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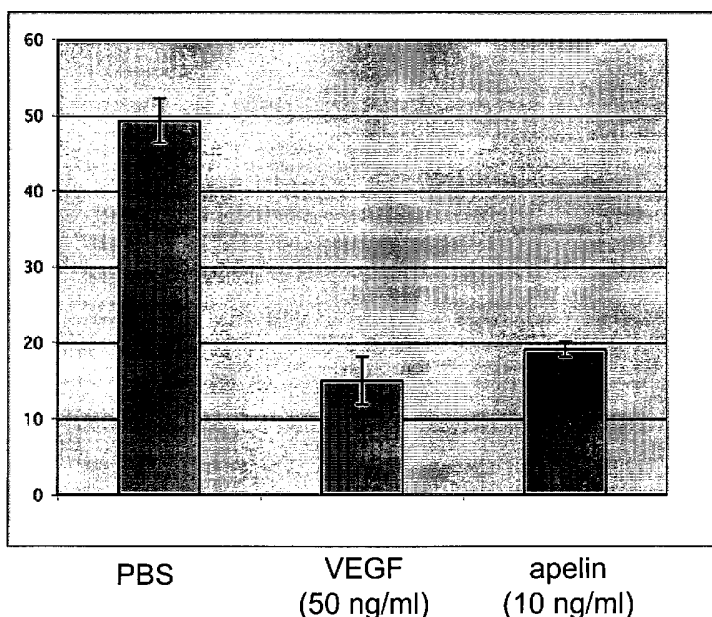
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(54) Title: METHODS FOR MODULATING ANGIOGENESIS AND APOPTOSIS WITH APELIN COMPOSITIONS

% TUNEL
Positive cells



(57) Abstract: Novel methods of inhibiting angiogenesis or tumorigenesis or promoting apoptosis with compositions that inhibit the apelin/APJ signaling pathway are provided. Also provided are methods of promoting angiogenesis or inhibiting apoptosis with compositions comprising an apelin polypeptide or small molecule agonist. The present invention further provides methods for identifying therapeutic agents that affect angiogenesis and/or apoptosis.

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METHODS FOR MODULATING ANGIOGENESIS AND APOPTOSIS WITH APELIN COMPOSITIONS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit of U.S. Provisional Application Serial No. 60/803,807 filed August 23, 2004, which is hereby incorporated in its entirety.

ACKNOWLEDGMENT OF FEDERAL RESEARCH SUPPORT

[0002] This invention was made, at least in part, with funding from National Institutes of Health (NIH Grant Number HL64763 and HL74184 to PK). Accordingly, the United States Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Field of the Invention

[0003] This invention relates generally to methods for modulating angiogenesis, tumorigenesis, apoptosis, and/or vascular permeability. In particular, this invention relates to methods for the use of compositions that affect apelin signaling to treat patients suffering from various angiogenesis-related and/or apoptosis-related diseases or conditions.

Background Art

[0004] Under normal physiological conditions, humans and animals undergo angiogenesis only in very specific situations. For example, angiogenesis is normally observed in wound healing, fetal and embryonic development, and formation of the corpus luteum, endometrium, and placenta. Unregulated angiogenesis occurs in a number of diseases and conditions, such as tumor growth, metastasis, obesity, and wet age-related macular degeneration (AMD). Both controlled and unregulated angiogenesis are thought to proceed in a similar manner. Endothelial cells and pericytes, surrounded by a basement membrane, form capillary blood vessels. Angiogenesis begins with the erosion of the basement membrane by enzymes released by endothelial cells and leukocytes. The endothelial cells, which line the lumen of blood vessels, then protrude through the basement membrane. Angiogenic stimulants induce the endothelial cells to migrate through the eroded basement membrane. The migrating cells form a "sprout" off the parent blood vessel, where the endothelial cells

undergo mitosis and proliferate. The endothelial sprouts merge with each other to form capillary loops, creating the new blood vessel.

[0005] APJ is a cell surface receptor belonging to the G protein-coupled receptor family and has seven transmembrane domains. APJ is related to the angiotensin II receptor and has been described as being a coreceptor involved in the mediation of HIV-1 neuropathogenesis. A natural ligand of APJ was identified and named apelin (APJ endogenous ligand). The apelin polypeptide is initially produced as a 77 amino acid protein (preproapelin) that is cleaved to produce cleavage products of 36 amino acids, 17 amino acids, and 13 amino acids. The peptide consisting of the C-terminal 13 amino acids of the apelin polypeptide is necessary and sufficient for the ability of an apelin polypeptide to interact with APJ.

[0006] Some methods have been achieved which effectively modulate angiogenesis under certain conditions. For example, Avastin[®] is an anti-VEGF antibody produced by Genentech that is currently in clinical trials for the treatment of breast cancer, colorectal cancer, small cell lung cancer, and renal cancer, and that has been shown to have an anti-angiogenic effect on certain tumor types. Avastin[®] has received FDA approval and is now being marketed for use in patients having a colorectal cancer that has disseminated. Similarly, Macugen[®], developed and marketed by Eyetech/Pfizer, is an aptamer that targets VEGF and that has a demonstrated anti-angiogenic effect with regard to wet age-related macular degeneration (AMD) (Eyetech Study Group, 2003, Ophthalmology 110(5):979-86). In addition, Genentech and Novartis Ophthalmics have developed Lucentis[®], a humanized monoclonal antibody that binds and inhibits VEGF-A. Lucentis[®] has produced significant results for the treatment of AMD in clinical trials.

[0007] To date, various polypeptides have been described that stimulate angiogenesis (e.g. VEGFs, FGFs, PDGFB, EGF, LPA, HGF, PD-ECF, IL-8, angiogenin, TNF-alpha, TGF-beta, TGF-alpha, proliferin, and PLGF) or inhibit angiogenesis (e.g. endostatin[®], angiostatin[®], and thrombospondin). Although some methods have been achieved which effectively modulate angiogenesis in certain situations, clearly more therapeutics are needed to treat a broader range of diseases and conditions, as well as to increase the efficacy of the methods that already exist. Therefore, what is needed in the art are new compositions and methods for modulating angiogenesis to inhibit the undesired growth of blood vessels associated with certain diseases and conditions. What is also needed are methods and compositions for

modulating tumorigenesis, apoptosis, and/or permeability of a tumor. What is further needed are new methods for promoting angiogenesis and/or inhibiting apoptosis in patients suffering from diseases or conditions that are indicated by decreased vascularization. Moreover, what is also needed are methods for identifying therapeutic agents capable of modulating angiogenesis and/or apoptosis effectively and safely in a patient.

