigel plug was determined fluorometrically after i.v. injection of 100  $\mu$ l of dextran conjugated with fluorescein isothiocyanate (FITC-dextran; MW 250,000, Sigma). Five days after injection, the plugs were removed and subjected to mincing of tissue which releases vascular FITC-dextran into the supernatant. Samples of supernatant were subjected to fluorimetry as a measure of vascularity of the plug. The amount of vasculature (neovessels) is directly proportional to the fluorescent signal which reflects the total vascular blood volume of the plug. The results are shown in Table 7, below and expressed as arbitrary fluorescence units or as % inhibition relative to the negative control.

**Table 7: Inhibition of Matrigel Plug Angiogenesis *In Vivo* by ATN-310, ATN-311 and ATN-312**

	Saline Control	ATN310	ATN311	ATN312
<b>Mean<sup>1</sup></b>	<b>0.2259</b>	<b>0.02760</b>	<b>0.0277</b>	<b>0.0413</b>
$\pm$ SD	0.0206	0.0070	0.0110	0.0089
<b>% Inhibition</b>	<b>0</b>	<b>87.8</b>	<b>87.7</b>	<b>81.7</b>

<sup>1</sup> Arbitrary fluorescence units

It was concluded that at this concentration, all three peptides were very effective inhibitors of angiogenesis. Inhibition of angiogenesis was also observed at lower concentrations of these cyclic peptides.

The references cited above are all incorporated by reference, whether specifically incorporated or not (as are the references cited therein).

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

**CLAIMS**

1. An isolated tropomyosin (Tpm) -related anti-angiogenic receptor polypeptide or peptide which,

- (a) is a fragment of a full length native Tpm protein expressed on the surface of endothelial cells or a variant of said fragment,
- (b) has a molecular mass of about 17 kDa and corresponds in its sequence to, or is a variant of, an internal fragment of a native Tpm isoform which is a binding site for antiangiogenic polypeptide agents, and
- (c) binds to said antiangiogenic polypeptide agents which bind to said native Tpm internal fragment binding site;

wherein

said peptide has between about 4 and about 40 amino acids; and

said variant of the polypeptide or peptide is a conservative substitution variant of a native Tpm sequence ; and

said isolated anti-angiogenic receptor polypeptide, peptide or variant has substantially the same biochemical activity of binding to said antiangiogenic polypeptide agents as does said native Tpm internal fragment.

2. The isolated polypeptide, peptide or variant of claim 1 wherein the native Tpm isoform has an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, and SEQ ID NO:19.

3. The isolated polypeptide or peptide or variant of claim 1, wherein the internal fragment of said native Tpm has an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, and SEQ ID NO:20.

4. The isolated polypeptide, peptide or variant of claim 1 wherein the Tpm isoform is a human Tpm isoform.

5. The isolated polypeptide, peptide or variant of claim 1-4 wherein said antiangiogenic polypeptide agent which binds to said isolated polypeptide or peptide is selected from the group consisting of:

- (a) human histidine-proline rich glycoprotein (HPRG);
- (b) rabbit HPRG;
- (c) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of human or rabbit HPRG;
- (d) two chain human kininogen human kininogen (HK<sub>a</sub>);
- (e) the D5 domain of HK<sub>a</sub>; and
- (f) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of said HK<sub>a</sub> or said D5 domain thereof.

6. The isolated polypeptide, peptide or variant of claim 5 that binds to one or more of SEQ ID NO:21, 22, 23, 24, 25 and 26.

7. A peptide or variant according to any of claims 1-4 which is capped at its N-terminus, its C-terminus, or both its N- and its C-terminus.

8. An antibody or an antigen-binding fragment (ABF) thereof which is specific for an epitope of a Tpm isoform expressed on the surface an activated endothelial cell, which antibody or ABF has:

- (a) antiangiogenic activity in that it binds to said activated endothelial cell, causing the generation of an antiangiogenic signal in said cell, resulting in (i) inhibition of migration, invasion, proliferation or angiogenesis, or (ii) apoptosis;  
or
- (b) proangiogenic activity in that it binds to Tpm on said endothelial cell and inhibits the binding to said cell of a Tpm -binding antiangiogenic agent, thereby permitting or promoting migration, invasion, proliferation or angiogenesis that would otherwise be inhibited by said antiangiogenic agent.

9. An antiangiogenic antibody or ABF according to claim 8.

10. A proangiogenic antibody or ABF according to claim 8.

11. The antibody or ABF of any of claims 8-10, wherein the epitope for which said antibody or ABF is specific is present in, or formed by a polypeptide or peptide of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, or SEQ ID NO:20.

12. The antibody or ABF of any of claims 8, 10 or 11 wherein the Tpm -binding antiangiogenic agent is selected from the group consisting of:

- (a) human HPRG;
- (b) rabbit HPRG;
- (c) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of human or rabbit HPRG;
- (d) two chain human kininogen human kininogen (HK<sub>a</sub>);
- (e) the D5 domain of HK<sub>a</sub>; and
- (f) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of said HK<sub>a</sub> or said D5 domain thereof.

13. The antibody of any of claims 8-10 which is a monoclonal antibody.

14. The antibody of claim 13 that is a human or humanized monoclonal antibody.

15. An antibody useful for detecting a Tpm polypeptide or peptide that serves as an anti-angiogenic receptor on endothelial cells, comprising the antibody or ABF of any of claims 8-10, which is detectably labeled with a detectable label.

16. An antibody useful for detecting a Tpm polypeptide or peptide that serves as an anti-angiogenic receptor on endothelial cells, comprising the antibody or ABF of claim 11, which is detectably labeled with a detectable label.

17. An antibody useful for detecting a Tpm polypeptide or peptide that serves as an anti-angiogenic receptor on endothelial cells, comprising the antibody or ABF of claim 12, which is detectably labeled with a detectable label.

18. An antibody useful for detecting a Tpm polypeptide or peptide that serves as an anti-angiogenic receptor on endothelial cells, comprising the antibody or ABF of claim 13, which is detectably labeled with a detectable label.

19. An antibody useful for detecting a Tpm polypeptide or peptide that serves as an anti-angiogenic receptor on endothelial cells, comprising the antibody or ABF of claim 14, which is detectably labeled with a detectable label.

20. The antibody of claims 15 wherein the detectable label is a radionuclide, a PET-imageable agent, an MRI-imageable agent, a fluorescer, a fluorogen, a chromophore, a chromogen, a phosphorescer, a chemiluminescer or a bioluminescer.

21. A diagnostically useful Tpm-binding antibody composition comprising:
  - (a) the detectably labeled antibody or ABF of claim 15 or 16,; and
  - (b) a diagnostically acceptable carrier.
22. The composition of claim 17, wherein the detectable label is a radionuclide selected from the group consisting of  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{35}\text{S}$ ,  $^{67}\text{Ga}$ ,  $^{68}\text{Ga}$ ,  $^{72}\text{As}$ ,  $^{89}\text{Zr}$ ,  $^{97}\text{Ru}$ ,  $^{99}\text{Tc}$ ,  $^{111}\text{In}$ ,  $^{123}\text{I}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{169}\text{Yb}$  and  $^{201}\text{Tl}$ .
23. The composition of claims 17 wherein the detectable label is a fluorescer or fluorogen selected from the group consisting of fluorescein, rhodamine, dansyl, phycoerythrin, phycocyanin, allophycocyanin, *o*-phthaldehyde, fluorescamine, a fluorescein derivative, Oregon Green, Rhodamine Green, Rhodol Green and Texas Red.
24. A therapeutically useful antiangiogenic antibody or ABF that targets Tpm or an epitope thereof and inhibits angiogenesis *in vitro* or *in vivo*, comprising the antibody or ABF of any of claims 8 or 9 to which is optionally bound, directly or indirectly, a therapeutically active moiety.
25. A therapeutically useful antiangiogenic antibody or ABF that targets Tpm or an epitope thereof and inhibits angiogenesis *in vitro* or *in vivo*, comprising the antibody or ABF of claim 11 to which is optionally bound, directly or indirectly, a therapeutically active moiety.
26. A therapeutically useful antiangiogenic antibody or ABF that targets Tpm or an epitope thereof and inhibits angiogenesis *in vitro* or *in vivo*, comprising the antibody or ABF of claim 12 to which is optionally bound, directly or indirectly, a therapeutically active moiety.
27. A therapeutically useful antiangiogenic antibody or ABF that targets Tpm or an epitope thereof and inhibits angiogenesis *in vitro* or *in vivo*, comprising the antibody or ABF of claim 13 to which is optionally bound, directly or indirectly, a therapeutically active moiety.
28. A therapeutically useful antiangiogenic antibody or ABF that targets Tpm or an epitope thereof and inhibits angiogenesis *in vitro* or *in vivo*, comprising the antibody or ABF of claim 14 to which is optionally bound, directly or indirectly, a therapeutically active moiety.

29. A therapeutic antiangiogenic pharmaceutical composition that inhibits angiogenesis *in vitro* or *in vivo*, comprising:

- (a) an effective amount of the antibody or ABF of claim 24; and
- (b) a pharmaceutically acceptable carrier.

30. The composition of claim 29 wherein the therapeutically active moiety is bound directly to said antibody.

31.. The therapeutic pharmaceutical composition of claim 30 wherein the therapeutically active moiety is a radionuclide, drug or toxin.

32. The therapeutic pharmaceutical composition of claim 31, wherein the moiety is a radionuclide is selected from the group consisting of  $^{47}\text{Sc}$ ,  $^{67}\text{Cu}$ ,  $^{90}\text{Y}$ ,  $^{109}\text{Pd}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{186}\text{Re}$ ,  $^{188}\text{Re}$ ,  $^{199}\text{Au}$ ,  $^{211}\text{At}$ ,  $^{212}\text{Pb}$  and  $^{217}\text{Bi}$ .

33. The therapeutic antibody or pharmaceutical composition of any of claims 24 - 32, in a form suitable for injection.

34. A therapeutically useful proangiogenic antibody or ABF that targets Tpm or an epitope of Tpm and stimulates angiogenesis *in vitro* or *in vivo*, comprising the antibody or ABF of claim 8 or 10.

