Materials and Methods

Cell isolation and culture: Mouse Lung Endothelial Cells (EC) were isolated from wild-type (WT), *Thbs4*$^{-/-}$, and mutant A387P TSP-4 knock-in (mutTSP 4 KI) mice as described by Mahabaleshwar and colleagues [1]. EC were cultured in DMEM/F-12 medium (Sigma) supplemented with 20% fetal bovine serum (HiClone), 80 mg/L heparin (Sigma), and 40 mg/L endothelial cell growth supplement (ECGS) purified from bovine brain tissue. Cells were grown in 6-, 12- or 24-well clusters until they became confluent.

The EMT6 mouse breast cancer cell line was purchased from ATCC (Manassas, VA) and grown according to the ATCC recommendations until harvested.

Adhesion assay: Adhesion assays were performed as previously described [2, 3]. The cultured EC were trypsinized, counted, and $5x10^5$ cells were added in complete medium with 5% serum to the wells of 24-well plates (CoStar, Corning, New York, #3526) pre-coated with fibronectin (Sigma Aldrich, St. Louis, Missouri, #F1141) along with recombinant TSP-4 (r-TSP-4) or recombinant A387P TSP-4 (r-mutTSP-4) overnight at 4°C. r-TSP-4 and r-mutTSP-4 were added to the coating solution at the concentration 10 µg/ml as was done previously [4] and fibronectin was used at the concentration 10 µg/ml. Plates were incubated for 1 h at 37 °C, then washed with PBS once and stored at -80 °C for 3 h. After thawing the plates, CyQuant reagent (Invitrogen, Grand Island, New York, #C35007) was used to quantify cell DNA in the wells.

Migration assay: Migration assays were performed using trans-well chambers (Fisher, Corning, New York, #07200149). The bottom of the trans-well chambers were coated with r-TSP-4 or r-mutTSP-4 overnight at 4 °C [2]. EC (10^6 cells) were resuspended in the serum-free DMEM, transferred to the trans-well chambers, and incubated at 37 °C for 4 hours. Complete EC medium with 20% serum was used as a chemoattractant. To identify the receptors for TSP-4 involved in EC migration on TSP-4 substratum, we used the following function blocking antibodies: anti-integrin β1 antibody (Millipore, #MAB2253Z), anti-integrin β3 antibody (Millipore, #MAB2023Z), anti-integrin $\alpha_V\beta_3$ antibody (Millipore, #MAB1976Z), anti-integrin $\alpha_V\beta_5$ antibody (Millipre, #MAB1961Z), anti-integrin α_2 antibody (Millipore, #MAB1950Z), and anti-CD47 antibody (Millipore, #ab3283). Gabapentin (Sigma, #G-007) was used to block TSP-4 interaction with gabapentin receptor $\alpha_2\delta$-1. After plating, the cells were treated with the selected antibodies (20 µg/ml) and incubated at 37 °C for 4 hours. After 4 hours, the medium was aspirated, and cells were removed from the inside surface of the upper chamber using cotton swabs. The plates were frozen in -80 °C for 3 hours, and the DNA of migrated cells was quantified using CyQuant reagent.

Proliferation assay: Proliferation assays were performed in 12-well cell culture plates (Co Star, St. Louis, Missouri, #3513) coated with fibronectin with or without added r-TSP-4 or r-mutTSP-4 as described for adhesion assays. 5,000 cells were plated per well in complete DMEM medium with 5% serum, and cells were left to grow for 4, 24, 48 and 72 h. At each time point, the wells were washed with PBS once and stored at -80 °C for at least 3 h, and then DNA in the wells was quantified using CyQuant reagent.

Recombinant TSP-4: recombinant WT TSP-4 (r-TSP-4) and r-mutTSP-4 bearing the A387P substitution were purified from the culture media of mammalian cells stably transfected with THBS4 cDNA as previously described [2, 4-7].