SUMMARY OF THE INVENTION

[0008] This invention fulfills in part the need to identify new, unique methods for modulating angiogenesis, tumorigenesis, apoptosis, and/or tumor permeability. In particular, the present invention describes methods for inhibiting angiogenesis or tumorigenesis in a biological sample, comprising providing a biological sample; and combining the sample with an angiogenesis-inhibiting or tumorigenesis-inhibiting amount of a composition comprising an inhibitor of apelin activity. In one embodiment, the composition comprising an inhibitor of apelin activity decreases the vascular permeability of a biological sample. In another embodiment, the composition comprising an inhibitor of apelin activity increases apoptosis in a biological sample. In a preferred embodiment, the composition interferes with the interaction of an apelin polypeptide or apelin peptide with a receptor for apelin. In a more preferred embodiment, the composition interferes with the interaction of apelin with APJ. In another preferred embodiment, the composition comprises an anti-apelin antibody or fragment thereof. In a more preferred embodiment, the antibody binds a polypeptide or peptide selected from the group consisting of a polypeptide as defined in SEQ ID NO:1; a polypeptide as defined in SEQ ID NO:2; a polypeptide as defined in SEQ ID NO:3; a polypeptide as defined in SEQ ID NO:4; a polypeptide as defined in SEQ ID NO:5; and a polypeptide having at least 80% sequence identity with any of the polypeptides or peptides above. In yet another preferred embodiment, the inhibitor of apelin activity is selected from the group consisting of apelin antisense nucleic acid, receptor decoy, ribozyme, sense polynucleotide, double stranded RNA, RNAi, aptamer, and small molecule antagonist.

[0009] The present invention provides that in some embodiments, the methods for inhibiting angiogenesis or increasing apoptosis are used to treat a patient with disease or condition that involves angiogenesis or decreased apoptosis. The present invention also provides that in some embodiments, the compositions comprise a

combination of anti-angiogenic molecules, including a molecule that inhibits apelin activity and a molecule that inhibits another angiogenic factor. In other embodiments, the methods further comprise administering to the patient a therapeutically effective amount of an anti-cancer agent, wherein the anti-cancer agent is selected from the group consisting of a chemotherapeutic agent, a radiotherapeutic agent, an anti-angiogenic agent, and an apoptosis-inducing agent.

[0010] The present invention also provides methods for promoting angiogenesis or decreasing apoptosis in a biological sample, comprising providing a biological sample; and combining the sample with a biologically effective amount of an angiogenesis promoting or apoptosis inhibiting composition comprising apelin. In a preferred embodiment, the composition comprises a polypeptide or peptide selected from the group consisting of a polypeptide as defined in SEQ ID NO:1; a polypeptide as defined in SEQ ID NO:2; a polypeptide as defined in SEQ ID NO:3; a polypeptide as defined in SEQ ID NO:4; a polypeptide as defined in SEQ ID NO:5; and a polypeptide that has at least 80% sequence identity with any of the polypeptides or peptides above and that interacts with APJ. The present invention further provides methods of promoting angiogenesis in a patient that has a disease or condition that is indicated by decreased vascularization. The present invention also provides that in some embodiments, the compositions comprise a combination of angiogenic molecules, including apelin or an apelin agonist and another angiogenic factor.

[0011] The present invention also provides methods for identifying a modulator of angiogenesis or apoptosis, comprising providing an angiogenesis promoting or apoptosis inhibiting composition comprising apelin; combining a putative modulator of angiogenesis or apoptosis with the composition; introducing the composition or the combination of the putative modulator and the composition to an angiogenesis or apoptosis predictive model; and comparing the amount of vascular branching or intact cells in the model in the presence and absence of the putative modulator. In preferred embodiments, the apelin composition comprises a polypeptide or peptide selected from the group consisting of a polypeptide as defined in SEQ ID NO:1; a polypeptide as defined in SEQ ID NO:2; a polypeptide as defined in SEQ ID NO:3; a polypeptide as defined in SEQ ID NO:4; a polypeptide as defined in SEQ ID NO:5; and a polypeptide that has at least 80% sequence identity with any of the polypeptides or peptides above and that interacts with APJ.

[0012] The present invention also provides methods of inhibiting angiogenesis in a biological sample, comprising providing a biological sample, and introducing to the sample an angiogenesis inhibiting amount of a composition comprising an inhibitor of apelin activity, wherein the biological sample comprises adipose tissue. In one embodiment, the composition interferes with the interaction of an apelin polypeptide or apelin peptide with a receptor polypeptide. In a preferred embodiment, the composition interferes with the interaction of an apelin polypeptide or apelin peptide with APJ. In another preferred embodiment, the composition comprises an anti-apelin antibody or fragment thereof. In one embodiment, the antibody or fragment thereof binds a polypeptide that is selected from the group consisting of: a polypeptide as defined in SEQ ID NO:1; a polypeptide as defined in SEQ ID NO:2; a polypeptide as defined in SEQ ID NO:3; a polypeptide as defined in SEQ ID NO:4; a polypeptide as defined in SEQ ID NO:5; and a polypeptide that has at least 80% sequence identity with the polypeptide of a) through e) above and that interacts with APJ. In another embodiment, the antibody or fragment thereof binds a polypeptide that has at least 90% sequence identity with the polypeptide or peptide of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5 and that interacts with APJ. The present invention also provides that the inhibitor of apelin activity may be an anti-APJ antibody or fragment thereof. In a preferred embodiment, the APJ antibody or fragment thereof binds a polypeptide as defined in SEQ ID NO:17 or a polypeptide that has at least 90% sequence identity with the polypeptide as defined in SEQ ID NO:17 and that interacts with apelin. In another embodiment, the inhibitor of apelin activity is selected from the group consisting of an apelin or APJ antisense nucleic acid, receptor decoy, ribozyme, sense polynucleotide, double stranded RNA, RNAi, aptamer, and small molecule antagonist. In yet another embodiment, the inhibitor of apelin activity is an inhibitor of a serine protease that cleaves a polypeptide specifically after an arginine residue.

[0013] The present invention also provides that the inhibitor of apelin activity may be an inhibitor of an insulin transduction pathway. In one embodiment, the inhibitor of apelin activity is an inhibitor of Phosphatidyl Inositol 3 Kinase (PI3K), Protein Kinase C (PKC), or Mitogen Activated Protein Kinase (MAPK). In another embodiment, the inhibitor of apelin activity is selected from a group consisting of wortmannin, LY294002, GF109203X, and PD098059. In a preferred embodiment, the biological sample is a human biological sample. In one embodiment, the biological

sample is in a patient. The present invention provides that the composition may be introduced to a patient by a route selected from the group consisting of subcutaneous injection, intravenous injection, intraocular injection, intradermal injection, intramuscular injection, intraperitoneal injection, intratracheal administration, epidural administration, inhalation, intranasal administration, oral administration, sublingual administration, buccal administration, rectal administration, vaginal administration, and topical administration. The present invention provides that in one embodiment, the patient has a disease or condition selected from a group consisting of obesity and an obesity-associated disorder.