35. A therapeutically useful proangiogenic antibody or ABF that targets Tpm or an epitope of Tpm and stimulates angiogenesis *in vitro* or *in vivo*, comprising the antibody or ABF of claim 11.

36. A therapeutically useful proangiogenic antibody or ABF that targets Tpm or an epitope of Tpm that stimulates angiogenesis *in vitro* or *in vivo*, comprising the antibody or ABF of claim 12.

37. A therapeutically useful proangiogenic antibody or ABF that targets Tpm or an epitope of Tpm that stimulates angiogenesis *in vitro* or *in vivo*, comprising the antibody or ABF of claim 13.

38. A therapeutically useful proangiogenic antibody or ABF that targets Tpm or an epitope of Tpm that stimulates angiogenesis *in vitro* or *in vivo*, comprising the antibody or ABF of claim 14.

39. A pharmaceutical proangiogenic pharmaceutical composition, comprising:  
(a) an effective amount of the antibody or antibody fragment of claim 35; and  
(b) a pharmaceutically acceptable carrier.

40. The therapeutic antibody or pharmaceutical composition of any of claims 34-39 in a form suitable for injection.

41. A cyclic peptide of between about 4 and about 20 amino acids which binds to the D5 domain of HK<sub>a</sub> and inhibits angiogenesis in an *in vitro* or *in vivo* assay of angiogenesis.

42. The cyclic peptide of claim 41 selected from the group consisting of:

CGPNWAGDGTYLGGGGPC (SEQ ID NO:37)

CGPNTPDPDGFWWVDGPC (SEQ ID NO:38)

CGPTIYKTDGGGETTGPC (SEQ ID NO:39)

43. An *in vitro* method for inhibiting endothelial cell migration, invasion, proliferation or angiogenesis, or for inducing endothelial cell apoptosis, comprising contacting endothelial cells with an effective amount of a antiangiogenic polypeptide or peptide that binds to Tpm expressed on the surface of activated endothelial cells, and thereby causes said inhibition or said apoptosis.

44. The method of claim 43 wherein the Tpm-binding polypeptide is selected from the group consisting of:

- (a) human histidine-proline rich glycoprotein (HPRG);
- (b) rabbit HPRG;
- (c) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of human or rabbit HPRG;
- (d) two chain human kininogen human kininogen (HK<sub>a</sub>);
- (e) the D5 domain of HK<sub>a</sub>;
- (f) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of said HK<sub>a</sub> or said D5 domain thereof;
- (g) troponin T;

- (h) tropomodulin;
- (i) caldesmon;
- (j) actin;
- (k) calponin;
- (l) pEL98;
- (m) glutamic dehydrogenase; and
- (n) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of any of (g)-(m).

45. The composition of claim 29 for use in treating a subject having a disease or condition associated with undesired cell migration, invasion, proliferation, or angiogenesis, in a method comprising administering to the subject an effective angiogenesis-inhibiting amount of the composition.

46. The composition of claim 30 for use in treating a subject having a disease or condition associated with undesired cell migration, invasion, proliferation, or angiogenesis, in a method comprising administering to the subject an effective angiogenesis-inhibiting amount of the composition.

47. The composition of claim 31 for use in treating a subject having a disease or condition associated with undesired cell migration, invasion, proliferation, or angiogenesis, in a method comprising administering to the subject an effective angiogenesis-inhibiting amount of the composition.

48. The composition of claim 32 for use in treating a subject having a disease or condition associated with undesired cell migration, invasion, proliferation, or angiogenesis, in a method comprising administering to the subject an effective angiogenesis-inhibiting amount of the composition.

49. The composition of claim 45 wherein said subject has a tumor, and said angiogenesis inhibition results in reduction in size or growth rate of said tumor or destruction of said tumor.

50. The composition of claim 49 wherein said subject is a human.

51. The composition of claim 39 for use in stimulating angiogenesis in a subject in need of enhanced angiogenesis, in a method comprising administering to said subject an effective amount of the composition.

52. A method for detecting in a biological sample the presence of Tpm of an isoform expressed on the surface of activated endothelial cells, comprising the steps of:

- (a) contacting *in vitro* the sample with the antibody or ABF of claim 15; and
- (b) detecting *in vitro* the presence of the label associated with the sample.

53. A method for detecting the presence of Tpm in a biological sample, comprising the steps of:

- (a) contacting *in vitro* the sample with the a detectably labeled antiangiogenic polypeptide or peptide that binds to Tpm expressed on the surface of activated endothelial cells; and
- (b) detecting *in vitro* the presence of the label associated with the sample.

54. The method of claim 53 wherein said antiangiogenic polypeptide or peptide is selected from the group consisting of

- (a) human histidine-proline rich glycoprotein (HPRG);
- (b) rabbit HPRG;
- (c) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of human or rabbit HPRG;
- (d) two chain human kininogen human kininogen (HK<sub>a</sub>);
- (e) the D5 domain of HK<sub>a</sub>; and
- (f) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of said HK<sub>a</sub> or said D5 domain thereof.

55. The method of any of claims 52-53, wherein the sample is plasma, serum, cells, a tissue, an organ, or an extract of said cells, tissue or organ.

56. A screening test to identify a test compound as a candidate antiangiogenic molecule that binds to Tpm, comprising

- (a) adding the test compound to a mixture of a source of Tpm and a Tpm -binding antiangiogenic polypeptide or peptide agent or anti-Tpm antibody, wherein at least one of (i) said Tpm or (ii) said agent or antibody is detectably labeled

- (b) in parallel, mixing similar amounts of said Tpm and said agent or antibody in the absence of said test compound; and
- (c) measuring the binding of said agent with said Tpm in (a) and (b); wherein, if the binding in (a) is less than the binding in (b), the test is considered positive for said test compound being an inhibitor of said binding,

thereby identifying said test compound as a candidate antiangiogenic molecule.

57. The screening test of claim 56, further comprising testing a test compound that has been identified as a candidate antiangiogenic molecule for its activity as an inhibitor of angiogenesis in an *in vitro* or *in vivo* angiogenesis assay.

58. The screening test of claim 56 or 57 wherein said agent is selected from the group consisting of:

- (a) human histidine-proline rich glycoprotein (HPRG);
- (b) rabbit HPRG;
- (c) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of human or rabbit HPRG;
- (d) two chain human kininogen human kininogen (HK<sub>a</sub>);
- (e) the D5 domain of HK<sub>a</sub>; and
- (f) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of said HK<sub>a</sub> or said D5 domain thereof.

59. An affinity ligand useful for binding to or isolating a Tpm-binding antiangiogenic molecule or cells expressing the binding molecule, comprising the isolated polypeptide or peptide of any of claims 1-4, immobilized to a solid support or carrier.

60. An affinity ligand useful for binding to or isolating a Tpm-binding antiangiogenic molecule or cells expressing the binding molecule, comprising the isolated polypeptide or peptide of claim 5, immobilized to a solid support or carrier.

61. An affinity ligand useful for binding to or isolating a Tpm-binding antiangiogenic molecule or cells expressing the binding molecule, comprising the isolated polypeptide or peptide of claim 6, immobilized to a solid support or carrier.

62. A method for isolating a Tpm-binding antiangiogenic molecule from a complex mixture comprising:

- (a) contacting the mixture with the affinity ligand of claim 59;
- (b) allowing any material in the mixture to bind to the ligand;
- (c) removing unbound material from the ligand; and
- (d) eluting the bound Tpm -binding molecule.

63. The method of claim 62 wherein said anti-angiogenic receptor polypeptide or peptide:

- (i) has the sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, or SEQ ID NO:20;
- (ii) is a Tpm-binding peptide fragment of one of said sequences; or
- (iii) is a Tpm-binding conservative substitution variant of one of said sequences or of said peptide fragment.

64. An antiangiogenic polypeptide or peptide that binds to Tpm expressed on the surface of activated endothelial cells for use in inhibiting endothelial cell migration, invasion, proliferation or angiogenesis, or for inducing endothelial cell apoptosis, in a method comprising contacting endothelial cells with an effective amount of the polypeptide or peptide, and thereby causing said inhibition or said apoptosis.

65. The polypeptide or peptide of claim 64 which is selected from the group consisting of:

- (a) human histidine-proline rich glycoprotein (HPRG);
- (b) rabbit HPRG;
- (c) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of human or rabbit HPRG;
- (d) two chain human kininogen human kininogen (HK<sub>a</sub>);
- (e) the D5 domain of HK<sub>a</sub>;
- (f) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of said HK<sub>a</sub> or said D5 domain thereof;
- (g) troponin T;
- (h) tropomodulin;
- (i) caldesmon;

- (j) actin;
- (k) calponin;
- (l) pEL98;
- (m) glutamic dehydrogenase; and
- (n) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of any of (g)-(m).

66. The use of an antiangiogenic polypeptide or peptide that binds to Tpm expressed on the surface of activated endothelial cells in a method of making a medicament for use in a method for inhibiting endothelial cell migration, invasion, proliferation or angiogenesis, or for inducing endothelial cell apoptosis, comprising contacting endothelial cells with an effective amount of the polypeptide or peptide, and thereby causing said inhibition or said apoptosis.

67. The use of claim 66 wherein the Tpm-binding polypeptide is selected from the group consisting of:

- (a) human histidine-proline rich glycoprotein (HPRG);
- (b) rabbit HPRG;
- (c) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of human or rabbit HPRG;
- (d) two chain human kininogen human kininogen (HK<sub>a</sub>);
- (e) the D5 domain of HK<sub>a</sub>;
- (f) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of said HK<sub>a</sub> or said D5 domain thereof;
- (g) troponin T;
- (h) tropomodulin;
- (i) caldesmon;
- (j) actin;
- (k) calponin;
- (l) pEL98;
- (m) glutamic dehydrogenase; and
- (n) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of any of (g)-(m).