Animals: C57/BL6J male and female mice (Jackson Laboratories) were used in the described experiments (n≥10 per experimental point or condition as specified in the

Figure Legends). *Thbs4*$^{-/-}$ mice were described previously [5, 8, 9]. A387P TSP-4 knock-in mice (mutTSP-4 KI) were developed at Wyeth Pharmaceuticals. To create the A387P TSP-4 knock-in (mutTSP-4 KI) mouse, a point mutation was introduced in a genomic clone spanning 8-10 exons, the neo cassette was introduced into the construct, and the targeted mutant construct was electroporated into embryonic stem cells. These mice express P387 TSP-4 under the endogenous TSP-4 gene promoter (only a fragment of DNA where the SNP was found was substituted). The homology between human and mouse TSP-4 is close to 100%. Normally, the mouse TSP-4 gene codes for A387 TSP-4; thus, we decided to create a P387 TSP-4 KI mouse to explore the functional consequences of the SNP variation *in vivo*. Based on our results with human TSP-4 and the very high evolutionary conservation of the protein, such substitution in the mouse gene should result in consequences similar to the ones seen in human TSP-4. P387 TSP-4 is expressed in the same pattern as the naturally occurring A387 variant in WT mice (data not shown). The KI mouse colony has been maintained for more than 5 years in C57Bl/6 background. The mice develop and reproduce normally, and do not have an overt phenotype without challenge. Animals were housed and cared for in the AAALAC-approved animal facilities of the Cleveland Clinic. All animal studies were approved by the Institutional Animal Care and Use Committee, and all experiments were conducted in strict accordance with the National Institutes of Health and institutional guidelines. Ketamine (80 mg/kg)/Xylazine (5 mg/kg) mixture was used for anesthesia to immobilize the mice for subcutaneous Matrigel or cancer cell injections.

Matrigel plug angiogenesis assay: Twenty-six- to twenty-seven-week-old mice were anesthetized by IP injection of Ketamine (80 mg/kg)/Xylazine (5mg/kg) mixture, and the neck area was shaved and swabbed with 70% ethanol. Mice were injected subcutaneously in the upper dorsal side with 750 µL of Matrigel Matrix Basement Membrane (BD Biosciences) supplemented with bFGF (750 ng/mL, R&D Systems) and heparin (26 U/mL, Sigma). Subcutaneous plugs were removed 7 days later and frozen in OCT in liquid nitrogen. Sections (10 μm) of the plugs were stained using antibodies against markers of blood vessels as described [10] using antibodies against CD31, laminin-1, and α-actin.

Cancer angiogenesis assay: Fifteen- to sixteen-week-old mice were anesthetized by IP injection of Ketamine (80 mg/kg)/Xylazine (5 mg/kg) mixture. Mice were injected with 1.5 x 10^6 EMT6 cells in 100µl volume of PBS into the mammary fat pad. Ten days later, tumors were excised and frozen in OCT in liquid nitrogen. The characterization of angiogenesis in tumor sections was performed as described for Matrigel plugs.

Transduction of EMT6 cells with lentiviral particle containing shRNA for TSP-4 and development of EMT6 stably expressing TSP-4 shRNA: EMT6 cells were plated in 6 well plates and grown according to ATCC recommendations. After 24 h, cells were transduced with 10,000 IFU/ml lentiviral particles containing TSP-4 shRNA (Santa Cruz Biotechnology, Dallaz, TX) as per the manufacturer's directions. After 24 h, the transduction medium was changed to the growth medium for an additional 48 h. The cells were then treated with 10 µg/ml puromycin for 2 weeks. The cells expressing the construct were selected and maintained for the *in vivo* cancer angiogenesis experiments.

Retinal vasculature development in flat-mounted whole retinas was assessed in 5-day-old *Thbs4*$^{-/-}$, mutTSP-4 KI, and C57Bl/6 mice by staining of endothelial cells with the Alexa568-conjugated lectin (Isolectin GS-IB4 From *Griffonia simplicifolia*, I21412, Life Technologies) essentially as described elsewhere [11]. Briefly, the 5-day-old pups were

euthanized by CO_2 exposure. Eyes were removed and fixed in 4% paraformaldehyde for 30 min. Corneas were excised, lenses and vitreous were removed, retinas were dissected out and post-fixed for additional 2 hrs without flattening. Retinas were blocked and permeabilized in 1% BSA/5% goat serum with 0.5% Triton X-100 in PBS overnight, washed x3 in PBS, and incubated with GS-IB4-A568 (0.02 µg/µl in PBS containing 1% BSA/5% goat serum) overnight. The retinas were washed in PBS and flattened by four radial insisions, placed on a glass slide and mounted with Vectashield mounting media (Vector Labs). An image of each whole retinal flatmout was obtain by taking 9 overlapping microphotographs using Zeiss Axio Imager.Z1 with Plain Apochromat 5x objective, AxioCam MRc5 camera and AxioVision v4.8.2 software followed by merging corresponding microphotographs into one by using Adobe Photoshop CS5 (Adobe Systems).