[0014] The present invention further provides methods of inhibiting angiogenesis in a biological sample, comprising providing a biological sample, and introducing to the sample an angiogenesis inhibiting amount of a composition comprising an inhibitor of apelin activity, wherein the biological sample is in a patient that has wet age-related macular degeneration (AMD). In certain embodiments, these methods are used to inhibit the growth of abnormal blood vessels and/or to inhibit ocular angiogenesis under the macula in the patient. In another embodiment, the present methods are used to inhibit the leakage of fluid and/or blood from the patient's blood vessels under the macula. In one embodiment, the composition interferes with the interaction of an apelin polypeptide or apelin peptide with a receptor polypeptide. In a preferred embodiment, the composition interferes with the interaction of an apelin polypeptide or apelin peptide with APJ. In another preferred embodiment, the composition comprises an anti-apelin antibody or fragment thereof. In one embodiment, the antibody or fragment thereof binds a polypeptide that is selected from the group consisting of: a polypeptide as defined in SEQ ID NO:1; a polypeptide as defined in SEQ ID NO:2; a polypeptide as defined in SEQ ID NO:3; a polypeptide as defined in SEQ ID NO:4; a polypeptide as defined in SEQ ID NO:5; and a polypeptide that has at least 80% sequence identity with the polypeptide of a) through e) above and that interacts with APJ. In another embodiment, the antibody or fragment thereof binds a polypeptide that has at least 90% sequence identity with the polypeptide or peptide of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5 and that interacts with APJ. The present invention also provides that the inhibitor of apelin activity may be an anti-APJ antibody or fragment thereof. In a preferred embodiment, the APJ antibody or fragment thereof binds a polypeptide as defined in SEQ ID NO:17 or a polypeptide that has at least 90% sequence identity with the polypeptide as defined

in SEQ ID NO:17 and that interacts with apelin. In another embodiment, the inhibitor of apelin activity is selected from the group consisting of an apelin or APJ antisense nucleic acid, receptor decoy, ribozyme, sense polynucleotide, double stranded RNA, RNAi, aptamer, and small molecule antagonist. In yet another embodiment, the inhibitor of apelin activity is an inhibitor of a serine protease that cleaves a polypeptide specifically after an arginine residue.

[0015] These and other embodiments of the invention will become apparent to one of skill in the art upon review of the description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] Figure 1 is an amino acid sequence alignment of the C-terminal thirteen amino acids of the frog apelin polypeptide and human apelin polypeptide, showing that the C-terminal thirteen amino acids are identical between the two sequences.

[0017] Figure 2 is a schematic representation of the apelin/APJ signaling pathway.

[0018] Figure 3A are two photographs of electrophoretic gels, showing the RT-PCR detection of APJ transcripts in adult mouse tissue and a mouse endothelial cell line. Figure 3B is a photograph of an electrophoretic gel, showing the RT-PCR detection of apelin transcripts in an embryonic yolk sac and a mouse endothelial cell line.

[0019] Figure 4 is a photomicrograph of a frog embryo in which APJ transcripts were detected using an *in situ* hybridization protocol. These data reveal that APJ is strongly expressed in the developing vasculature. The “is” denotes intersomitic vessel, and the posterior cardinal vein is denoted “pcv” in this figure. APJ is also known as “X-msr” in *Xenopus*, as is indicated in this figure.

[0020] Figure 5 is a photomicrograph of an *in situ* hybridization of a frog embryo probed with either (A) apelin antisense sequences or (B) APJ antisense sequences. These data show that both apelin and APJ are expressed in the developing blood vessels, especially the growing intersomitic vessels. Panel A shows the expression of apelin, and panel B shows the expression of APJ.

[0021] Figure 6 is a photomicrograph of an *in situ* hybridization of a frog embryo stained with a vascular marker to visualize blood vessels, showing outgrowth of the vasculature toward the apelin-soaked beads. In this case, the probe detects transcripts for the vascular transcription factor *erg*. Beads soaked in the thirteen amino

acid apelin peptide were implanted into frog embryos and are indicated here by the arrows. Panel A and B show different magnifications of the same embryo at A) 50 x and B) 150 x. Panel C and D show different magnifications of the same embryo at C) 50 x and D) 150 x.

[0022] Figure 7 shows the results of chicken chorioallantoic membrane (CAM) assays. Panels A and B are photomicrographs of blood vessels that formed in association with membranes that had been treated with A) PBS buffer or B) 50 ng apelin-13. Panel C is a graph showing the quantitation of results from duplicate CAM assays (units are branches/unit area). The membranes had been treated with PBS buffer, 50 ng VEGF, or 50 ng apelin-13, as indicated. Panel B demonstrates that apelin results in an increase in blood vessel formation, as well as an increase in leakage from the blood vessels. These results indicate that apelin has an effect similar to that of VEGF, as an angiogenic factor and/or as a vascular permeability factor, as assayed using the chicken CAM procedure.

[0023] Figure 8A is a graph showing the increase in migration of bovine aortic endothelial (BAE) cells in response to 10 ng/ml apelin or VEGF. The blood vessel migration is measured by the number of cells per field. A total of four fields were counted for each membrane, and the experiments were performed in triplicate +/- s.e.m. Figure 8B is a graph showing the proliferation of bovine aortic endothelial (BAE) cells in response to VEGF or apelin, in the presence or absence of the VEGF pathway inhibitor SU1498. The results shown are the mean of triplicate experiments +/- the s.e.m. These data show that the VEGF pathway inhibitor SU1498 partially inhibits VEGF-mediated proliferation, but has no significant effect on apelin-mediated proliferation.

[0024] Figure 9 is photomicrograph of an *in situ* hybridization of a frog embryo stained to detect transcripts of the vascular marker *erg*, to visualize developing blood vessels. Panel A shows the uninjected side of the frog embryo. Panel B shows the side of the frog embryo that was injected with the ap1 apelin antisense morpholino. These data show that a reduction in apelin protein in the embryo causes a disruption in vascular growth.

[0025] Figure 10 shows expression of apelin mRNA in a significant portion of human tumors. The membrane (BD Biosciences, San Jose, CA, cat. # 7847-1) contained cDNA from 154 human tumors of different tissues, as well as a control sample corresponding to each of the tumor samples that was from non-tumor adjacent

tissue from the same individual. Figure 10A is an autoradiograph (17 hour exposure) of a dot blot membrane hybridized with an approximately 2 kb ^{32}P -labeled apelin probe. Figure 10B is an autoradiograph (17 hour exposure) of a dot blot membrane hybridized with an approximately 700 base ^{32}P -labeled VEGF-A probe.

[0026] Figure 11 is a schematic representation of the regulatory region of the human, mouse, and zebrafish apelin genes, depicting the putative HIF-1 α recognition sequences.

[0027] Figure 12 shows photographs of electrophoretic gels, demonstrating the effect of conditions that mimic hypoxia on gene expression. Primary rat cardiomyocyte cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum and penicillin/streptomycin at 37°C for four days. Medium was removed, and cells were treated with fresh media (U) or fresh media containing 150 μM Cobalt Chloride (Co) (Piret et al., 2002). Panel A shows the electrophoretic gel of PCR products generated using the GAPDH primer set; panel B shows the electrophoretic gel of PCR products generated using the GAPDH primer set; and panel C shows the electrophoretic gel of PCR products generated using the GAPDH primer set.

[0028] Figure 13 shows photomicrographs of an *in situ* hybridization of a frog embryo at an early (Panels A and D), middle (Panels B and E), or late developmental stage (Panels C and F), probed with either apelin antisense sequences (Panels A, B, and C) or APJ antisense sequences (Panels D, E, and F). These data show that both apelin and APJ are expressed in the developing blood vessels, especially the growing intersomitic vessels.

[0029] Figure 14 shows photomicrographs of an *in situ* hybridization of an E11.5 day mouse embryo probed with either APJ antisense sequences (Panel A) or apelin antisense sequences (Panel B). These data show that both apelin and APJ are expressed in the inter-segmental vessels and neural tube blood vessels of in an early embryo and that apelin and APJ are coexpressed to some extent in the same tissues.

[0030] Figure 15 shows photomicrographs of an *in situ* hybridization of an E11.5 day mouse embryo probed with either apelin antisense sequences (Panel A) or APJ antisense sequences (Panel B). The photomicrographs show a dorsal view of the developing neural tissues with blood vessels growing into new tissues. These data show that both apelin and APJ are coexpressed in developing blood vessels and that apelin is expressed in particular at the tips of the angiogenic vessels.