68. The antibody or ABF of claim 15 for use in a method for detecting in a biological sample the presence of Tpm of an isoform expressed on the surface of activated endothelial cells, comprising the steps of:

- (a) contacting the sample with the antibody or ABF; and
- (b) detecting the presence of the label associated with the sample.

69. A detectably labeled antiangiogenic polypeptide or peptide that binds to Tpm expressed on the surface of activated endothelial cells for use in a method for detecting the presence of Tpm in a biological sample, comprising the steps of:

- (a) contacting the sample with the polypeptide or peptide; and
- (b) detecting the presence of the label associated with the sample.

70. The polypeptide or peptide of claim 69 which is selected from the group consisting of

- (a) human histidine-proline rich glycoprotein (HPRG);
- (b) rabbit HPRG;
- (c) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of human or rabbit HPRG;
- (d) two chain human kininogen human kininogen (HK<sub>a</sub>);
- (e) the D5 domain of HK<sub>a</sub>; and
- (f) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of said HK<sub>a</sub> or said D5 domain thereof.

71. The antibody, ABF, polypeptide or peptide of any of claims 68-69, wherein the sample is plasma, serum, cells, a tissue, an organ, or an extract of said cells, tissue or organ.

72. The antibody, ABF, polypeptide or peptide of any of claims 68-69, wherein the contacting and the detecting are *in vitro*.

73. The antibody, ABF, polypeptide or peptide of any of claims 68-69 wherein the contacting is *in vivo* and the detecting is *in vitro*.

74. The antibody, ABF, polypeptide or peptide of any of claims 68-69, wherein the contacting and the detecting are *in vivo*.

75. The use of an antibody or ABF of claim 15 in a method of making a medicament for use in a method for detecting in a biological sample the presence of Tpm of an isoform expressed on the surface of activated endothelial cells, comprising the steps of:

- (a) contacting the sample with the antibody or ABF; and
- (b) detecting the presence of the label associated with the sample.

76. The use of a detectably labeled antiangiogenic polypeptide or peptide that binds to Tpm expressed on the surface on the surface of activated endothelial cells in a method of making a medicament for use in a method for detecting the presence of Tpm in a biological sample, comprising the steps of:

- (a) contacting the sample with the polypeptide or peptide; and
- (b) detecting the presence of the label associated with the sample.

77. The use of claim 76 wherein said antiangiogenic polypeptide or peptide is selected from the group consisting of

- (a) human histidine-proline rich glycoprotein (HPRG);
- (b) rabbit HPRG;
- (c) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of human or rabbit HPRG;
- (d) two chain human kininogen human kininogen (HK<sub>a</sub>);
- (e) the D5 domain of HK<sub>a</sub>; and
- (f) a Tpm-binding, antiangiogenic homologue, variant, domain or fragment of said HK<sub>a</sub> or said D5 domain thereof.

78. The use of any of claims 75-76, wherein the sample is plasma, serum, cells, a tissue, an organ, or an extract of said cells, tissue or organ.

79. The use of any of claims 75-76, wherein the contacting and the detecting are *in vitro*.

80. The use of any of claims 75-76 wherein the contacting is *in vivo* and the detecting is *in vitro*.

81. The use method of any of claims 75-76, wherein the contacting and the detecting are *in vivo*.

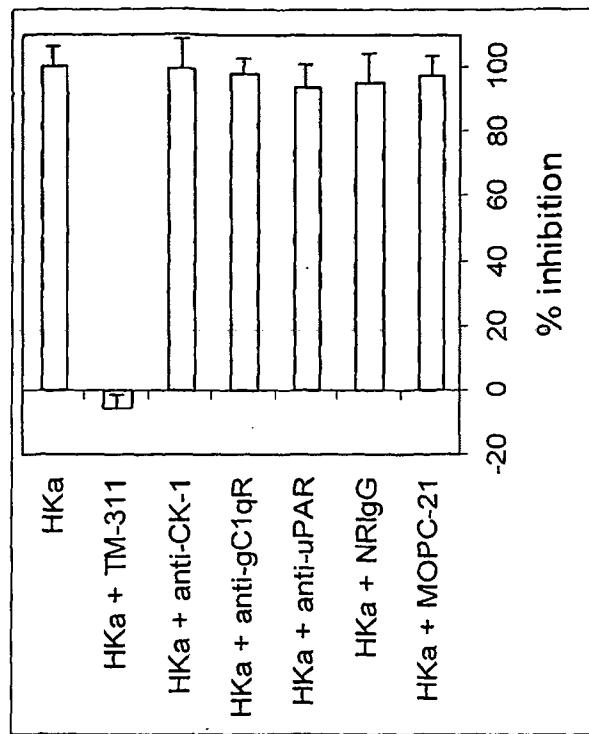


Fig. 1B

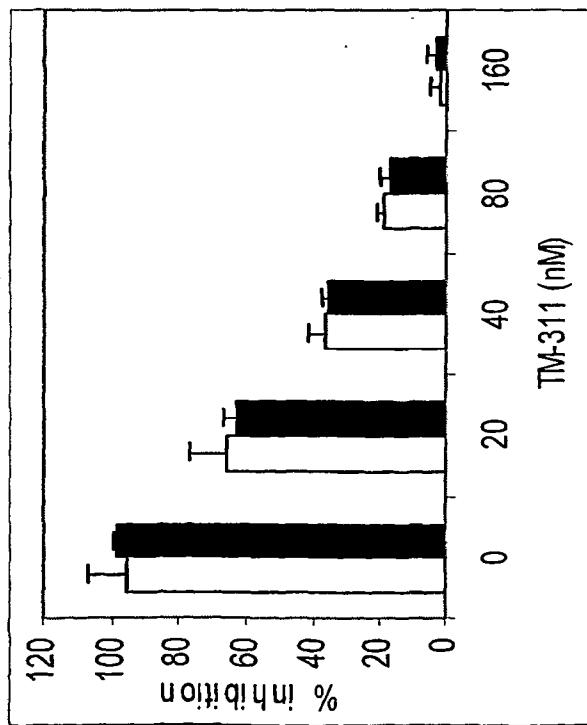
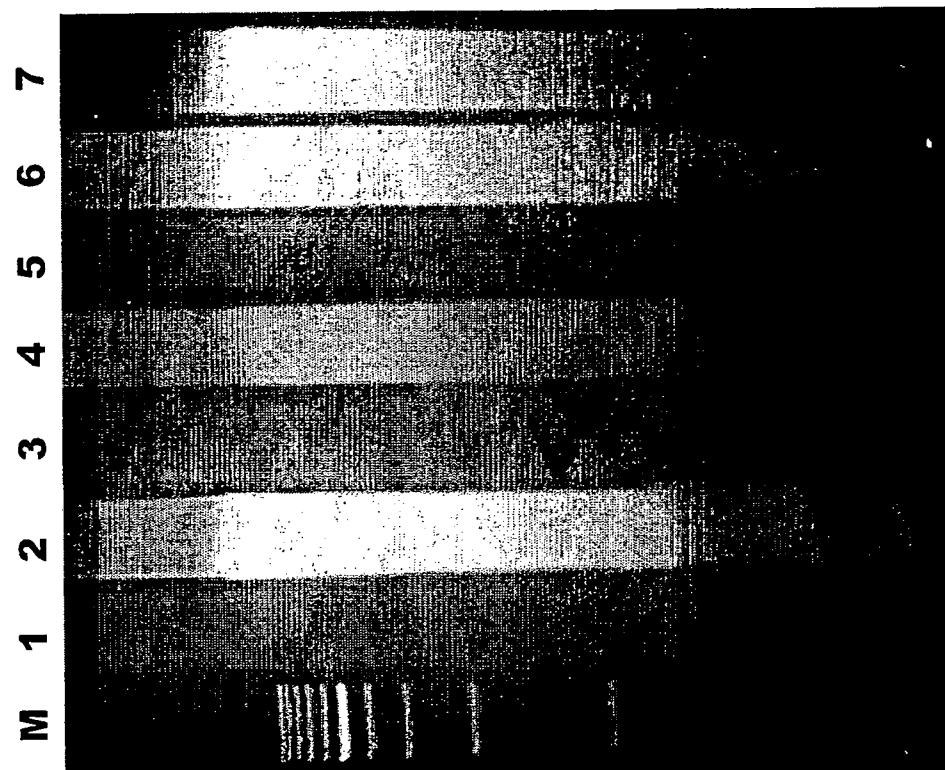


