The vascular permeabilizing factors histamine and serotonin induce angiogenesis through TR3/Nur77 and subsequently truncate it through thrombospondin-1

Liuliang Qin1*, Dezheng Zhao2,3,* Jianfeng Xu4,** Xianghui Ren4, Ernest F. Terwilliger4, Sareh Parangi5, Jack Lawler1,3, Harold F. Dvorak1,3, and Huiyan Zeng1,3,6,$

Departments of Pathology1 and Medicine (Gastroenterology Division2, Division of Experimental Medicine4 and Division of Molecular and Vascular Biology6), Center for Vascular Biology Research3, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston MA 02215. Department of Surgery5, Massachusetts General Hospital, Boston, MA

* These authors equally contributed to the work.

** Current Address: GlaxoSmithKline R&D China, Shanghai, China

Running title: Histamine- and serotonin-induced angiogenesis

$ Address for correspondence:

Huiyan Zeng, Ph.D.
Division of Molecular and Vascular Biology
Department of Medicine
Beth Israel Deaconess Medical Center, and Harvard Medical School
99 Brookline Ave. RN 270F
Boston, MA 02215
Telephone: 617-667-2329
Fax: 617-667-3591
E-mail: hzeng@caregroup.harvard.edu

Abbreviations: VEGF-A, vascular endothelial growth factor-A; VEGFR-2/KDR, vascular endothelial growth factor-A receptor 2; HUVEC, human umbilical vein endothelial cells; MV, mother vessels; TSP-1, thrombospondin-1; 3TSR, a recombinant protein containing all three of the type 1 repeats of TSP-1; AAV, adeno-associated virus.

Appropriate scientific category: Vascular Biology
**Key points:** Histamine and serotonin induce, but subsequently truncate, angiogenesis via a TSP-1-mediated negative feedback loop.

**Abstract:**

Angiogenesis plays an important role in cancer and in many other human diseases. Vascular endothelial growth factor-A (VEGF-A), the best known angiogenic factor, was originally discovered as a potent vascular permeability factor (VPF), suggesting that other vascular permeabilizing agents, such as histamine and serotonin, might also have angiogenic activity. We recently demonstrated that, like VEGF-A, histamine and serotonin upregulate the orphan nuclear receptor and transcription factor TR3 (mouse homologue Nur77) and that TR3/Nur77 is essential for their vascular permeabilizing activities. We now report that histamine and serotonin are also angiogenic factors that at low micromolar concentrations induce endothelial cell proliferation, migration and tube formation *in vitro*, and angiogenesis *in vivo*. All of these responses are mediated through specific histamine and serotonin receptors, are independent of VEGF-A, and are directly dependent on TR3/Nur77. Initially, the angiogenic response closely resembled that induced by VEGF-A, with generation of “mother” vessels. However, after ~10 days, mother vessels began to regress as histamine and serotonin, unlike VEGF-A, upregulated the potent angiogenesis inhibitor thrombospondin-1, thereby triggering a negative feedback loop. Thus, histamine and serotonin induce an angiogenic response that fits the time scale of acute inflammation.
Introduction

Histamine and serotonin (5-hydroxytryptamine (5-HT)) are biogenic amines with multiple essential functions in vivo and on cultured cells. Both have important roles in acute inflammation and are important neurotransmitters in the central nervous system. Histamine is widely expressed in mammalian tissues by neurons, mast cells and basophils, macrophages, parietal cells of the stomach, many cancer cells, etc. Histamine’s activities are mediated through four G-protein coupled receptors (H1-H4) that exhibit different tissue distributions. H1 is widely distributed, and, in addition to roles in the central nervous system, mediates histamine’s roles in immediate hypersensitivity reactions. In these reactions, histamine released from mast cells or basophils has potent effects on vascular smooth muscle cells, causing cell contraction and division, and on vascular endothelial cells (EC), inducing microvascular permeability, and, disputably, EC division. H2 also mediates neurotransmission, but, additionally, gastric acid secretion and T lymphocyte function. H3 serves primarily as a neurotransmitter, whereas the more recently discovered H4 is distributed in cells of hematopoietic lineage and mediates functions such as inflammatory cell chemotaxis, cytokine release, and T lymphocyte activation.

Like histamine, serotonin has multiple functions in the central nervous system and is concentrated peripherally in enterochromaffin cells of the gastrointestinal tract where it has a role in motility. More relevant to the present discussion, serotonin is also found in platelet dense granules, and, along with histamine, in mast cell granules. Upon mast cell or platelet degranulation, serotonin serves as a proinflammatory mediator that increases vascular permeability and is mitogenic for smooth muscle cells and vascular EC. Serotonin also activates monocytes, preventing apoptosis and modulating cytokine and chemokine production. Serotonin’s activities are mediated through seven classes of receptors (14 different proteins), as well as independently of receptors through the serotonin transporter, which facilitates reuptake of serotonin in neuronal presynapses. EC are reported to express several different serotonin receptors including 5-HT1, 5-HT2 and 5-HT4.

In addition to the multiple and varied functions listed above, there have been claims that histamine and serotonin have roles in angiogenesis. As early as 1969, Zauberman et al reported that both amines induced new blood vessel formation when introduced into the rabbit cornea. A number of papers have implicated histamine in pathological angiogenesis, but mechanistic studies to date have showed this action to be indirect through upregulation of VEGF-A expression. Less is known about a role for serotonin in angiogenesis, though serotonin does affect EC signaling in culture and serotonin deficient (tryptophan hydroxylase 1 null) mice exhibit decreased tumor angiogenesis. Further, Jackson et al reported a role for serotonin in the angiogenesis induced by metastatic carcinoids.
VEGF-A is the classic tumor angiogenesis factor but was originally discovered as a potent vascular permeability factor (VPF)\textsuperscript{21}. Recent studies have done much to elucidate the steps and mechanisms by which VEGF-A induces angiogenesis and enhances permeability\textsuperscript{22}. A number of signaling pathways have been implicated, but most, if not all, begin with activation of VEGF-A receptor 2 (VEGFR-2/KDR/Flk-1)\textsuperscript{23}. Activation of VEGFR-2, in turn, leads to the upregulation of the orphan nuclear receptor and transcription factor TR3 (human)/Nur77 (mouse), and TR3/Nur77 mediates most if not all of VEGF-A’s angiogenic and vascular permeabilizing activities\textsuperscript{24,25}. We recently reported that histamine and serotonin also upregulate TR3/Nur77 and that TR3/Nur77 is essential for their vascular permeabilizing activities\textsuperscript{25}. This finding was unexpected in that histamine and serotonin act through G-coupled receptors that are unrelated to VEGFR-2.