[0031] Figure 16 shows photomicrographs of an *in situ* hybridization of a frog embryo. In Panel A, the embryo was stained with a vascular marker to visualize blood vessels, showing outgrowth of the vasculature toward the apelin-soaked beads. In Panel B, the embryo was stained for VEGF mRNA expression. Beads soaked in the thirteen amino acid apelin peptide were implanted into frog embryos and are indicated here by the arrows. These data show that there is no upregulation of VEGF mRNA expression in the vicinity of the bead, suggesting that apelin's stimulation of angiogenic growth does not proceed via VEGF expression.

[0032] Figure 17 is a graph showing the cellular proliferation of the mouse endothelial cell line that constitutively expresses APJ. These data show that bEnd3 cells proliferate upon the addition of VEGF or apelin in concentrations ranging from 0.1 ng/ml to 1 µg/ml as is indicated on the graph.

[0033] Figure 18 is a graph showing the proliferation of mouse endothelial cells (bEnd3) in response to VEGF or apelin, in the presence or absence of the VEGF pathway inhibitor SU1498. The results shown are the mean of three separate experiments, each of which was conducted in triplicate +/- the s.e.m. Apelin was provided at a concentration of 10 ng/ml; VEGF was provided at a concentration of 50 ng/ml; and SU1498 was provided at a 25 µM concentration. These data show that the VEGF pathway inhibitor SU1498 partially inhibits VEGF-mediated proliferation, but has no significant effect on apelin-mediated proliferation.

[0034] Figure 19 is a graph showing the proliferation of mouse endothelial cells (bEnd3) in response to FGF or apelin, in the presence or absence of the FGF pathway inhibitor SU5402. The results shown are the mean of two separate experiments, each of which was conducted in triplicate +/- the s.e.m. Apelin was provided at a concentration of 10 ng/ml; bFGF was provided at a concentration of 50 ng/ml; and SU5402 was provided at a 20 µM concentration. These data show that the FGF pathway inhibitor SU5402 partially inhibits FGF-mediated proliferation, but has no significant effect on apelin-mediated proliferation.

[0035] Figure 20 shows the effect of apelin on endothelial cell migration. Panels A-C are photomicrographs of the endothelial cells present in a view of the underside of a trans-welled chamber. Endothelial cells were placed in the upper chamber, and a serum control (Panel A), apelin (10 ng/ml) (Panel B), or VEGF (50 ng/ml) was added to the lower chamber. Cells that migrated from the upper chamber to

the lower chamber were visualized by the staining of the nuclei of the cells with a DAPI stain. These data demonstrate that apelin is a chemotactic agent for endothelial cells.

[0036] Figure 21 shows the results of TUNEL assays with endothelial cells. Panels A-C are photomicrographs of the endothelial cells stained with DAPI stain, and Panels D, E, and F are photomicrographs showing the amount of fragmented DNA present in the endothelial cells. Panels A and D show cells that were treated with PBS as a control. Panels B and E show cells that were treated with VEGF (50 ng/ml), and Panels C and F show cells that were treated with apelin (10 ng/ml). These data show that apelin is anti-apoptotic for endothelial cells.

[0037] Figure 22 is a graph showing the quantitation of the anti-apoptotic activity of apelin based on the TUNEL assay.

[0038] Figure 23 is a graph showing the increase in expression of apelin in response to hypoxic conditions. The expression of b-actin was used as a negative control, and the levels of expression of VEGF and GLUT1 were used as positive controls for being induced under hypoxic conditions.

[0039] Figure 24 is a graph showing that active apelin is secreted from transfected HT-29 cells. Conditioned media was used to stimulate bEnd.3 cell mitosis as assayed standard BUdR incorporation assay. Results were normalized against medium from HT-29 cells transfected with empty vector (HT-29 vector).

[0040] Figure 25 is a graph showing that apelin stimulates the rate of HT-29 cell tumor growth in the scid mouse xenograft model. Average tumor volume from apelin expressing cells and empty vector control cells is indicated (+/- SEM).

DETAILED DESCRIPTION OF THE INVENTION

[0041] The present invention may be understood more readily by reference to the following detailed description of the preferred embodiments of the invention and the Examples included herein. However, before the present methods are disclosed and described, it is to be understood that this invention is not limited to specific nucleic acids, specific polypeptides, specific cell types, specific host cells, specific conditions, or specific methods, etc., as such may, of course, vary, and the numerous modifications and variations therein will be apparent to those skilled in the art. It is also to be understood that the terminology used herein is for the purpose of describing specific embodiments only and is not intended to be limiting. It is further to be understood that

unless specifically defined herein, the terminology used herein is to be given its traditional meaning as known in the relative art.

[0042] The present invention describes for the first time that the apelin peptide is useful in modulating angiogenesis and apoptosis. In certain preferred embodiments, the present invention describes methods for inhibiting angiogenesis, inhibiting tumorigenesis, promoting apoptosis, or decreasing vascular permeability in a biological sample, comprising providing a biological sample; and combining the sample with an angiogenesis-inhibiting, tumorigenesis-inhibiting, apoptosis-promoting, and/or vascular permeability-decreasing amount of a composition comprising an inhibitor of apelin activity. As used herein, the term "angiogenesis" refers to the generation of new blood vessels, and the term "tumorigenesis" refers to the growth of a tumor. In one embodiment, the term "angiogenesis" involves the vascularization of adipose tissue. As also used herein, the phrase "methods for inhibiting angiogenesis or tumorigenesis" is intended to include methods that inhibit angiogenesis or tumorigenesis by decreasing vascular permeability. In one embodiment, the "methods for inhibiting angiogenesis or tumorigenesis" are used to inhibit angiogenesis in a biological sample comprising adipose tissue. As used herein, the term "apoptosis" refers to the programmed death of a cell. As also used herein, the term "apelin polypeptide" is intended to refer to a polypeptide that comprises the C-terminal 13 amino acids of apelin (i.e. the polypeptide of SEQ ID NO:4). In a preferred embodiment, the composition interferes with the interaction of an apelin polypeptide or apelin peptide with a receptor for apelin. In another preferred embodiment, the composition interferes with the interaction of an apelin polypeptide or apelin peptide with APJ or a polypeptide having at least 80% sequence identity with APJ. In another preferred embodiment, the composition comprises an anti-apelin antibody or fragment thereof. In a preferred embodiment, the antibody binds a polypeptide or peptide selected from the group consisting of a polypeptide as defined in SEQ ID NO:1; a polypeptide as defined in SEQ ID NO:2; a polypeptide as defined in SEQ ID NO:3; a polypeptide as defined in SEQ ID NO:4; a polypeptide as defined in SEQ ID NO:5; and a polypeptide having at least 80% sequence identity with any of the polypeptides or peptides above. As used herein, the phrase "antibody fragment" is intended to include various fragments of an antibody including, but not limited to, F_{ab} fragments, $F_{ab'}$ fragments, $F_{(ab')_2}$ fragments, and smaller fragments such as the domain antibodies described by Domantis. In another preferred embodiment, the composition comprises an apelin antisense nucleic acid.