Fig. 1A



**Fig. 2**



**Fig 3A**

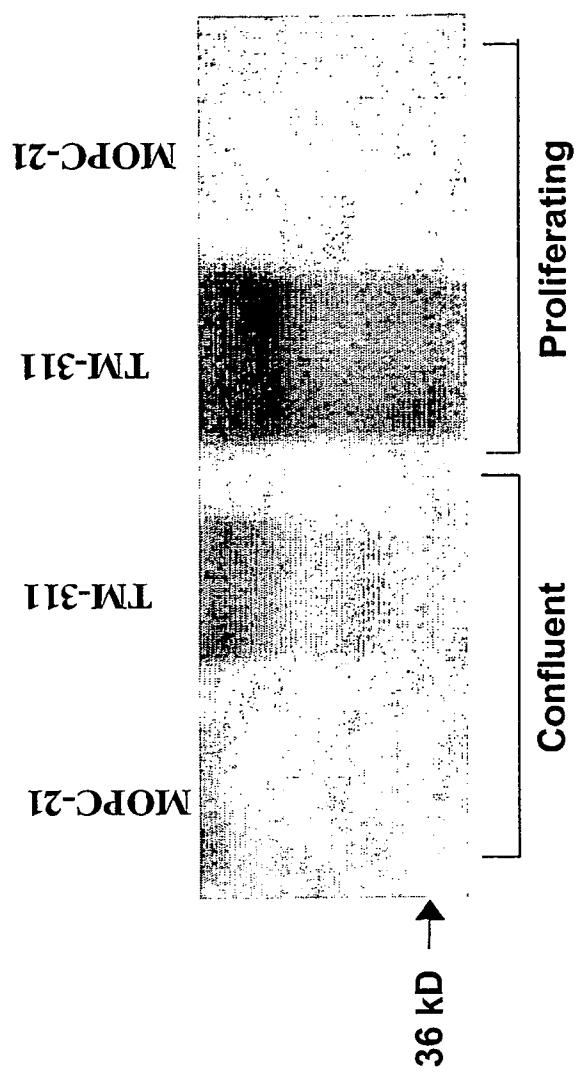


**Fig 3B**

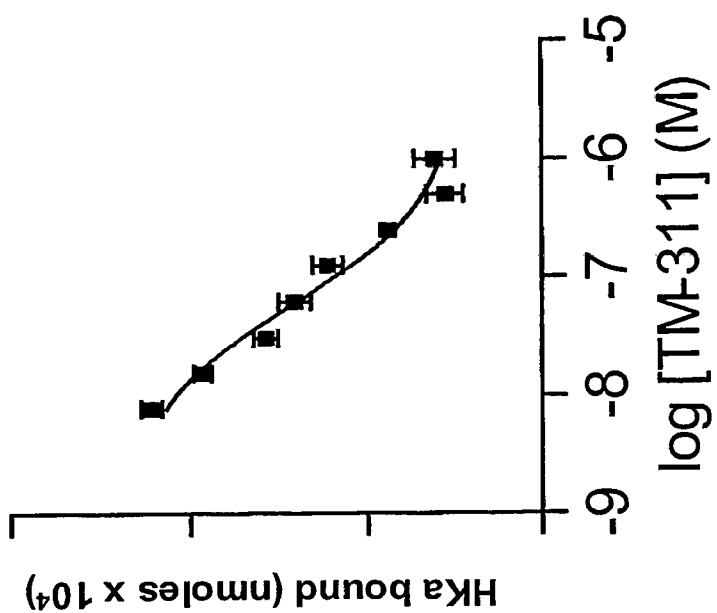


**Fig 3C**

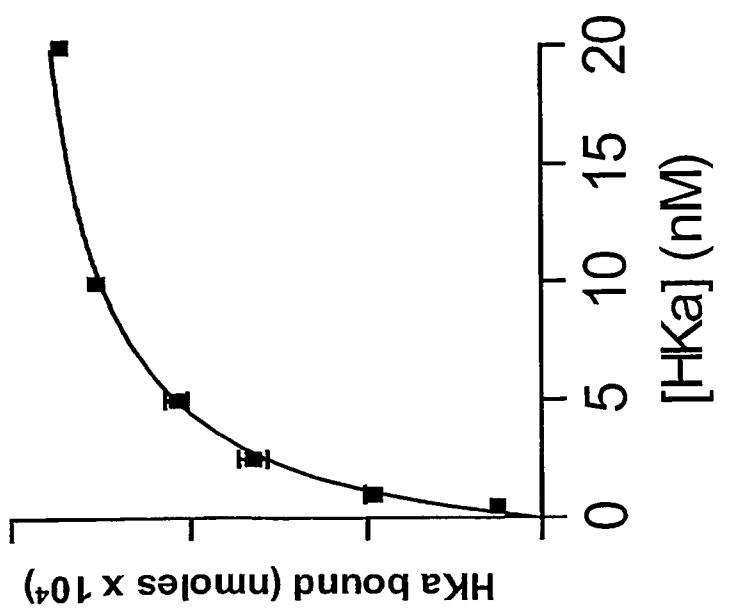
**Immunoprecipitation of biotinylated endothelial cell surface proteins by mAb TM-311**



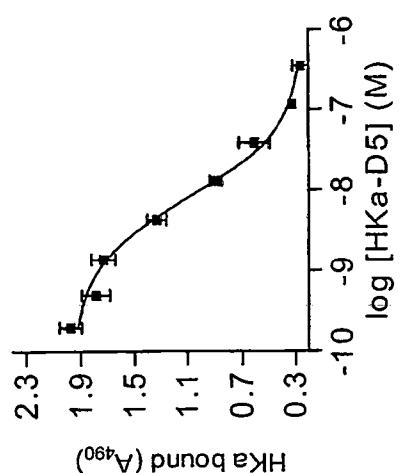
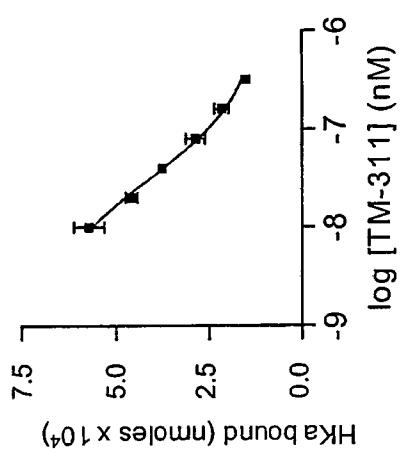
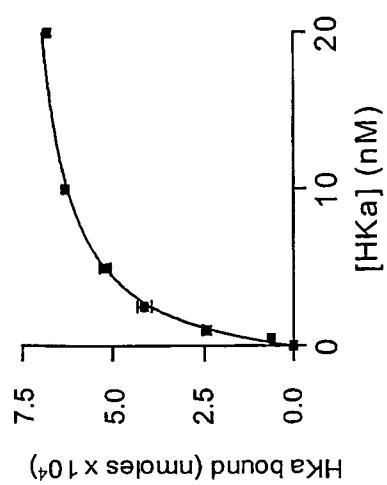
**Fig. 4**