Together these findings prompted us to investigate whether histamine and serotonin induced angiogenesis and, if so, by what mechanisms. Here, we report that both histamine and serotonin, acting through their specific receptors and independent of VEGF-A and VEGFR-2, induce HUVEC proliferation, migration and tube formation \textit{in vitro} and angiogenesis \textit{in vivo}. Moreover, like VEGF-A, these actions are mediated through TR3/Nur77. As in response to VEGF-A, the first new blood vessels formed \textit{in vivo} were “mother” vessels, greatly enlarged, highly permeable, pericyte-poor sinusoids. Histamine or serotonin, acting on endothelial cells in culture or on tissue sites \textit{in vivo}, initially reduced expression of thrombspondin-1 (TSP-1), a potent angiogenesis inhibitor\textsuperscript{26}, thus favoring angiogenesis. Subsequently, however, histamine and serotonin, unlike VEGF-A, upregulated the TSP-1 promoter and restored TSP-1 levels to normal. This triggered a negative feedback loop that caused vascular regression and thus imposed a temporal limit on the angiogenic response induced by histamine and serotonin.

Materials and Methods

\textbf{Materials:} VEGF-A\textsuperscript{165} (human) and VEGF-A\textsuperscript{164} (mouse) were purchased from R&D Systems (Minneapolis, MN). Histamine (2-(4-Imidazolyl)ethylamine); serotonin (5-hydroxytryptamine); the VEGFR-2 (KDR) receptor inhibitor SU1498; the H1 histamine receptor antagonist, mepyramine (N-(4-Methoxyphenyl)methyl-N\textsubscript{2},N\textsubscript{2}-dimethyl-N-(2-pyridinyl)-1,2-ethanedianime); the H2 histamine receptor antagonist, Zolantidine (N-[3-[3-(1-piperidinyl)methyl]phenoxy]propyl]-2-benzothiazolamine); the H4 histamine receptor antagonist, JNJ777120; the serotonin (5HT) receptor 1,6,7 antagonist, M149 (1-[10,11-dihydro-8-(methylthio)dibenzo[b,f] thiepin-10-yl]-4-methylpiperazine mesylate); the serotonin (5HT) receptor 2 antagonist, S006 (3-(2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl)-2,4(1H,3H)-quinazolinedione (+)-tartrate); and the serotonin (5HT) receptor 3 antagonist, T113 (3-tropanylindole-3-carboxylate methiodide); were purchased from Sigma (St. Louis, MO). Antibodies against TR3/Nur77 and mouse CD31 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).
An antibody prepared in chickens against mouse thrombospondin-1 was used for immunoblots, as described previously.  

**Cell culture and assays:** Human umbilical vein endothelial cells (HUVEC, Lonza Inc. Walkersville, MD) were cultured and transduced with retroviruses carrying various genes as previously described. At 80% confluence, HUVEC were incubated in 0.1% fetal bovine serum (FBS)-containing EBM medium for 24 h and then treated, in all assays described below, with VEGF-A (10 ng/ml), histamine (10 μM), or serotonin (1 μM) as previously described. In all of the in vitro assays that follow, receptor antagonists (40 μg/ml H1 antagonist, 10 μM H2 antagonist, 3 μM H4 antagonist, 0.25 μM serotonin (5HT) receptor 1,6,7 antagonist, 10 μg/ml serotonin (5HT) receptor 2 antagonist, and 3 μg/ml serotonin (5HT) receptor 3 antagonist) were added 10 min before addition of VEGF-A, histamine or serotonin, whereas 3TSR (50 nM) was added 30 min before.

**Proliferation assay:** HUVEC (2 x 10^3) were seeded in 24-well plates. After 2 days, cells were serum starved (0.1% serum) for 24 h, pretreated with antagonists or inhibitors, and then stimulated with VEGF-A, histamine or serotonin for 20 h. Thereafter, 1 μCi ³H-thymidine was added to each well and 4 h later cells were washed with cold PBS 3x, fixed with 100% cold methanol for 15 min at 4°C, precipitated with 10% cold trichloracetic acid for 15 min at 4°C, washed with water 3x, and lysed with 200 μl 0.1N NaOH for 30 minutes at room temperature for scintillation counting.

**Transwell Migration Assay:** HUVEC serum starved as above were washed 2x with PBS and incubated for 20 min at 37°C with 4 ml collagenase solution (0.2 mg/ml collagenase (Sigma), 0.2 mg/ml soybean trypsin inhibitor, 1 mg/ml BSA, 2 mM EDTA in PBS). Cells were detached by gentle scraping and centrifuged at 1100 rpm for 3 min, washed 2x with EBM containing 1% BSA, seeded (1 x 10^5 cells per well) on transwell filters coated with Pure Col (30 μg/ml, Advanced BioMatrix, San Diego, CA), and inserted in 24-well plate wells containing 1 ml of the same medium. Cells were incubated at 37°C for 1 hour to allow cell adhesion, after which antagonists or inhibitors were added followed by addition of VEGF-A, histamine, or serotonin to the bottom well. After 2h incubation, cells remaining on the upper surface of the transwell filter membrane were wiped off with a cotton tip and the membranes were incubated with Cyquant DNA stain overnight at 4°C. After warming to room temperature, stained cells were counted in a spectrofluorometer (SpectraFluor; TECAN) using Delta Soft 3 software. A standard curve was prepared using cells seeded over a range of 3 x10^3 to 1 x 10^5 cells per well in a 96-well plate, incubated and stained as described for stimulated cells.

**Monolayer migration (wound healing) assay:** The HUVEC wound healing assay was carried out as described. Confluent HUVEC on 24-well plates were subjected to serum starvation as above. The monolayer was then wounded with a single pass of a 200 μl pipette tip, washed with PBS, photographed with Axiovert 35 microscope.
pretreated with or without antagonists or inhibitors, incubated for 16 hours in the presence or absence of VEGF-A\textsuperscript{165}, histamine or serotonin. Thereafter, the monolayer was washed with PBS and photographed (Microscope: ). Data are expressed as number of cells migrating into the wounded area (20 views from three independent experiments).