[0043] In another preferred embodiment, the inhibitor of apelin activity is selected from the group consisting of apelin antisense nucleic acid, receptor decoy, ribozyme, sense polynucleotide, double stranded RNA, RNAi, aptamer, and small molecule antagonist. As used herein, the term “receptor decoy” refers to a molecule that will bind to an apelin polypeptide and prevent the apelin polypeptide from participating in its native signaling pathway(s). A receptor decoy is intended to encompass a soluble receptor for apelin (or fragment thereof) which is capable of binding apelin in the serum and inhibiting apelin from signaling through its native signaling pathway(s). As used herein, “RNAi” or “RNA interference” refers to a technique in which double-stranded RNA or dsRNA derived from the gene to be analyzed is introduced into the host cell. As used herein, “dsRNA” refers to RNA that is partially or completely double stranded. The dsRNA may have a single stranded overhang at either or both ends of the molecule. This dsRNA is processed into relatively small fragments and can subsequently become distributed throughout the host cell. The dsRNA fragments interact, in a cell, with the corresponding endogenously produced messenger RNA, resulting in the endogenous transcript being specifically broken down (Zamore et al., 2000, Cell 101:25-33). This process leads to a loss-of-function mutation having a phenotype that may closely resemble the phenotype arising from a complete or partial deletion of the target gene.

[0044] The present invention also provides that in some embodiments, the compositions comprise a combination of anti-angiogenic molecules, including a molecule that inhibits apelin activity and a molecule that inhibits another angiogenic factor. As used herein, the term “angiogenic factor” is intended to include molecules that promote angiogenesis, such as, for example, VEGFs, FGFs, PDGFB, EGF, LPA, HGF, PD-ECF, IL-8, angiogenin, TNF-alpha, TGF-beta, TGF-alpha, proliferin, and PLGF.

[0045] In other preferred embodiments of the present invention, the compositions used inhibit apelin activity by interfering with a receptor for apelin. In one embodiment, the receptor for apelin is APJ. In certain preferred embodiments, the inhibitor of apelin activity is an anti-APJ antibody or fragment thereof. In a more preferred embodiment, inhibitor of apelin activity is an antibody or fragment thereof that binds a polypeptide as defined in SEQ ID NO:17. In other preferred embodiments, the inhibitor of apelin activity is selected from the group consisting of an APJ antisense

nucleic acid, receptor decoy, ribozyme, sense polynucleotide, double stranded RNA, RNAi, aptamer, and small molecule antagonist.

[0046] In certain preferred embodiments of the present invention, the compositions comprise a combination of anti-angiogenic molecules, including a molecule that inhibits APJ activity and a molecule that inhibits another angiogenic factor. In a more preferred embodiment, the angiogenic factor is selected from the group consisting of VEGFs, FGFs, PDGFB, EGF, LPA, HGF, PD-ECF, IL-8, angiogenin, TNF-alpha, TGF-beta, TGF-alpha, proliferin, and PLGF.

[0047] In certain preferred embodiments of the present invention, the methods for inhibiting angiogenesis or tumorigenesis or for promoting apoptosis are used to treat a patient with disease or condition that involves angiogenesis or tumorigenesis or decreased apoptosis. As used herein, the phrase "disease or condition involving angiogenesis" is intended to include, but is not limited to, stroke, hemangioma, solid tumors, leukemias, lymphomas, myelomas, metastasis, telangiectasia psoriasis scleroderma, pyogenic granuloma, Myocardial angiogenesis, plaque neovascularization, coronary collaterals, ischemic limb angiogenesis, corneal diseases, rubeosis, neovascular glaucoma, diabetic retinopathy, retrolental fibroplasia, arthritis, diabetic neovascularization, macular degeneration, wound healing, peptic ulcer, fractures, keloids, vasculogenesis, hematopoiesis, ovulation, menstruation, placentation, polycystic ovary syndrome, dysfunctional uterine bleeding, endometrial hyperplasia and carcinoma, endometriosis, failed implantation and subnormal foetal growth, myometrial fibroids (uterine leiomyomas) and adenomyosis, ovarian hyperstimulation syndrome, ovarian carcinoma, obesity, and obesity-associated disorders. As used herein, the term "macular degeneration" refers to wet age-related macular degeneration (AMD), a condition indicated by the growth of abnormal blood vessels and/or ocular angiogenesis under the macula, wherein the blood vessels leak fluid and blood and cause scar tissue that destroys the macula, resulting in the patient's deterioration of sight. As also used herein, the phrase "obesity and obesity-associated disorders" refers to diseases or conditions involving the improper proliferation of adipocytes or preadipocytes and/or improper levels of angiogenesis in adipose tissue.

[0048] In one embodiment, the present invention provides methods of inhibiting angiogenesis in a biological sample, comprising providing a biological sample, and introducing to the sample an angiogenesis inhibiting amount of a composition comprising an inhibitor of apelin activity, wherein the biological sample

comprises adipose tissue. In certain embodiments, the composition interferes with the interaction of an apelin polypeptide or apelin peptide with a receptor polypeptide. In a preferred embodiment, the composition interferes with the interaction of an apelin polypeptide or apelin peptide with APJ. In another preferred embodiment, the composition comprises an anti-apelin antibody or fragment thereof. In one embodiment, the antibody or fragment thereof binds a polypeptide that is selected from the group consisting of: a polypeptide as defined in SEQ ID NO:1; a polypeptide as defined in SEQ ID NO:2; a polypeptide as defined in SEQ ID NO:3; a polypeptide as defined in SEQ ID NO:4; a polypeptide as defined in SEQ ID NO:5; and a polypeptide that has at least 80% sequence identity with the polypeptide of a) through e) above and that interacts with APJ. In another embodiment, the antibody or fragment thereof binds a polypeptide that has at least 90% sequence identity with the polypeptide or peptide of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5 and that interacts with APJ. The present invention also provides that the inhibitor of apelin activity may be an anti-APJ antibody or fragment thereof. In a preferred embodiment, the APJ antibody or fragment thereof binds a polypeptide as defined in SEQ ID NO:17 or a polypeptide that has at least 90% sequence identity with the polypeptide as defined in SEQ ID NO:17 and that interacts with apelin. In another embodiment, the inhibitor of apelin activity is selected from the group consisting of an apelin or APJ antisense nucleic acid, receptor decoy, ribozyme, sense polynucleotide, double stranded RNA, RNAi, aptamer, and small molecule antagonist. In yet another embodiment, the inhibitor of apelin activity is an inhibitor of a serine protease that cleaves a polypeptide specifically after an arginine residue.

[0049] The present invention also provides that the inhibitor of apelin activity may be an inhibitor of an insulin transduction pathway. In one embodiment, the inhibitor of apelin activity is an inhibitor of Phosphatidyl Inositol 3 Kinase (PI3K), Protein Kinase C (PKC), or Mitogen Activated Protein Kinase (MAPK). In another embodiment, the inhibitor of apelin activity is selected from a group consisting of wortmannin, LY294002, GF109203X, and PD098059. In a preferred embodiment, the biological sample is a human biological sample. In one embodiment, the biological sample is in a patient. The present invention provides that the composition may be introduced to a patient by a route selected from the group consisting of subcutaneous injection, intravenous injection, intraocular injection, intradermal injection, intramuscular injection, intraperitoneal injection, intratracheal administration, epidural

administration, inhalation, intranasal administration, oral administration, sublingual administration, buccal administration, rectal administration, vaginal administration, and topical administration. The present invention provides that in one embodiment, the patient has a disease or condition selected from a group consisting of obesity and an obesity-associated disorder.