**Fig. 5B**



**Fig. 5A**

**Fig. 6C****Fig. 6B****Fig. 6A**

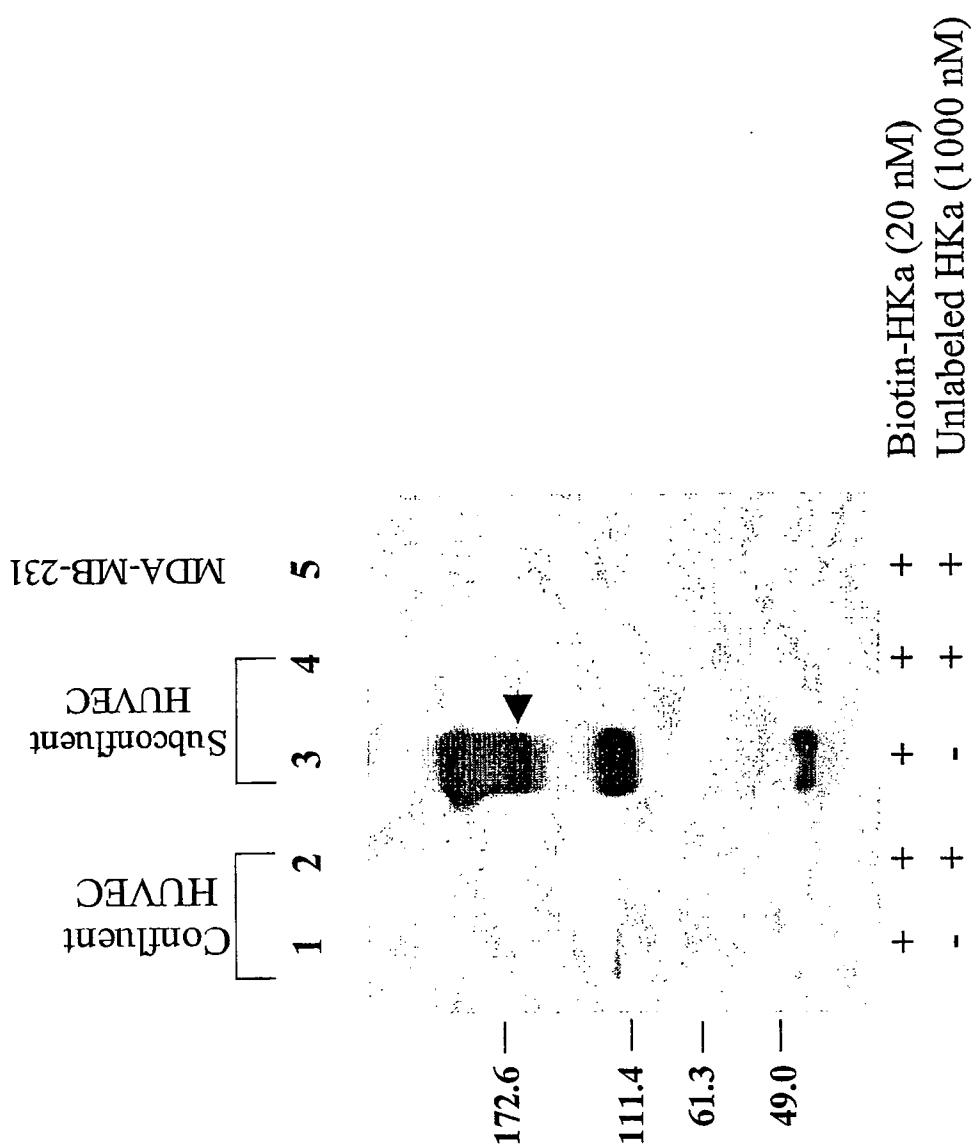


Fig. 7

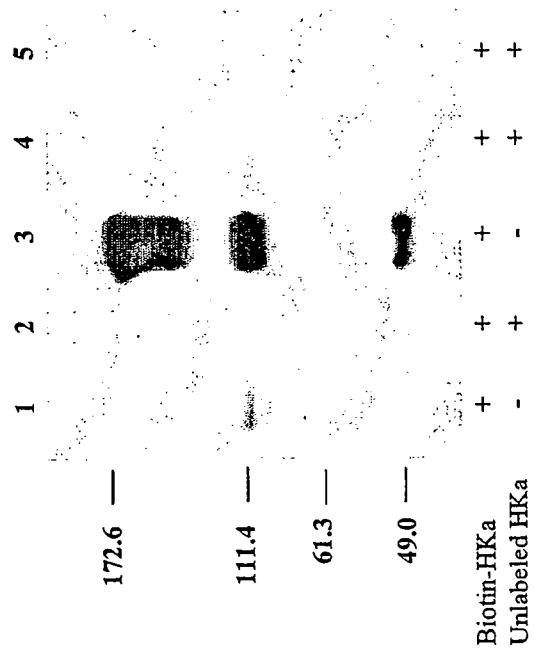
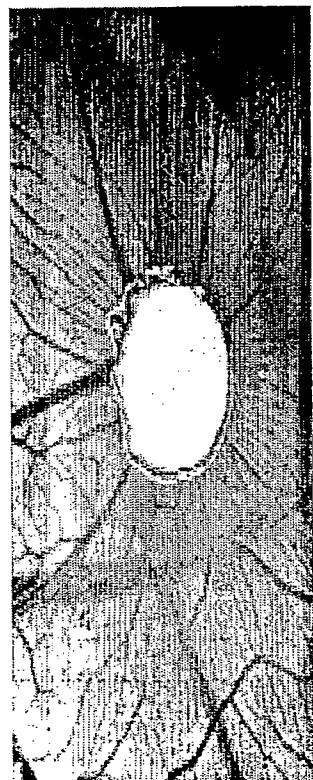
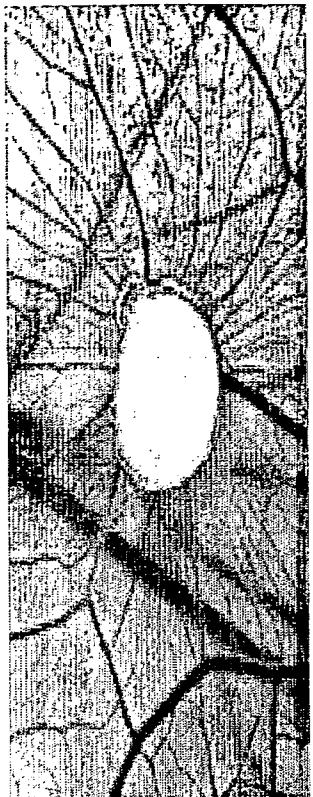


Fig. 7

**Fig. 8A**



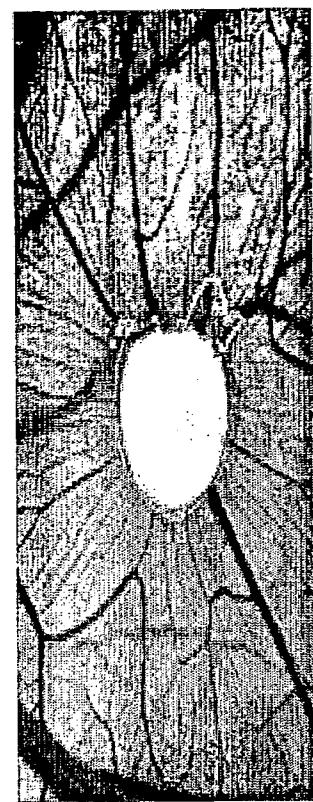
**Fig. 8B**

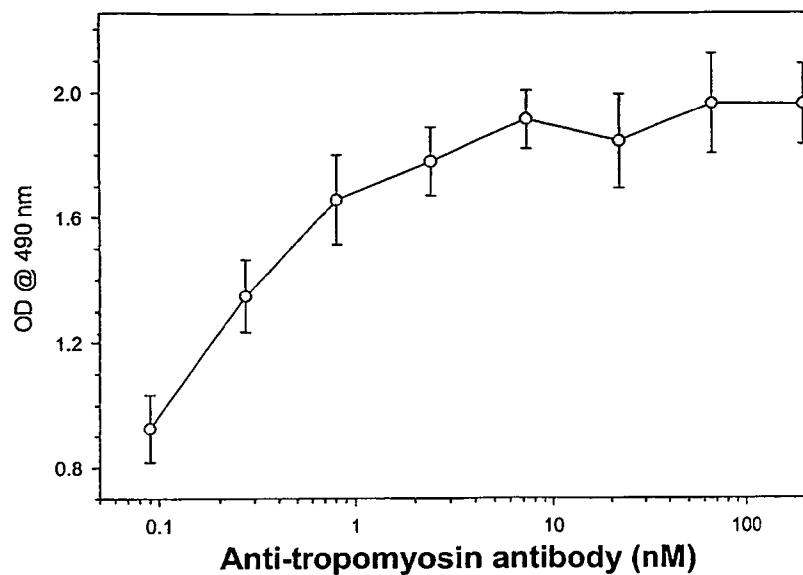
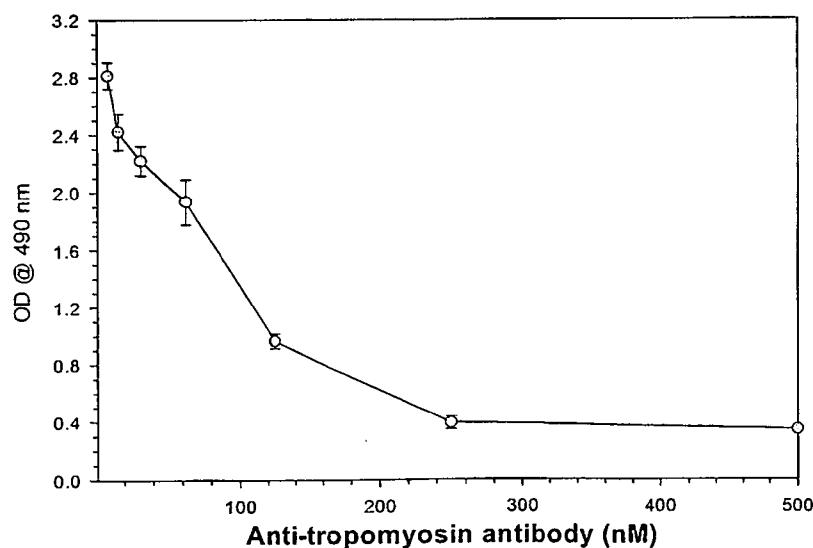


**Fig. 8C**

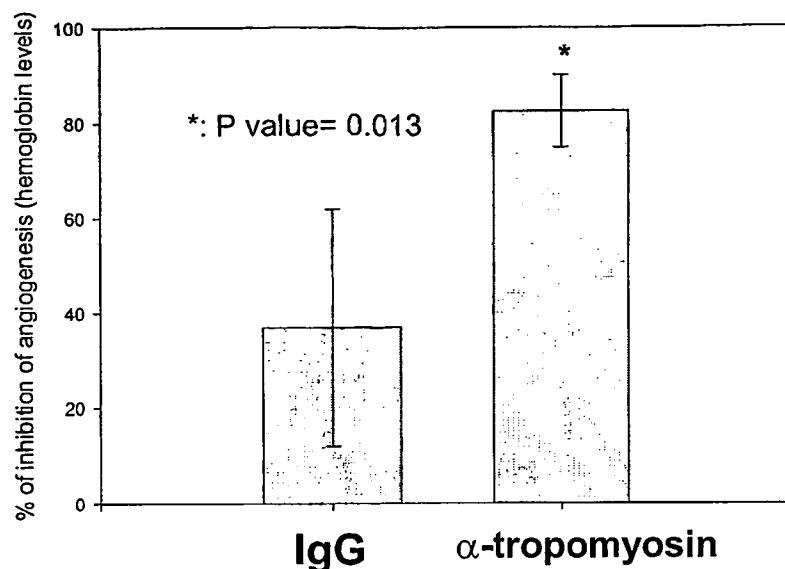


**Fig. 8D**



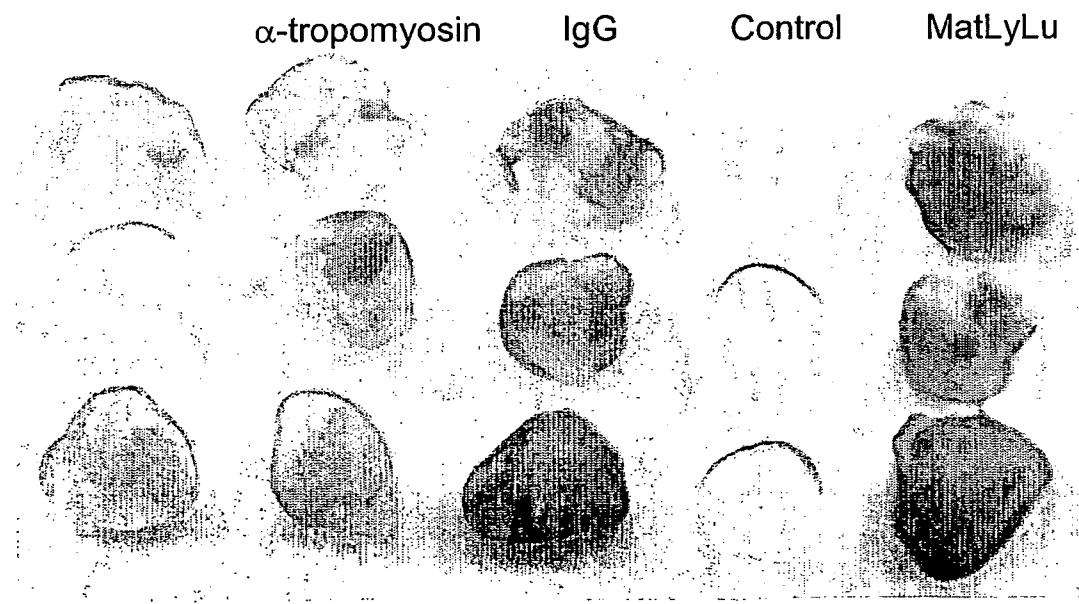
**Fig. 9****Binding competition of 10 nM biotin-HKa to tropomyosin by an anti-tropomyosin antibody****Fig. 10**