**Endothelial cell tube formation assay:** Serum-starved HUVEC were seeded at a density of 1x10\textsuperscript{5} cells in 12-well plates that were pre-coated with 0.5 ml growth-factor reduced Matrigel, pretreated with or without inhibitors, incubated for 6 h in the presence or absence of VEGF-A\textsuperscript{165}, histamine or serotonin, washed with PBS and photographed. Pictures were taken with Axiovert 35 microscope (Zeiss, Oberkochen, Germany), DFC350FX camera (Leica, Allendale, NJ) with Leica FireCam software. Figures were analyzed with Image J software and total tube lengths were calculated in 20 views from each of the three independent experiments (20x microscopic fields).

**Animals:** Female, 4-5 week Nu/Nu mice were purchased from NIH and C57Bl/6 wild type mice from Charles River Laboratories. Nur77\textsuperscript{-/-} mice were obtained originally from Dr. Jeffrey Milbrandt (Washington University School of Medicine, St. Louis, MO)\textsuperscript{30} and have subsequently been bred in house. All animal experiments were performed in compliance with the Beth Israel Deaconess Medical Center’s Institutional Animal Care and Use Committee.

**Adeno- and adeno-associated viruses:** An adenovirus expressing vascular permeability factor/vascular endothelial growth factor (VEGF-A\textsuperscript{164}, 1x 10\textsuperscript{7} pfu in 10 \textmu l) was injected to induce angiogenesis in the ears of Nu/Nu mice as previously described\textsuperscript{31}. An adeno-associated virus (AAV1) expressing 3TSR was generated to test the effects of TSP-1 on histamine- and serotonin-induced angiogenesis\textsuperscript{32}.

**Angiogenesis induced with histamine- and serotonin- releasing pellets:** Flank hair was clipped as necessary one day before experiments. Nude, C57Bl/6 wild type and Nur77/-/- mice were implanted s.c. with control pellets or pellets that released histamine or serotonin continuously over 21 days (Innovative Research of America, Sarasota, FL). At indicated times tissues were dissected and photographed (RGB) with Wild M400 Karema Umrustkit (ProMicron, Kirchheim, Neckar), SPOT 14.2 color Mosaic camera (Spot Imaging Solutions, Sterling Heights, MI) and Spot software, or were homogenized in ice-cold T-PER tissue protein extraction reagent (Pierce Biotechnology, Inc. Rockford, IL 61105) containing PMSF (1mM), Na\textsubscript{3}VO\textsubscript{4} (1mM), EGTA (1 mM), leupeptin (1 mM), aprotinin (1 mg/ml) and pepstatin A (2 ng/ml). In some experiments, histamine or serotonin receptor antagonists or a VEGF-A\textsuperscript{165} receptor inhibitor were administered as well.
**Histology and immunohistochemistry:** Tissues were fixed and prepared for Giemsa-stained, 1 μm thick Epon sections as previously described. For immunohistochemistry, tissues were fixed in 4% paraformaldehyde and embedded in OCT compound. Frozen sections were treated with 0.3% H2O2 for 5 min. and were subjected to antigen retrieval with 100 mM Tris-HCl in boiling water for 15 min. Sections were blocked with PBS-5% donkey serum at room temperature for 1 hr, incubated with rat anti-mCD31 (1:100 dilution, BD Biosciences, San Jose, CA) in PBS-5% donkey serum (200 μl) at 4°C overnight, rinsed with PBS 3x, incubated for 45 min with donkey anti rat IgG in PBS-5% donkey serum (200 μl) at room temperature for 1h, rinsed with PBS 3x and stained with Vectastain ABC Kit (Vector Laboratories, Inc. Burlingame, CA). Pictures (RGB) were taken with Axio Imager A1 Microscope (Zeiss, Oberkochen, Germany), AxioCam (Zeiss, Oberkochen, Germany) and Axio Vision Rel 4.8 Software. Vascular density was measured with Image J software based on counts of ten 20x microscopic fields.

**Vascular permeability assays:** Flank hair skin was clipped and depilated one day before mice were anesthetized with Avertin (tribromoethanol, 200 mg/kg), injected i.v. with 0.2 ml of Evans blue dye (0.5 mg/ml in saline), and injected i.d. in flank skin with pellet extracts or HBSS. Flank injection sites were photographed 30 min later. Dye was extracted with formamide and quantified in a spectrophotometer at 620 nm in a Thermo Max microplate reader (Molecular Devices, Menlo Park, CA) using Softmax 881 software.

**TSP-1 promoter activity assay:** HUVEC (1 × 10^5 cells/well) were cultured in 12-well plates for 24h transfected with TSP-1 reporter plasmids and a control luciferase plasmid (pRL-tk) using FuGENE-6 transfection reagent (Roche Diagnostics, Indianapolis, IN). Cells were then washed 2x with PBS. Two microliters of Fugene were added to 50 μl OPTI-MEM1 medium and incubated for 5 min. TSP-1 promoter plasmid (0.5 μg) and pRL-tk (0.2 μg) were added to the mixture and incubated for 15 min, and then added to cells in 300 μl of culture medium. Twenty-four hours after transfection, cells were stimulated with histamine, serotonin or VEGF-A for indicated times, washed with PBS, and lysed with 100 μl passive lysis buffer (Dual-luciferase Reporter Assay System, Promega, Madison, WI) at room temperature. Luciferase activity was assayed and normalized to equal internal control luciferase activity according to the manufacturer’s protocol.