[0050] The present invention further provides methods of inhibiting angiogenesis in a biological sample, comprising providing a biological sample, and introducing to the sample an angiogenesis inhibiting amount of a composition comprising an inhibitor of apelin activity, wherein the biological sample is in a patient that has wet age-related macular degeneration (AMD). In certain embodiments, these methods are used to inhibit the growth of abnormal blood vessels and/or to inhibit ocular angiogenesis under the macula in the patient. In another embodiment, the present methods are used to inhibit the leakage of fluid and/or blood from the patient's blood vessels under the macula. In one embodiment, the composition interferes with the interaction of an apelin polypeptide or apelin peptide with a receptor polypeptide. In a preferred embodiment, the composition interferes with the interaction of an apelin polypeptide or apelin peptide with APJ. In another preferred embodiment, the composition comprises an anti-apelin antibody or fragment thereof. In one embodiment, the antibody or fragment thereof binds a polypeptide that is selected from the group consisting of: a polypeptide as defined in SEQ ID NO:1; a polypeptide as defined in SEQ ID NO:2; a polypeptide as defined in SEQ ID NO:3; a polypeptide as defined in SEQ ID NO:4; a polypeptide as defined in SEQ ID NO:5; and a polypeptide that has at least 80% sequence identity with the polypeptide of a) through e) above and that interacts with APJ. In another embodiment, the antibody or fragment thereof binds a polypeptide that has at least 90% sequence identity with the polypeptide or peptide of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5 and that interacts with APJ. The present invention also provides that the inhibitor of apelin activity may be an anti-APJ antibody or fragment thereof. In a preferred embodiment, the APJ antibody or fragment thereof binds a polypeptide as defined in SEQ ID NO:17 or a polypeptide that has at least 90% sequence identity with the polypeptide as defined in SEQ ID NO:17 and that interacts with apelin. In another embodiment, the inhibitor of apelin activity is selected from the group consisting of an apelin or APJ antisense nucleic acid, receptor decoy, ribozyme, sense polynucleotide, double stranded RNA, RNAi, aptamer, and small molecule antagonist. In yet another embodiment, the

inhibitor of apelin activity is an inhibitor of a serine protease that cleaves a polypeptide specifically after an arginine residue.

[0051] In other preferred embodiments of the present invention, the methods for inhibiting angiogenesis or tumorigenesis or for promoting apoptosis are used to treat a patient with disease or condition that involves tumorigenesis. In other preferred embodiments of the present invention, the methods for inhibiting angiogenesis or tumorigenesis or promoting apoptosis are used to treat a patient with disease or condition that involves inappropriate leakage of blood vessels. In a more preferred embodiment, the apelin-inhibiting composition used in the methods for inhibiting angiogenesis or tumorigenesis or promoting apoptosis acts by decreasing vascular permeability.

[0052] In certain preferred embodiments of the present invention, the methods further comprise administering to the patient a therapeutically effective amount of an anti-cancer agent, wherein the anti-cancer agent is selected from the group consisting of a chemotherapeutic agent, a radiotherapeutic agent, an anti-angiogenic agent, an apoptosis-inducing agent. As used herein, a “therapeutically effective amount” is an amount that has a negative effect on angiogenesis or tumor growth. As also used herein, an “anti-cancer agent” refers to a molecule that has a negative effect on angiogenesis or tumor growth. In one embodiment, the anti-cancer agent is an anti-angiogenic agent that inhibits the expression or activity of an angiogenic factor selected from the group consisting of VEGFs, FGFs, PDGFB, EGF, LPA, HGF, PD-ECF, IL-8, angiogenin, TNF-alpha, TGF-beta, TGF-alpha, proliferin, and PLGF. In another embodiment, the anti-cancer agent is an anti-angiogenic agent selected from the group consisting of an agent that inhibits the expression or activity of a matrix metalloproteinase; an agent that interacts with a cell adhesion molecule; and an agent that inhibits the activity of a urokinase; and an agent that inhibits angiogenesis through another mechanism.

[0053] The present invention also provides methods for promoting angiogenesis or apoptosis in a biological sample, comprising providing a biological sample; and combining the sample with a biologically effective amount of an angiogenesis promoting or apoptosis inhibiting composition comprising apelin. In a preferred embodiment, the composition comprises a polypeptide or peptide selected from the group consisting of a polypeptide as defined in SEQ ID NO:1; a polypeptide as defined in SEQ ID NO:2; a polypeptide as defined in SEQ ID NO:3; a polypeptide

as defined in SEQ ID NO:4; a polypeptide as defined in SEQ ID NO:5; and a polypeptide having at least 80% sequence identity with any of the polypeptides or peptides above. The present invention also provides that in some embodiments, the compositions comprise a combination of angiogenic molecules, including apelin (or an apelin agonist) and another angiogenic factor.

[0054] In certain preferred embodiments of the present invention, the methods for promoting angiogenesis or decreasing apoptosis are used to treat a patient with disease or condition that is indicated by decreased vascularization or increased apoptosis. As used herein, the phrase “disease or condition involving angiogenesis or increased apoptosis” is intended to include, but is not limited to, diabetes, arthritis, ischemia, anemia, a wound, gangrene, or necrosis.

[0055] As used herein, the terms “peptide,” “polypeptide,” and “protein” refer to a chain of at least four amino acids joined by peptide bonds. The chain may be linear, branched, circular, or combinations thereof. Accordingly, the present invention provides methods for the use of isolated apelin polypeptides and APJ polypeptides, and polypeptides having at least 80%, 85%, 90%, 95% or greater sequence identity with apelin or APJ polypeptides. In preferred embodiments, the apelin polypeptide is defined in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5; and the APJ polypeptide is defined in SEQ ID NO:17.

[0056] The apelin polypeptide of the present invention, and fragments thereof, are preferably synthesized chemically by standard peptide synthesis techniques. For the purposes of this invention, when chemically synthesized, the apelin-13 peptide may comprise a pyroglutamate rather than a glutamic acid residue in the N-terminal position. As an alternative to standard peptide synthesis, an apelin polypeptide, or peptide thereof, can be produced using standard recombinant DNA techniques. For example, a nucleic acid molecule encoding the polypeptide is cloned into an expression vector, the expression vector is introduced into a host cell, and the apelin polypeptide is expressed in the host cell. The apelin polypeptide can then be isolated from the cells by an appropriate purification scheme using standard polypeptide purification techniques. For the purposes of the invention, the term “recombinant polynucleotide” refers to a polynucleotide that has been altered, rearranged, or modified by genetic engineering. Examples include any cloned polynucleotide, and polynucleotides that are linked or joined to heterologous sequences. The term “recombinant” does not refer to alterations to polynucleotides that result from naturally occurring events, such as spontaneous

mutations. The apelin polypeptide of the present invention, and fragments thereof, are preferably produced using standard recombinant techniques. Moreover, native apelin or APJ polypeptide can be isolated from cells, for example, using an anti-apelin or anti-APJ antibody, respectively.