**An anti-tropomyosin monoclonal antibody inhibits angiogenesis in the Matrigel plug**



**Fig 11**

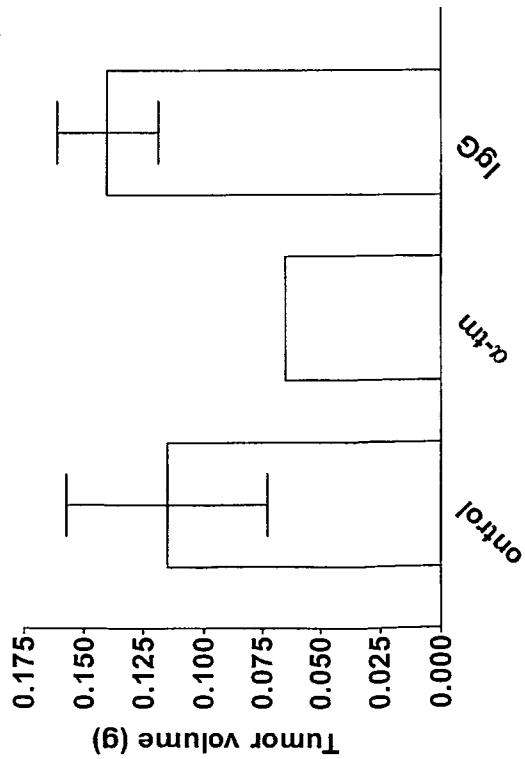
**Matrigel/MatLyLu model:  
Treat with anti-tropomyosin antibody**



**Fig. 12**

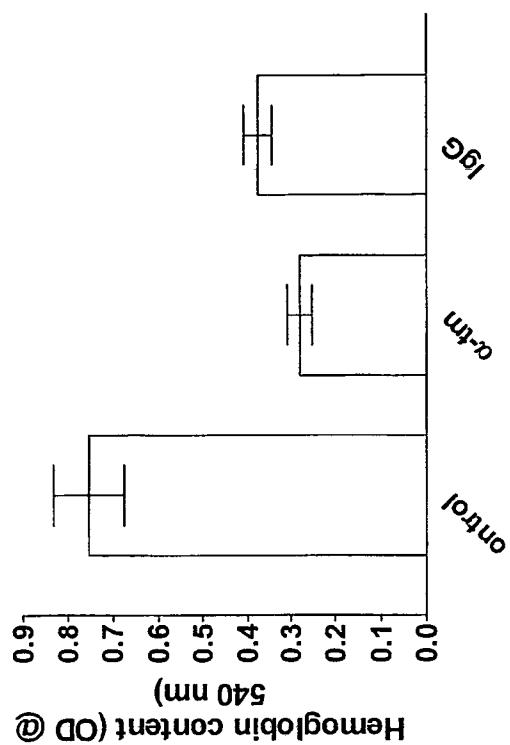
**Matrigel/MatLyLu Model: Treatment with Anti-Tropomyosin Antibody**

**Tumor growth (Weight)**



**Fig 13A**

**Hemoglobin  
Hb content**



**Fig 13B**

HK-D5 and/ HPRG-H/P have approximately 1,000-fold higher affinity for immobilized tropomyosin than does endostatin

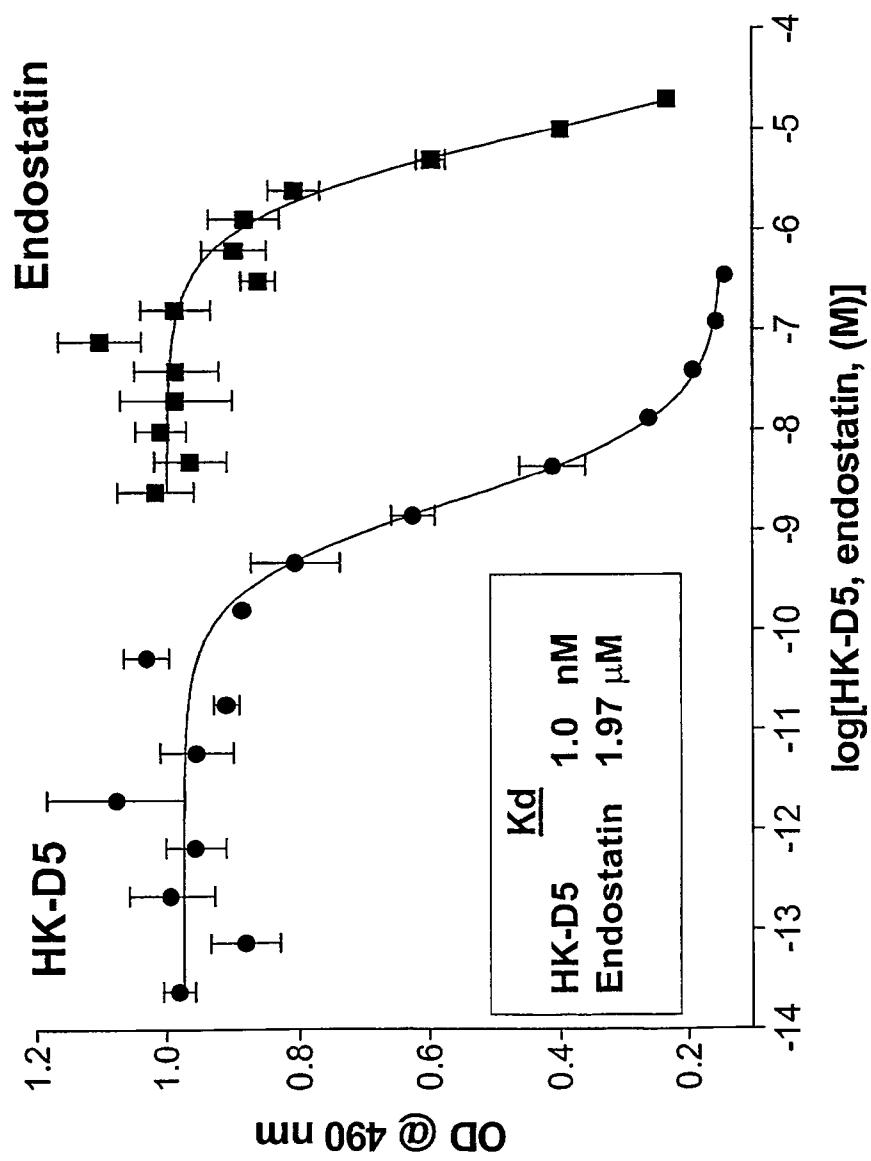
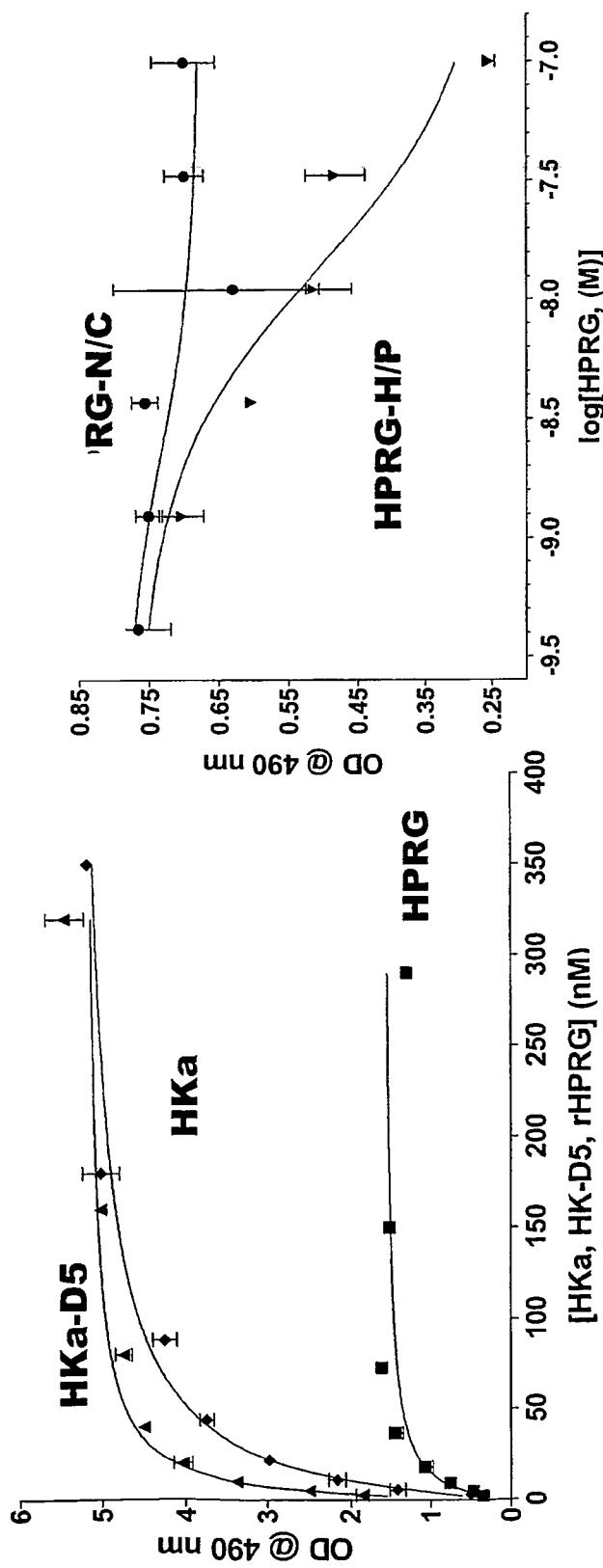