**Statistics:** Statistics were performed with the Tukey-Kramer or Kruskal-Wallis multiple comparison tests, or with the Student t test, as indicated, using inStat software.
Results

**Histamine and serotonin induce HUVEC proliferation, migration and tube formation in vitro**

We used several different assays to assess the ability of histamine and serotonin to modify the behavior of cultured endothelial cells (EC). As shown in Fig. 1, both amines significantly stimulated HUVEC incorporation of $^3$H thymidine, a measure of cell proliferation (Fig. 1A, a, b); HUVEC migration in transwell chambers (Fig. 1B, a, b); HUVEC migration in the scratch monolayer wound healing assay (Fig. 1C, a, b, Supplemental Fig. 1S. A); and HUVEC tube formation on Matrigel (Fig. 1D, a, b, Supplemental Fig. 1S, B). In all of these assays, histamine and serotonin performed about as well as VEGF-A$_{165}$ (Fig. 1A-D, c). SU1489, a selective inhibitor of VEGFR-2, did not inhibit the activities of either histamine or serotonin in any of these assays, though it potently inhibited the activity of VEGF-A$_{165}$ (Fig. 1A-D, c). On the other hand, selective inhibitors of the various histamine and serotonin receptors effectively inhibited histamine and serotonin functions in each of these assays, though different receptor inhibitors were active in different assays (Fig. 1, Table 1).

**Histamine- and serotonin-slow release pellets induce angiogenesis in vivo**

Biodegradable, slow release pellets containing varying amounts of histamine or serotonin were implanted in the subcutaneous space of Nu/Nu mice where they induced angiogenesis in a dose-dependent manner (Fig. 2A, B). New blood vessel formation was apparent as early as 3 days, whereas control pellets induced no significant angiogenesis. Based on these preliminary experiments, we chose pellets containing 10 μg histamine and 1μg serotonin for further study. These pellets are reported to release histamine and serotonin continuously and at constant rates (i.e., 20 and 2 ng/hour, respectively) for a period of 21 days and therefore are expected to achieve tissue concentrations of these amines that are well within the pathophysiological range. H1 and H2 antagonists, but not the H4 antagonist, greatly inhibited histamine pellet-induced angiogenesis (Fig. 2C, a-d). Antagonists of the 5-HT2 and 5-HT3 serotonin receptors inhibited serotonin-induced angiogenesis, whereas an antagonist of the 5-HT1, 6, and 7 serotonin receptors, had no effect (Fig. 2C, f-i). Again, the VEGFR-2 kinase inhibitor SU1498, which potently inhibits VEGF-A$_{165}$-induced angiogenesis and microvascular hyperpermeability, had no effect on histamine- or serotonin-induced angiogenesis and microvessel permeability (Fig. 2C, e and j). Together these data demonstrate that histamine and serotonin induce angiogenesis directly through their specific receptors.

**Histamine- and serotonin-induced angiogenesis is dependent on TR3/Nur77**

We recently reported that the orphan nuclear receptor TR3 (mouse analogue Nur77) is required for the vascular permeabilizing activities of VEGF-A, histamine, and serotonin, in addition to its requirement for VEGF-A-induced HUVEC proliferation, tube formation and angiogenesis in Matrigel. We therefore considered the
possibility that histamine and serotonin might also require TR3/Nur77 for their angiogenic activities. Initially we took an antisense approach. We showed that transduction of HUVEC with TR3 antisense cDNA reduced TR3 expression by >90%, whereas transduction with LacZ had no effect on TR3 expression. Further, TR3 antisense DNA almost completely inhibited VEGF-A\textsuperscript{165}-stimulated HUVEC proliferation \textit{in vitro} and Matrigel angiogenesis \textit{in vivo}, whereas transduction with LacZ had no effect. We have now extended these findings to histamine- and serotonin-induced stimulation of cultured EC. HUVEC transduced with TR3 antisense cDNA exhibited greatly reduced incorporation of \textsuperscript{3}H thymidine, migration in transwell chambers and in scratch wound healing assays, and tube formation in response to VEGF-A\textsuperscript{165}, histamine or serotonin (Fig. 3A-D, Supplemental Fig. 2S, A, B). In addition, almost no angiogenesis developed when histamine or serotonin pellets were implanted in Nur77\textsuperscript{-/-} mice (Fig. 3E).

\textbf{The angiogenic responses induced by histamine and serotonin initially resemble that induced by VEGF-A\textsuperscript{165}, but are transitory}

The angiogenic responses induced by histamine and serotonin progressed through about day 10, but then began to regress so that by 21 days very few of the newly formed blood vessels remained (Fig. 4A). This finding is in contrast to that in tumors in which angiogenesis, induced primarily by the overexpression of VEGF-A continues progressively. A similar, continuously progressive angiogenic response can be induced in nude mice by an adenoviral vector engineered to express VEGF-A\textsuperscript{164} (Ad-VEGF-A\textsuperscript{164}).\textsuperscript{22} Ad-VEGF-A\textsuperscript{164} induces a succession of new, abnormal blood vessels which derive from preexisting venules and capillaries. “Mother” vessels (MV) form first and over time evolve into several types of “daughter” vessels which persist for many months.\textsuperscript{22}

The histology of the angiogenic response induced by histamine and serotonin pellets in both nude and wild-type C57Bl/6 mice was at first very similar to that induced by Ad-VEGF-A\textsuperscript{164}.\textsuperscript{22} Large numbers of MV formed initially, reaching a maximum at \textasciitilde10 days (Fig. 4B, a, b, d, e). Thereafter, however, as predicted by the macroscopic appearance (Fig. 4A), MV regressed and only small numbers of slightly enlarged capillaries remained at 15 and 21 days (Fig. 4B, c, f). Vascular density counts quantified these findings (Fig. 4C).

To be certain that the regression of angiogenesis after day 10 was not attributable to a failure of histamine or serotonin content or release, we recovered pellets from mice at 14 and 21 days after implantation and suspended them in 100\textmu l HBSS overnight to extract histamine or serotonin. Similar pellets that had not been implanted were extracted in the same way and served as positive controls. We then tested these extracts for activity in the Miles vascular permeability assay. We found that substantial histamine and serotonin remained in the pellets and were readily extracted at both 14 and 21 days after implantation (Supplemental Fig. 3S).
Histamine and serotonin affect TSP-1 expression

Unlike the angiogenic response induced by Ad-VEGF-A\textsuperscript{164}, that induced by histamine- and serotonin-releasing pellets was transitory (Fig. 4), despite continuing release of histamine and serotonin (Supplemental Fig. 3S). Since angiogenesis is controlled by a balance of pro- and anti-angiogenic factors, we considered the possibility that histamine and serotonin might also affect the activity of an angiogenic inhibitor, such as TSP-1. Testing this possibility, we found that both histamine and serotonin induced a biphasic response in TSP-1 expression in HUVEC. Initially (1-12h), histamine and serotonin induced a profound reduction in TSP-1 expression, but, at later times, TSP-1 levels returned to near normal levels (Fig. 5A,C). VEGF-A\textsuperscript{165} also downregulated TSP-1 expression, but with slower kinetics; also, TSP-1 levels did not return to normal but remained at reduced plateau levels (Fig. 5A,C).