[0057] As used herein, the term “nucleic acid” and “polynucleotide” refer to RNA or DNA that is linear or branched, single or double stranded, or a hybrid thereof. The term also encompasses RNA/DNA hybrids. These terms also encompass untranslated sequence located at both the 3' and 5' ends of the coding region of the gene: at least about 1000 nucleotides of sequence upstream from the 5' end of the coding region and at least about 200 nucleotides of sequence downstream from the 3' end of the coding region of the gene. Less common bases, such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine, and others can also be used for antisense, dsRNA, and ribozyme pairing. For example, polynucleotides that contain C-5 propyne analogues of uridine and cytidine have been shown to bind RNA with high affinity and to be potent antisense inhibitors of gene expression. Other modifications, such as modification to the phosphodiester backbone, or the 2'-hydroxy in the ribose sugar group of the RNA can also be made. The antisense polynucleotides and ribozymes can consist entirely of ribonucleotides, or can contain mixed ribonucleotides and deoxyribonucleotides. The polynucleotides of the invention may be produced by any means, including genomic preparations, cDNA preparations, *in vitro* synthesis, RT-PCR, and *in vitro* or *in vivo* transcription.

[0058] A nucleic acid molecule of the present invention, or a portion thereof, can be isolated using standard molecular biology techniques and the sequence information provided herein. For example, an apelin cDNA can be isolated from a cDNA library using all or portion of a polynucleotide sequence encoding one of the polypeptides of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5. Moreover, a nucleic acid molecule encompassing all or a portion of an apelin polypeptide can be isolated by the polymerase chain reaction (PCR) using oligonucleotide primers designed based upon the sequences provided herein. For example, mRNA can be isolated from a cell, and synthetic oligonucleotide primers for PCR amplification can be designed based upon a polynucleotide sequence encoding one of the polypeptide sequences shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:17. A nucleic acid molecule of the invention can be amplified using cDNA or, alternatively, genomic DNA, as a

template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid molecule so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to an apelin nucleotide sequence can be prepared by standard synthetic techniques, e.g., using an automated DNA synthesizer.

[0059] As used herein, the term “biologically active portion of” an apelin is intended to include a portion, e.g., a domain/motif of an apelin that participates in the interaction with APJ and/or the modulation of APJ activity. Biologically active portions of an apelin include peptides comprising amino acid sequences derived from the amino acid sequence of an apelin, e.g., an amino acid sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5, or the amino acid sequence of a polypeptide identical to an apelin, which includes fewer amino acids than a full length apelin or the full length polypeptide which is identical to an apelin polypeptide, and exhibits at least one activity of an apelin polypeptide. As also used herein, the term “biologically active portion of” APJ is intended to include a portion, e.g., a domain/motif of APJ that participates in the interaction of APJ with apelin and/or the modulation of the apelin/APJ signaling pathway. Biologically active portions of APJ include peptides comprising amino acid sequences derived from the amino acid sequence of APJ, e.g., an amino acid sequence of SEQ ID NO:17, or the amino acid sequence of a polypeptide identical to APJ, which includes fewer amino acids than a full length APJ or the full length polypeptide which is identical to an APJ polypeptide, and exhibits at least one activity of an APJ polypeptide. Typically, biologically active portions (e.g., peptides which are, for example, 5, 10, 15, 20, 30, 35, 36, 37, 38, 39, 40, or more amino acids in length) comprise a domain or motif with at least one activity of an apelin polypeptide. As used herein, the terms “apelin activity” and “APJ activity” are intended to refer to the promotion of angiogenesis or tumorigenesis or inhibition of apoptosis. As also used herein, the term “apelin/APJ signaling pathway” refers to the signal transduction pathway involving the interaction of apelin and APJ by which angiogenesis is promoted and/or apoptosis is inhibited. For the purposes of the present invention, modulation of apelin or APJ activity refers to at least a 10% increase or decrease in angiogenesis or apoptosis in the presence of a composition as compared to angiogenesis in the absence of the composition.

[0060] The invention also provides the use of apelin or APJ chimeric or fusion polypeptides. For example, as used herein, an apelin “chimeric polypeptide” or “fusion

polypeptide” comprises an apelin polypeptide or peptide operatively linked to a non-apelin polypeptide. An apelin polypeptide refers to a polypeptide having an amino acid sequence corresponding to an apelin polypeptide, whereas a non-apelin polypeptide refers to a polypeptide having an amino acid sequence corresponding to a polypeptide which is not substantially identical to the apelin polypeptide, e.g., a polypeptide that is different from the apelin and is derived from the same or a different organism. With respect to the fusion polypeptide, the term “operatively linked” is intended to indicate that the apelin polypeptide and the non-apelin polypeptide are fused to each other so that both sequences fulfill the proposed function attributed to the sequence used. The non-apelin polypeptide can be fused to the N-terminus or C-terminus of the apelin polypeptide. For example, in one embodiment, the fusion polypeptide is a GST-apelin fusion polypeptide in which the apelin sequences are fused to the C-terminus of the GST sequences. Such fusion polypeptides can facilitate the purification of recombinant apelin polypeptides. In another embodiment, the fusion polypeptide is an apelin polypeptide containing a heterologous signal sequence at its N-terminus. In certain host cells (e.g., mammalian host cells), expression and/or secretion of an apelin polypeptide can be increased through use of a heterologous signal sequence.

[0061] Preferably, an apelin or APJ chimeric or fusion polypeptide of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers that give rise to complementary overhangs between two consecutive gene fragments that can subsequently be annealed and re-amplified to generate a chimeric gene sequence (See, for example, Current Protocols in Molecular Biology, Eds. Ausubel et al. John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (e.g., a GST polypeptide). An apelin encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the apelin polypeptide.

[0062] In addition to fragments and fusion polypeptides of the apelin polypeptides described herein, the present invention includes homologs and analogs of naturally occurring apelin or APJ polypeptides and apelin or APJ encoding nucleic acids in the same or other organisms. "Homologs" are defined herein as two nucleic acids or polypeptides that have similar or "identical," nucleotide or amino acid sequences, respectively. Homologs include allelic variants, orthologs, paralogs, agonists, and antagonists of apelin or APJ as defined hereafter. The term "homolog" further encompasses nucleic acid molecules that differ from a first nucleic acid (and portions thereof) due to degeneracy of the genetic code and thus encode the same polypeptide. As used herein, a "naturally occurring" apelin or APJ polypeptide refers to an apelin or APJ amino acid sequence that occurs in nature. Preferably, a naturally occurring apelin comprises an amino acid sequence as defined in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5; and a naturally occurring APJ comprises the amino acid sequence as defined in SEQ ID NO:17.

[0063] An agonist of the apelin or APJ polypeptide can retain substantially the same, or a subset, of the biological activities of the apelin or APJ polypeptide. An antagonist of the apelin or APJ polypeptide can inhibit one or more of the activities of the naturally occurring form of the apelin or APJ polypeptide. For example, the apelin antagonist can competitively bind to a downstream or upstream member of the cell membrane component metabolic cascade that includes the apelin polypeptide.

[0064] In another embodiment of the present invention, the compositions used may inhibit or promote apelin activity indirectly. For example, the compositions may comprise a specific endopeptidase or endopeptidase inhibitor. In particular, the endopeptidase to be used belongs to the subtilisin family of serine proteases (Barr, 1991, Cell 66:1-3). These enzymes cleave specifically after arginine residues (e.g. KR, RR, KXKR, RXRR, KKRR, RRRR, KXXR, and RXXR) and are likely involved in the cleavage of apelin to the 13 amino acid and 17 amino acid peptides.