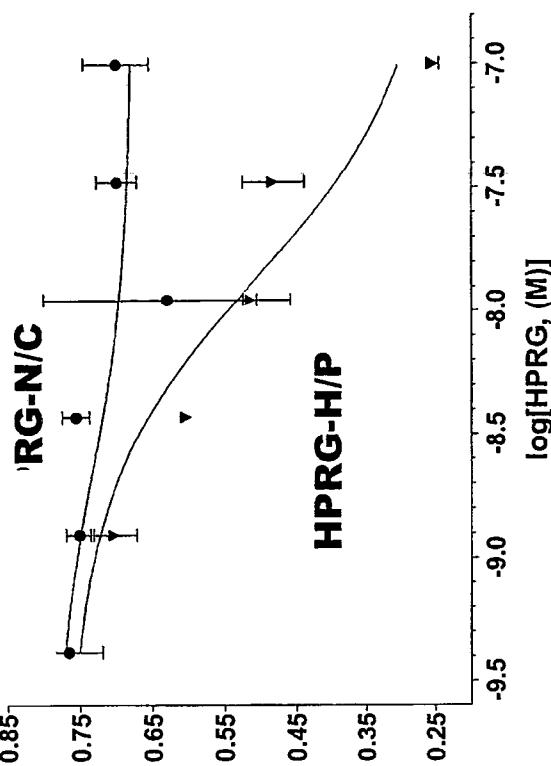
Fig 14

**HPRG binds to immobilized chicken gizzard tropomyosin through its H/P domain**

**Fig. 15A**



**Fig. 15B**



ATN228 but not ATN 246 binds to immobilized tropomyosin

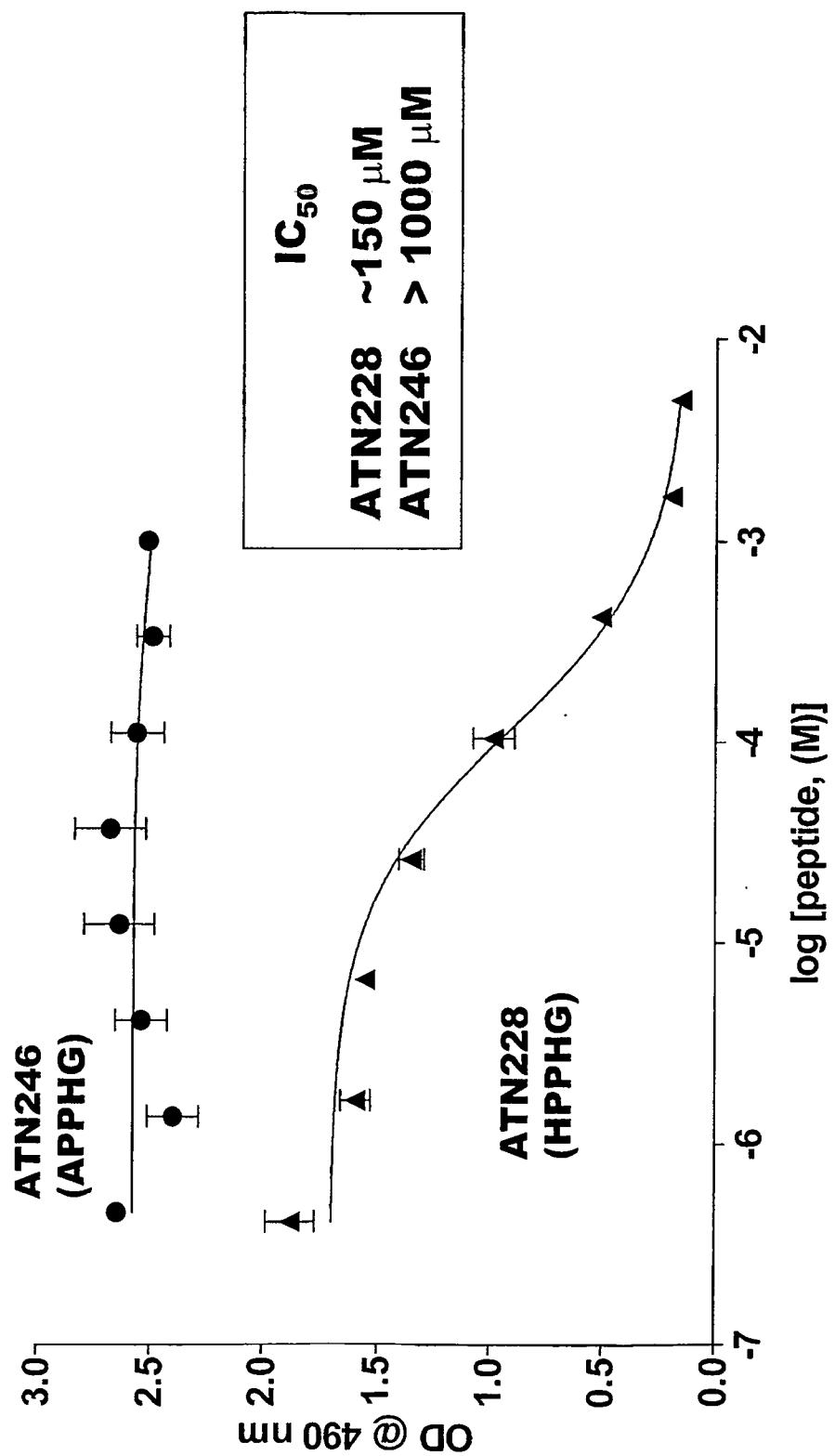
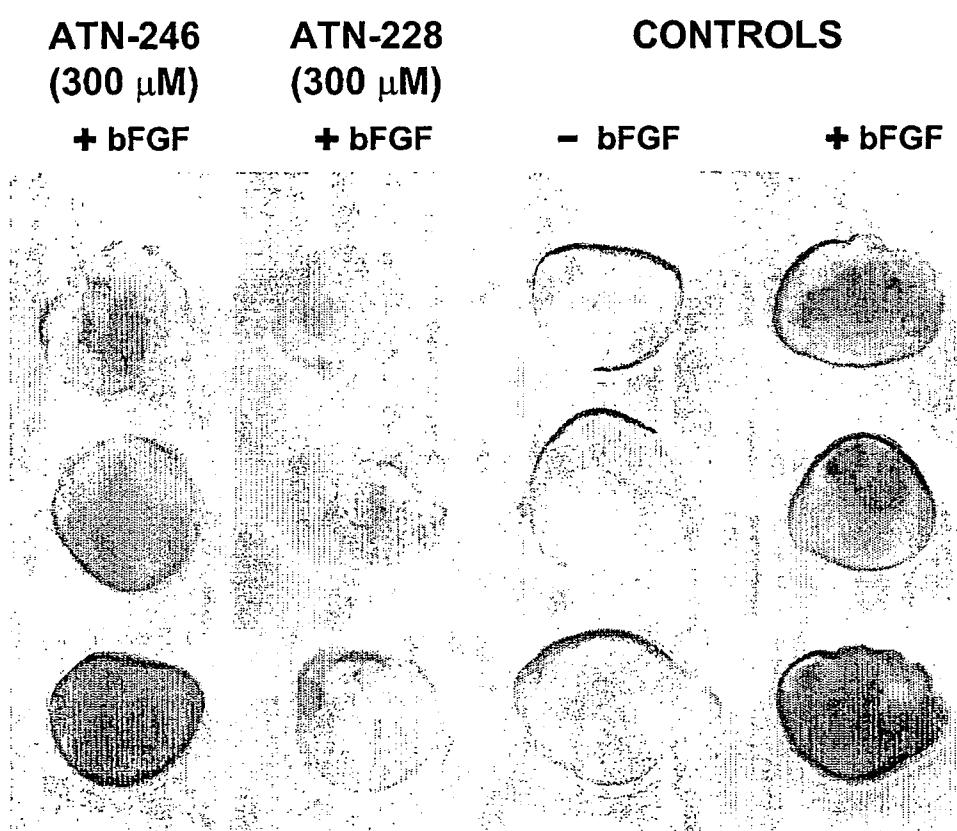


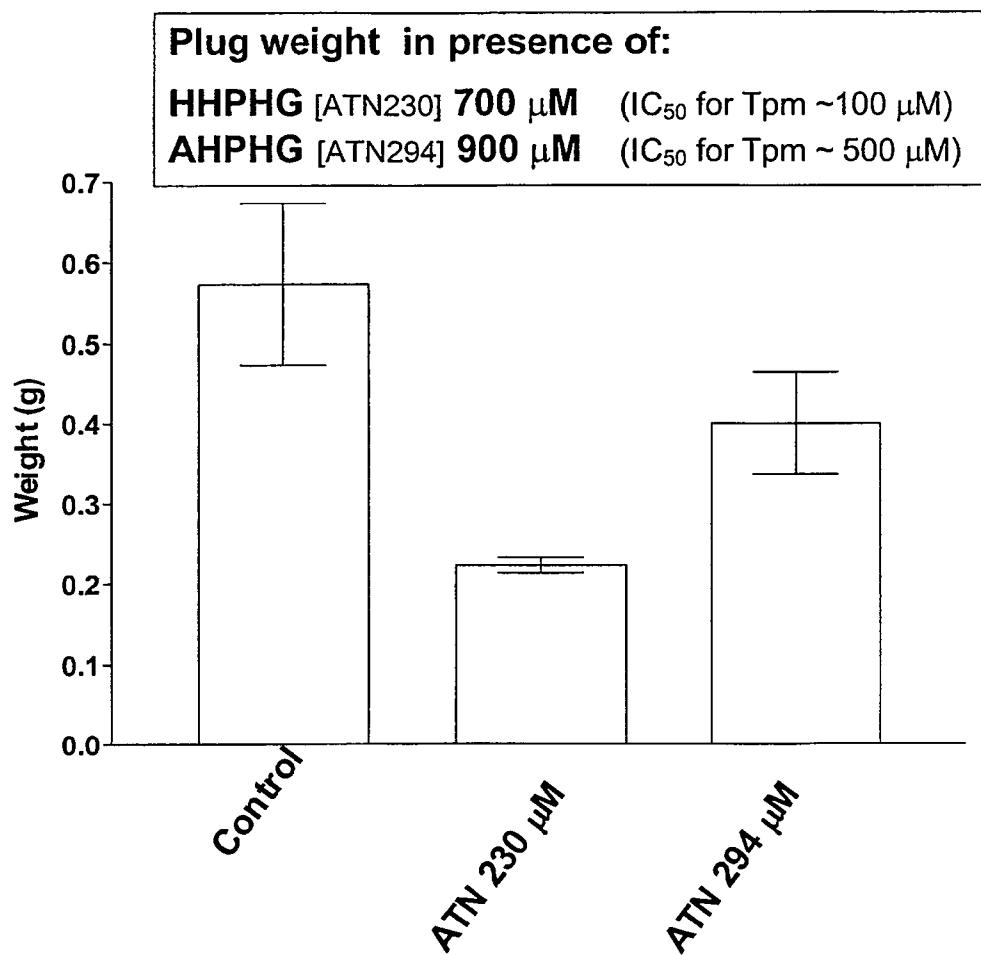
Fig 16

**ATN228 but not ATN246 Inhibits  
Angiogenesis in Matrigel Plug**



**Fig 17**

**ATN230, but not ATN294, inhibits MatLyLu growth in  
Matrigel model/MLL.**



**Fig. 18**

## Tropomyosin digestion by Chymotrypsin. Time course

Time: 10' 20' 30' 40' 50' 60' 70' 80' 110'