We also measured levels of TSP-1 in tissue extracts harvested from sites immediately adjacent to histamine and serotonin pellet implants, and, for comparison, extracts prepared from skin sites of Ad-VEGF-A\textsuperscript{164} injection (Fig. 5B,C). TSP-1 protein levels fell by 88-93\% and by 70-98\% at histamine and serotonin injections sites, respectively, over the period of 4-10 days when angiogenesis was developing; levels then rebounded in temporal accord with angiogenesis regression (Fig. 4). In contrast, TSP-1 levels did not change significantly at Ad-VEGF-A\textsuperscript{164} injected skin sites over the period of 4-21 days.

3TSR recombinant protein inhibits the histamine- and serotonin-induced responses of cultured HUVEC and angiogenesis in vivo.

The anti-angiogenic activity of TSP-1 is largely mediated by that portion of the molecule referred to as type 1 repeats, and one of us (Lawler) has prepared a recombinant protein, designated 3TSR, that contains all three of the type 1 repeats and that mimics thrombospondin-1’s anti-angiogenic action\textsuperscript{36}. We therefore tested whether 3TSR could inhibit histamine or serotonin responses on cultured EC. Serum-starved HUVEC were pretreated with 50 nM 3TSR for 30 min and then stimulated with histamine (10 \( \mu \text{M} \)) or serotonin (1 \( \mu \text{M} \)) to evaluate cell proliferation, migration, and tube formation. 3TSR completely inhibited all of these effects (Fig. 6A-D, Supplemental Fig. 4S, A, B).

We then asked whether the 3TSR recombinant protein could inhibit histamine- or serotonin-induced angiogenesis in vivo. 1 x 10\textsuperscript{11} pfu of an adeno-associated virus (AAV) expressing the 3TSR recombinant protein was injected into the flank skin of Nu/Nu mice and the site marked with indelible ink. Two weeks later, histamine or serotonin pellets were implanted subcutaneously immediately beneath AAV-injected sites and tissues were collected 10 days later. As shown in Fig. 6E, histamine- and serotonin-induced angiogenesis was largely inhibited in the AAV-3TSR pretreated mice.
Histamine and serotonin regulate TSP-1 promoter activity, but not directly through TR3.

We next investigated whether histamine or serotonin acted on the TSP-1 promoter. Serum-starved HUVEC were transfected with a TSP-1 promoter-luciferase reporter or control luciferase vector and were then stimulated with histamine, serotonin or VEGF-A\(^{165}\). Histamine upregulated TSP-1 promoter activity significantly at 4h, and serotonin did so at both 6 and 16h (Fig. 7A), timing that correlates well with the return of TSP-1 protein expression at 12-24h (Fig. 5A). In contrast, VEGF-A\(^{165}\) had no effect on luciferase expression over the 16 h time course (Fig. 7 A).

TR3 is a transcription factor. Therefore, we analyzed the transcriptional regulatory elements in the TSP-1 promoter but did not find TR3 conserved regulatory elements \(^{37}\). We also tested whether overexpression of TR3 had an effect on TSP-1 expression. Cell extracts from HUVEC that were not transduced, or were transduced with TR3 or with Lac Z cDNAs were subjected to immunoblot analysis with an antibody against TSP-1. TSP-1 expression was not affected by overexpression of TR3 (Fig. 7B, top panel). We had shown previously that TR3 downregulated VE-cadherin expression \(^{25}\). Therefore, to demonstrate that the TR3 in our assay was functional, we measured expression of VE-cadherin from the same extracts and found that it was downregulated as expected by overexpression of TR3 (Fig. 7B, middle panel). Thus, histamine and serotonin regulate TSP-1 promoter activity, but TR3, if involved, acts on TSP-1 by an indirect mechanism.

Discussion

The data presented here provide definitive evidence that histamine and serotonin are potent angiogenic factors at low micromolar concentrations, and that, like VEGF-A\(^{165}\), they exert this activity directly through the orphan nuclear receptor and transcription factor TR3/Nur77. Histamine and serotonin both stimulated HUVEC incorporation of \(^{3}H\) thymidine, migration and tube formation (Figs. 1, A-D). Further, these responses were inhibited by different selective antagonists of histamine or serotonin receptors, but not by the VEGFR-2 inhibitor SU1498 (Fig. 1, Table 1) and were greatly reduced in HUVEC in which TR3 was knocked down by \((\sim 90\%\) (Fig. 3, A-D). Likewise, histamine and serotonin slow-releasing pellets induced angiogenesis when implanted in nude mice and the responses were inhibited by selective histamine or serotonin receptor antagonists but not by SU1498 (Fig. 2). Of note, angiogenesis did not develop when pellets were implanted in Nur77\(^{-/-}\) mice (Fig. 3E). Though the angiogenic responses induced by histamine and serotonin pellets initially mimicked that induced by VEGF-A, they were transitory and began to decline after about 10 days. Investigating the mechanisms responsible, we found that histamine and serotonin downregulated TSP-1 expression for a time in both cultured HUVEC (Fig. 5 A,C) and \textit{in vivo} in the tissues surrounding implanted pellets (Fig. 5B,C). The mechanism of TSP-1 downregulation is unknown but could reflect changes in mRNA stability. Subsequent restoration of TSP-1 to normal levels, both in cultured HUVEC and \textit{in vivo}, was attributable to activation of the
TSP-1 promoter by both histamine and serotonin (Fig. 7A). VEGF-A also downregulated TSP-1 expression in cultured HUVEC, but had little or no effect on TSP-1 expression at skin sites injected with Ad-VEGF-A164 (Fig. 5. A-C).