Quantitative vascular analysis: Analysis of retinal vasculature in flat-mounts stained with Alexa-568-labeled GS-IB4 lectin was performed using customized, automated algorithms and scripts written for ImagePro Plus (v7.0, Media Cybernetics, Silver Spring, MD). Briefly, for each retinal image, the tissue boundary (i.e. Region of Interest, ROI) was segmented using a combination of lo-pass and connected components algorithms to provide a measure of *Total Retinal Area*. Subsequently, a "flattening" filter was applied to compensate for any unevenness in the image resulting from lens distortion. The ROI for vessel extension from the optic nerve was then delineated using a combination of intensity thresholding, morphological "closing" operations, and largest connected component analysis. This area within this ROI was calculated and exported as the *Vessel ROI Area*. To segment vessels in the retina, the vasculature in the processed lectin images was enhanced and equalized using spectral filters and subsequently thresholded to generate a binary vessel mask. *Vessel Area* was then calculated by summing pixels in this mask. Similarly, the *Vascular Length* parameter was calculated by summing pixels in this mask after "skeletonization" or thinning of vessels to a 1-pixel thick midline representation. Using this skeletonized vessel mask, the 8-pixel neighborhood around each skeletal pixel was examined; any pixel with 3 or more skeletal pixels in its neighborhood (constituting a vessel branching point) was summed to provide the *Total Nodes* in the retina. Lastly, using the mask generated for *Vessel ROI Area* analysis (vessel extension mask), 360 vectors were extended from the center of the optic nerve to the vessel mask periphery (1 degree per rotation). For each of these vectors, the distance to the edge of the vessel periphery was calculated, summed and divided by 360 to derive *Mean Vessel Extension*. To enable visual verification of algorithm performance, a pseudo-colored overlay was generated for each image analyzed. This overlay image is comprised of a grayscale representation of the original lectin image in which a yellow outline delineates the segmented retinal tissue, a blue outline delineates the segmented vessel extension ROI, the skeletonized segmented vasculature is indicated in red, and green pixels represent the vascular branch nodes.

Skin wound healing: Excisional wounds were made using the Acu-Punch (Acuderm Inc., Ft. Lauderdale, Florida, #CE0403), scissors, and forceps. Wound pictures were taken, and the area of the wounds was measured on the day of surgery and on days 1, 3, 7, 10, 11, 12, 13, and 14 followeing the surgery. The images of the wound area were analyzed and quantified using Adobe Photoshop software.

Immunohistochemistry: Sections of Matrigel plugs, skin, and tumors were processed as previously described [5, 8, 10, 12] and stained using Vecta Stain ABC Kit according to the manufacturer's instructions to detect the expression of angiogenesis markers. EC were

visualized in Matrigel plug sections using anti-vWF antibody (Accurate Chem, NY), and Biotinylated rat anti-mouse CD31 (BD Biosciences, 1:100), rabbit polyclonal anti-α-actin (Abcam, 1:200), and rabbit polyclonal anti-laminin-1 (Abcam, 1:300) were used to visualize EC, smooth muscle cells/pericytes, and basement membrane protein, respectively. Goat monoclonal anti-TSP-4 (R&D systems; 1:100) was used to examine TSP-4 protein expression. Visualization after staining with antibodies was performed using a high-resolution slide scanner (Leica SCN400FL, Leica microsystems, GmbH, Wetzlar, Germany) at 20X magnification. High-resolution images of whole sections were generated and quantified to determine the percentage of the stained area and the intensity of staining using ImagePro 6.1 software.

Statistical analysis: The analyses of the data were performed using the programs in Sigma Plot Software: one-way ANOVA was used to determine the significance of parametric data, and the Wilcoxon test was used for non-parametric data sets. The significance level was set at $p<0.05$, and the significant differences in experimental groups is marked in the figures. Error bars in all figures represent SEM. The number of mice or mouse retinas in each group or the number of independent *in vitro* experiments is indicated in Figure legends as n.

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