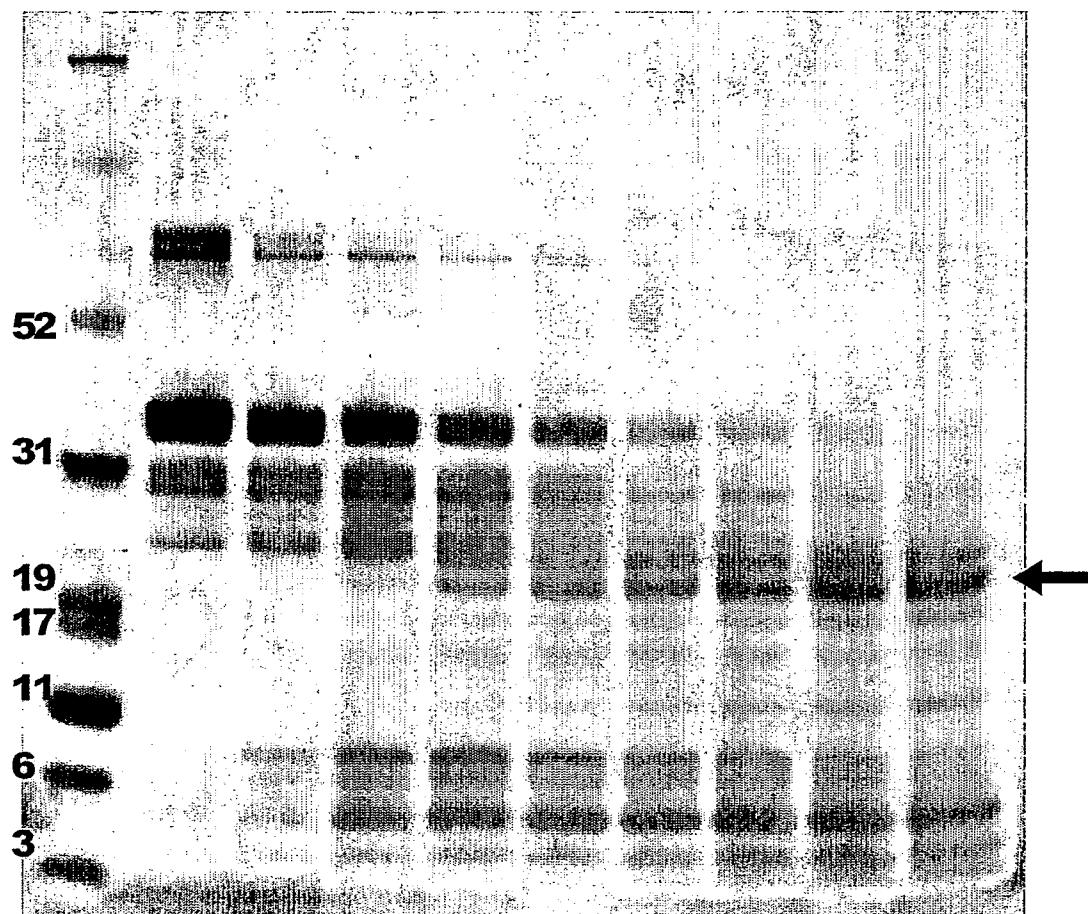
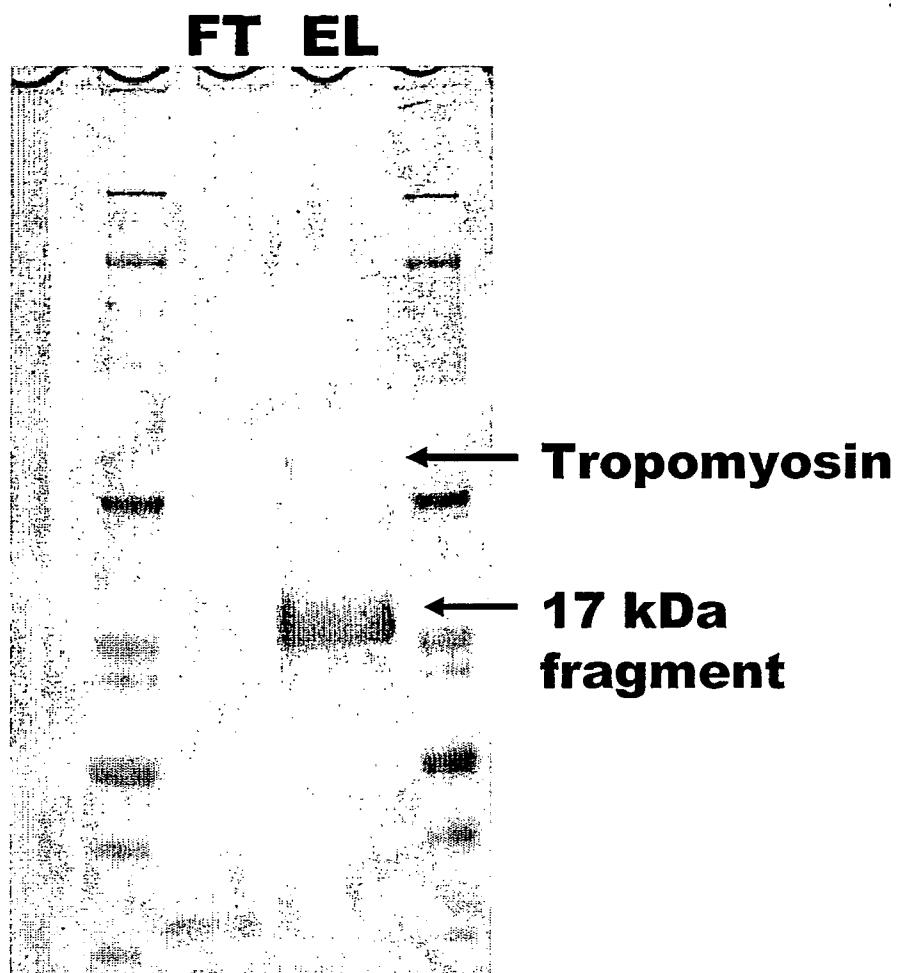


Fig. 19

## **SDS-PAGE**



**Fig 20**

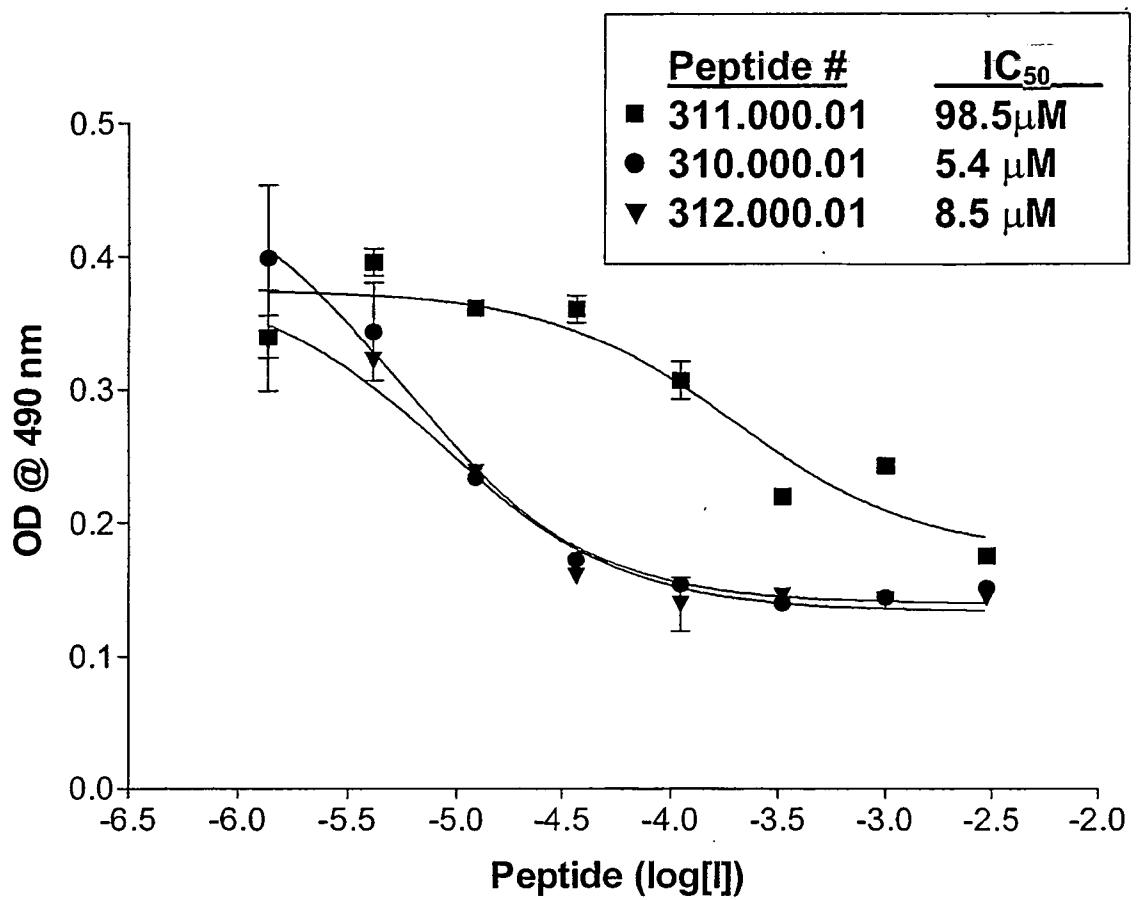


Fig 21