The anti-angiogenic recombinant TSP-1 derived protein, 3TSR, strikingly inhibited both histamine- and serotonin-induced HUVEC proliferation, migration and tube formation (Fig. 6, A-D). Also, histamine- and serotonin-induced angiogenic responses were strikingly inhibited by local injection of an adeno-associated virus expressing 3TSR (Fig. 6E). Nonetheless, the relationships between histamine, serotonin, TR3/Nur77 and TSP-1 expression remain elusive. Whereas TR3/Nur77 was found to downregulate VE-cadherin expression and so modulate vascular permeability 25, overexpression of TR3 had no effect on TSP-1 expression (Fig. 7B). Further work will be required to elucidate the pathways by which histamine and serotonin regulate TSP-1 expression.

While there is very little data implicating serotonin in angiogenesis since the early report of Zauberman 14, a number of papers have reported a role for histamine in the angiogenesis associated with acute inflammation. The Norrby group 15-17 reported that compound 48/80, a mast cell degranulating agent, induced angiogenesis in peritoneal mesenteries. Mast cells express and, upon degranulation release, many bioactive molecules besides histamine, including VEGF-A165 38, but a role for histamine was strongly supported by the finding that compound 48/80-induced angiogenesis was inhibited by H1 and H2 histamine receptor antagonists. These results are similar to ours in which both H1 and H2 antagonists inhibited histamine-induced HUVEC proliferation, migration and tube formation, as well as histamine pellet-induced angiogenesis (Figs.1, 2). The kinetics of angiogenesis induced by histamine releasing pellets are also consistent with earlier reports of angiogenesis induced by compound 48/80 in peritoneal mesenteries. Jakobsson et al 39 demonstrated that vascular density and vascular surface area in mesenteries were maximal at 9 days after i.p. injection of compound 48/80 and had regressed by day 16, timing similar to that of the histamine pellet-induced angiogenesis we observed (Fig. 4).

Ghosh et al 18 also provided evidence that histamine had a role in inflammatory angiogenesis. Using a cotton string implantation model, they found that angiogenesis was greatly reduced in mice null for histidine decarboxylase (HDC), the enzyme responsible for histamine synthesis. They also reported that the H2 blocker cimetidine inhibited angiogenesis in this model; however, in contrast to our work and that of Norrby et al, an H1 receptor inhibitor was without effect. Also, Ghosh et al 18 found that anti-VEGF-A165 antibodies inhibited the angiogenic response in the string assay. They therefore attributed histamine’s angiogenic effect to an accumulation of HDC-expressing macrophages which secreted histamine and so induced VEGF-A165 formation. These data are not in conflict with ours in that histamine- and serotonin-secreting pellets elicited very few macrophages (Fig. 4B). Thus, histamine may induce angiogenesis by two mechanisms, both of which involve
TR3/Nur77; i.e., upregulation of TR3/Nur77 directly or indirectly by first inducing VEGF-A\(^165\) expression which subsequently upregulates TR3/Nur77 \(^24, 25\).

In addition to their long-studied roles in acute inflammation, histamine and serotonin and their receptors have recently been implicated in cancer \(^8, 19, 20, 40-43\). Many reports indicate that both amines are tumor cell mitogens, but in at least some cases they have been implicated in tumor angiogenesis. For example, Natori et al \(^43\) demonstrated that cimetidine, a histamine H2 receptor inhibitor, inhibited the angiogenic response induced by syngeneic CMT93 colon cancer cells. Also, Nocito et al reported decreased angiogenesis and consequent reduced colon cancer growth in serotonin-deficient mice, which they attributed to an induction of the angiogenesis inhibitor angiostatin \(^8\).

In sum, our data add another layer to the complexity of pathological angiogenesis. Histamine and serotonin, in addition to their many other functions, induce angiogenesis directly by activating the transcription factor TR3/Nur77. After a time, however, histamine and serotonin also inhibit angiogenesis by promoting expression of the angiogenic inhibitor TSP-1. These findings are consistent with the long-held view that histamine and serotonin have roles in acute inflammation and immediate hypersensitivity and suggest that, by truncating angiogenesis, one of their functions may be to prevent these reactions from becoming chronic. On the other hand, to the extent that they also stimulate VEGF-A\(^165\) expression in inflammatory cells \(^18\), histamine and serotonin may also have roles in promoting the angiogenesis of chronic inflammation.

**Acknowledgments:**

This study was supported in part by research funding from NIH grants K01 CA098581 and R01CA133235 to H. Zeng; CA-142262; P01 CA-92644 and a contract from the National Foundation for Cancer Research to H. F. Dvorak; and CA-130895 and NS071197 to J. Lawler.


**Conflict-of-interest disclosure:** The authors declare no competing financial interests.
References


37. http://www.genomatix.de/cgi-bin/matinspector_prof/mat_fam.pl?s=58ca979d74b6b0826f55e75611310172.


Table 1. Summary of effects of various antagonists on histamine, serotonin or VEGF-A stimulation in different *in vitro* and *in vivo* angiogenesis assays.*

<table>
<thead>
<tr>
<th>Assay</th>
<th>Histamine Receptor Inhibitors</th>
<th>Serotonin Receptor Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inhibition</td>
<td>No Inhibition</td>
</tr>
<tr>
<td>³H thymidine incorporation</td>
<td>H1; H2</td>
<td>H4</td>
</tr>
<tr>
<td>Transwell Migration</td>
<td>H4</td>
<td>H1; H2</td>
</tr>
<tr>
<td>Wound Healing Migration</td>
<td>H1; H4</td>
<td>H2</td>
</tr>
<tr>
<td>Tube Formation</td>
<td>H1; H2; H4</td>
<td>- -</td>
</tr>
<tr>
<td>Angiogenesis in vivo</td>
<td>H1; H2</td>
<td>H4</td>
</tr>
</tbody>
</table>

*SU1489, an inhibitor of VEGFR-2, fully inhibited VEGF-A stimulation, but not that of histamine or serotonin, in all of the above assays.
Figure Legends

**Figure 1. Histamine and serotonin induce \(^3\)H thymidine incorporation (A), transwell and scratch monolayer migration (B, C) and tube formation (D) in cultured endothelial cells.** Serum-starved HUVEC, with or without pretreatment with indicated inhibitors or antagonists, were stimulated with histamine (10 μM), serotonin (1 μM) or VEGF-A\(^{165}\) (10 ng/ml), as described in Methods. Results are summarized in Table 1. (A) \(^3\)H thymidine incorporation assay (n=4, data are representative of 3 independent experiments). (B) Transwell migration (n=4, data are representative of 3 independent experiments); (C) Scratch monolayer migration (total n=20 from three independent experiments); (D) Tube formation (n=20 from three independent experiments). *, p<0.05; **, p<0.01; ***, p<0.001. Tukey-Kramer multiple comparisons test (A, B, D); Kruskal-Wallis test with Dunn’s multiple comparison (C).

**Figure 2. Histamine and serotonin induce angiogenesis in vivo.** (A) Female, 4-5 week Nu/Nu mice were implanted s.c. with control pellets or pellets that contained indicated amounts of histamine or serotonin. Tissues were dissected and photographed 3 days later; (B) Tissues adjacent to pellet implant sites were immunostained with an antibody against CD31. Mean vessel density was calculated with Image J software as described in Methods (n=10 from 4 animals per group). ***, p<0.001, Student t test; (C) Effect of various histamine and serotonin receptor antagonists on angiogenesis induced in Nu/Nu mice by histamine- or serotonin-releasing pellets. Inhibitors (1mg/kg) were injected i.p. daily, beginning when the pellets were implanted; in addition, a single injection of inhibitor (1 micromole in 50 μl or HBSS as control) was administered intradermally daily directly over the pellet implant site. Tissues were harvested and photographed on day 6. Histamine-induced angiogenesis was strongly inhibited by H1 and H2 antagonists but not by an antagonist of H4. Serotonin-induced angiogenesis was strongly inhibited by an antagonist of serotonin receptor types 2 and 3, but not by antagonists of type 1, 6, 7. SU1498, an inhibitor of VEGFR-2, had no effect on angiogenesis induced by either histamine or serotonin. Data are representative of 8 mice in each group.

**Figure 3. Histamine- and serotonin-induced angiogenesis require TR3/Nur77.** (A-D) Effects of TR3-antisense vs Lac-Z, as control, on histamine-, serotonin- and VEGF-A\(^{165}\)-induced \(^3\)H thymidine incorporation (n=4, experiments repeated 3x), transwell migration (n=4, experiments repeated 3x), scratch wound healing assay migration (n=20, 3 independent experiments) and tube formation (n=4, experiments repeated 3x). Assays were performed as in Fig. 1A-D. ***, p<0.01 and ***, p<0.001, Student t test. (E) Histamine- and serotonin-releasing pellets were harvested 6 days after implantation in Nur77\(^{-/-}\) vs. Nur77\(^{+/+}\) wild type mice. Angiogenesis with mother vessels (red arrows) developed in Nur77\(^{+/+}\) but not in Nur77\(^{-/-}\) mice. Data are representative of experiments performed on 8 mice in each group.
**Figure 4.** The angiogenic responses induced by histamine and serotonin initially resembled that induced by VEGF-A^{165} but were transient

(A) Macroscopic appearance of progression and subsequent regression of angiogenesis over time in Nu/Nu mice implanted with histamine- and serotonin-containing pellets vs control pellets; (B) (a-c) Angiogenic responses induced by histamine at 1, 5 and 15 d after implantation, respectively. Note characteristic mother vessels (MV) at 1 d, vessel remodeling at 5 d, and striking vessel regression at 15 d. (d-f) Angiogenic responses induced by serotonin at 3, 7 and 21 d after implantation, respectively. Prominent mother vessels (MV) are present at 3 d which thereafter undergo remodeling (7 d) and regression (21 d). One micron thick, Giemsa-stained Epon sections \^{31}. Magnification bars, 50μm. Data are representative of 4 mice in each group. (C) Tissues adjacent to pellet implant sites were immunostained with an antibody against CD31. Mean vessel density was calculated with Image J software (ten 20x fields, 4 animals per group). ***, p< 0.001; **, p<0.01; *, p<0.05, Student t test.

**Figure 5. Effects of histamine and serotonin on TSP-1 expression.** (A) Serum-starved HUVEC were stimulated with 10 μM histamine, 1 μM serotonin or 10 ng/ml VEGF-A^{165} for indicated times. Cell extracts were immunoblotted with a polyclonal chicken antibody specific for mouse TSP-1 (left panel). Membranes were stripped and reprobed with an antibody against β-actin to confirm equal protein loading (right panel). Data are representative of three separate experiments. (B) Nu/Nu mice were implanted subcutaneously with pellets containing 10μg histamine or 1μg serotonin, or were injected intradermally with 1x 10^7 pfu Ad-VEGF-A^{164}. At indicated times, tissue adjacent to pellets was collected and extracted and immunoblots were prepared using the same anti-mouse TSP-1 antibody used in (A). Data are representative of 4 separate experiments. (C) Quantification of immunoblots from three independent HUVEC experiments and four separate in vivo experiments with Image J software. Data are presented as % change (mean +/- std. error) from time zero and were analyzed with the Kruskal-Wallis test. With regard to the HUVEC data, TSP-1 levels were significantly depressed from 4-8h, but not thereafter, in the case of both histamine and serotonin treatments, whereas TSP-1 was not significantly depressed in response to VEGF until 12h. With regard to the histamine and serotonin in vivo data (B), TSP-1 was significantly reduced over the period of 4-8 days, corresponding to the period of angiogenesis; thereafter, TSP-1 levels rebounded, correlating with angiogenesis regression (Fig. 4). Ad-VEGF-A^{164} did not lead to reduced TSP-1 expression at any time point.

**Figure 6. Modulation of histamine- and serotonin-induced EC proliferation and angiogenesis by 3TSR peptide.** (A-D) Histamine- and serotonin-induced ^3H thymidine incorporation, transwell and scratch assay migration, and tube formation by serum-starved HUVEC that had been pretreated for 10 min. with 50 nM 3TSR peptide. (n=4 and experiments were repeated three times, A, B and D. In C, n=20 from three independent
experiments. ***, p < 0.001 vs. control, Student t test. (E) Nu/Nu mice received intradermal injections of 1 x 10^{11} pfu AAV-TSR in 100 μl HBSS or HBSS alone. Two weeks later, histamine or serotonin pellets were implanted immediately beneath AAV-TSR (b, d) or HBSS (a, c) injection sites and tissues were collected 10 days later. Data are representative of experiments performed on 8 mice/group.

**Figure 7. Histamine and serotonin, but not VEGF-A_{165}, activate the TSP-1 promoter, but not directly through TR3.**

(A) HUVEC were transduced with TSP-1 promoter luciferase and internal luciferase constructs and then stimulated with histamine (10 μM) (Left panel), serotonin (10 μM) (Right panel) or VEGF-A_{165} (10 ng/ml) (Both panels) as indicated. (n=6, experiments were repeated 3 times) ***, p < 0.001 vs control, Tukey-Kramer multiple comparisons test. (B) Cell extracts from HUVEC that were not transduced or transduced with Lac Z (control) or with TR3 cDNAs were subjected to immunoblotting with antibodies against TSP-1 (top panel), VE-cadherin (middle panel) and β-actin for protein equal loading control (bottom panel). Data are representative of three independent experiments.
For personal use only.

**Fig. 3**

**A** Proliferation

[Graph showing [3H] Thymidine Incorporation (Fold) across different treatments for Lac Z and TR3-AS.]

**B** Transwell Migration

[Graph showing the number of migrating cells across different treatments for Lac Z and TR3-AS.]

**C** Scratch Wound Healing

[Graph showing the number of migrating cells across different treatments for Lac Z and TR3-AS.]

**D** Tube Formation

[Graph showing mean total tube length across different treatments for Lac Z and TR3-AS.]

**E** Histamine and Serotonin pellets implanted in Nur77+/+ or Nur77--/ mice

- **Histamine (10 µg)**
  - Nur77+/+
    - 2 mm
  - Nur77--/-

- **Serotonin (1 µg)**
  - Nur77+/+
  - Nur77--/-

- **Histamine (10 µg)**
  - Nur77+/+
  - Nur77--/-

- **Serotonin (1 µg)**
  - Nur77+/+
  - Nur77--/-

(Images and labels depict various experimental outcomes for each treatment group.)
Fig. 4 Control, histamine and serotonin pellets implanted s.c. in Nu/Nu mice.

A

<table>
<thead>
<tr>
<th></th>
<th>1D</th>
<th>4D</th>
<th>6D</th>
<th>10D</th>
<th>14D</th>
<th>21D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histamine (10 μg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serotonin (1 μg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>1 d</th>
<th>3 d</th>
<th>5 d</th>
<th>7 d</th>
<th>15 d</th>
<th>21 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine (10 μg)</td>
<td>a</td>
<td>d</td>
<td>b</td>
<td>e</td>
<td>c</td>
<td>f</td>
</tr>
<tr>
<td>Serotonin (1 μg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

![Bar chart](chart.png)

Mean vessel number per field

- Control
- Histamine
- Serotonin

**Control**: D4, D10, D21

**Histamine**: D4, D10, D21

**Serotonin**: D4, D10, D21

Significance:
- *P < 0.05
- **P < 0.01
- ***P < 0.001
Fig. 5

A  HUVEC

IB: TSP-1

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0</th>
<th>1</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>16</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serotonin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF-A$^{165}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IB: β-actin

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0</th>
<th>1</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>16</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serotonin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF-A$^{165}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B  Mouse skin tissues

IB: TSP-1

<table>
<thead>
<tr>
<th>days</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>16</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine (10 μg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serotonin (1 μg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad-VEGF-A$^{164}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IB: β-actin

<table>
<thead>
<tr>
<th>days</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>16</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine (10 μg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serotonin (1 μg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad-VEGF-A$^{164}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C  Quantification of Immunoblots

- Histamine
- Serotonin
- VEGF

HUVEC

Mouse skin tissues
Fig. 6

A  Proliferation

[3H] Thymidine Incorporation (Fold)

<table>
<thead>
<tr>
<th></th>
<th>HBSS</th>
<th>3TSR (50 nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td></td>
<td>***</td>
</tr>
<tr>
<td>Serotonin</td>
<td></td>
<td>***</td>
</tr>
</tbody>
</table>

B  Transwell Migration

No. of Migrating Cells

<table>
<thead>
<tr>
<th></th>
<th>HBSS</th>
<th>3TSR (50 nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td></td>
<td>***</td>
</tr>
<tr>
<td>Serotonin</td>
<td></td>
<td>***</td>
</tr>
</tbody>
</table>

C  Scratch Wound Healing

No. of Migrating Cells

<table>
<thead>
<tr>
<th></th>
<th>HBSS</th>
<th>3TSR (50 nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td></td>
<td>***</td>
</tr>
<tr>
<td>Serotonin</td>
<td></td>
<td>***</td>
</tr>
</tbody>
</table>

D  Tube Formation

Mean total tube length

<table>
<thead>
<tr>
<th></th>
<th>HBSS</th>
<th>3TSR (50 nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td></td>
<td>***</td>
</tr>
<tr>
<td>Serotonin</td>
<td></td>
<td>***</td>
</tr>
</tbody>
</table>

E  Effect of 3TSR on histamine- and serotonin-induced angiogenesis

HBSS  AAV-3TSR

Histamine (10 µg)

Serotonin (1 µg)

10 days
Fig. 7

A  TSP-1 promoter activity

<table>
<thead>
<tr>
<th>Time</th>
<th>Histamine</th>
<th>VEGF-A&lt;sup&gt;165&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>30 ± 5</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>4h</td>
<td>70 ± 10</td>
<td>50 ± 8</td>
</tr>
<tr>
<td>6h</td>
<td>40 ± 2</td>
<td>30 ± 5</td>
</tr>
<tr>
<td>16h</td>
<td>20 ± 1</td>
<td>10 ± 1</td>
</tr>
</tbody>
</table>

B  TR3 does not regulate TSP-1 expression

IB  | TSP-1 | VE-cadherin | β-actin |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>untransduced</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lac Z</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TR3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The vascular permeabilizing factors histamine and serotonin induce angiogenesis through TR3/Nur77 and subsequently truncate it through thrombospondin-1

Liuliang Qin, Dezheng Zhao, Jianfeng Xu, Xianghui Ren, Ernest F. Terwilliger, Sareh Parangi, Jack Lawler, Harold F. Dvorak and Huiyan Zeng