[0065] As stated above, the present invention includes apelin and APJ polypeptides and homologs thereof. To determine the percent sequence identity of two amino acid sequences (e.g., one of the sequences of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:17, and a mutant form thereof), the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of one polypeptide for optimal alignment with the other polypeptide or nucleic acid). The amino acid residues at corresponding amino acid

positions are then compared. When a position in one sequence (e.g., one of the sequences of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:17) is occupied by the same amino acid residue as the corresponding position in the other sequence (e.g., a mutant form of the sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:17), then the molecules are identical at that position. The same type of comparison can be made between two nucleic acid sequences.

[0066] The percent sequence identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., percent sequence identity = numbers of identical positions/total numbers of positions x 100). Preferably, the isolated amino acid homologs included in the present invention are at least about 50-60%, preferably at least about 60-70%, and more preferably at least about 70-75%, 75-80%, 80-85%, 85-90%, or 90-95%, and most preferably at least about 96%, 97%, 98%, 99%, or more identical to an entire amino acid sequence shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:17. In yet another embodiment, the isolated amino acid homologs included in the present invention are at least about 50-60%, preferably at least about 60-70%, and more preferably at least about 70-75%, 75-80%, 80-85%, 85-90%, or 90-95%, and most preferably at least about 96%, 97%, 98%, 99%, or more identical to an entire amino acid sequence encoded by a nucleic acid sequence shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:17. In other embodiments, the apelin or APJ amino acid homologs have sequence identity over at least 5 contiguous amino acid residues, more preferably at least 10 contiguous amino acid residues, and most preferably at least 13 contiguous amino acid residues of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:17.

[0067] In another preferred embodiment, an isolated nucleic acid homolog of the invention comprises a nucleotide sequence that is at least about 40-60%, preferably at least about 60-70%, more preferably at least about 70-75%, 75-80%, 80-85%, 85-90%, or 90-95%, and even more preferably at least about 95%, 96%, 97%, 98%, 99%, or more identical to a nucleotide sequence of the present invention, or to a portion comprising at least 39 consecutive nucleotides thereof.

[0068] For the purposes of the invention, the percent sequence identity between two nucleic acid or polypeptide sequences is determined using the Vector NTI 6.0 (PC)

software package (InforMax, 7600 Wisconsin Ave., Bethesda, MD 20814). A gap opening penalty of 15 and a gap extension penalty of 6.66 are used for determining the percent identity of two nucleic acids. A gap opening penalty of 10 and a gap extension penalty of 0.1 are used for determining the percent identity of two polypeptides. All other parameters are set at the default settings. For purposes of a multiple alignment (Clustal W algorithm), the gap opening penalty is 10, and the gap extension penalty is 0.05 with blosum62 matrix. It is to be understood that for the purposes of determining sequence identity when comparing a DNA sequence to an RNA sequence, a thymidine nucleotide is equivalent to a uracil nucleotide.

[0069] In another aspect, the invention provides an isolated nucleic acid comprising a polynucleotide that hybridizes to a polynucleotide of the present invention under stringent conditions. As used herein with regard to hybridization for DNA to a DNA blot, the term "stringent conditions" refers to hybridization overnight at 60°C in 10X Denhart's solution, 6X SSC, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA. Blots are washed sequentially at 62°C for 30 minutes each time in 3X SSC/0.1% SDS, followed by 1X SSC/0.1% SDS, and finally 0.1X SSC/0.1% SDS. As also used herein, "highly stringent conditions" refers to hybridization overnight at 65°C in 10X Denharts solution, 6X SSC, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA. Blots are washed sequentially at 65°C for 30 minutes each time in 3X SSC/0.1% SDS, followed by 1X SSC/0.1% SDS, and finally 0.1X SSC/0.1% SDS. Methods for nucleic acid hybridizations are described in Meinkoth and Wahl, 1984, *Anal. Biochem.* 138:267-284; *Current Protocols in Molecular Biology*, Chapter 2, Ausubel et al. Eds., Greene Publishing and Wiley-Interscience, New York, 1995; and Tijssen, 1993, *Laboratory Techniques in Biochemistry and Molecular Biology: Hybridization with Nucleic Acid Probes*, Part I, Chapter 2, Elsevier, New York, 1993.

[0070] Using the above-described methods, and others known to those of skill in the art, one of ordinary skill in the art can isolate homologs of the apelin or APJ polypeptides comprising amino acid sequences shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:17. One subset of these homologs is allelic variants. As used herein, the term "allelic variant" of apelin or APJ refers to a nucleotide sequence containing polymorphisms that lead to changes in the amino acid sequences of an apelin or APJ polypeptide and that exist within a natural population. Such natural allelic variations can typically result in 1-6% variance in an

apelin nucleic acid. Any and all such nucleic acid variations and resulting amino acid polymorphisms or variations in an apelin that are the result of natural allelic variation and that do not alter the functional activity of an apelin, are intended to be within the scope of the methods of the present invention.

[0071] Analogues, orthologs, and paralogs of a naturally occurring apelin or APJ polypeptide can differ from the naturally occurring apelin by post-translational modifications, by amino acid sequence differences, or by both. Post-translational modifications include *in vivo* and *in vitro* chemical derivatization of polypeptides, e.g., acetylation, carboxylation, phosphorylation, or glycosylation, and such modifications may occur during polypeptide synthesis or processing or following treatment with isolated modifying enzymes. In particular, orthologs of the invention will generally exhibit at least 80-85%, more preferably, 85-90% or 90-95%, and most preferably 95%, 96%, 97%, 98%, or even 99% identity, or 100% sequence identity, with all or part of a naturally occurring apelin or APJ amino acid sequence, and will exhibit a function similar to an apelin or APJ polypeptide. Preferably, an apelin ortholog of the present invention functions by interacting with APJ and by affecting angiogenesis or tumorigenesis.

[0072] In addition to naturally-occurring variants of an apelin or APJ sequence that may exist in the population, the skilled artisan will further appreciate that changes can be introduced by mutation into a nucleotide sequence encoding the polypeptides of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:17, thereby leading to changes in the amino acid sequence of the encoded apelin or APJ polypeptide, without altering the functional activity of the apelin or APJ. For example, nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues can be made in a sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:17. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of one of the apelin or APJ polypeptides without altering the activity of said apelin or APJ polypeptide, whereas an "essential" amino acid residue is required for apelin or APJ activity. Other amino acid residues, however, (e.g., those that are not conserved or only semi-conserved in the domain having apelin or APJ activity) may not be essential for activity and thus are likely to be amenable to alteration without altering apelin or APJ activity.

[0073] The present invention also includes polypeptides having a conservative amino acid substitution in one of the sequences of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:17. A “conservative amino acid substitution” is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine), and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in an apelin polypeptide is preferably replaced with another amino acid residue from the same side chain family. Alternatively, in another embodiment, mutations can be introduced randomly along all or part of an apelin coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for an apelin activity described herein to identify mutants that retain apelin activity.

[0074] Another aspect of the invention pertains to the use of isolated nucleic acid molecules that are antisense to an apelin coding sequence. Antisense polynucleotides are thought to inhibit gene expression of a target polynucleotide by specifically binding the target polynucleotide and interfering with transcription, splicing, transport, translation, and/or stability of the target polynucleotide. Methods are described in the prior art for targeting the antisense polynucleotide to the chromosomal DNA, to a primary RNA transcript, or to a processed mRNA. Preferably, the target regions include splice sites, translation initiation codons, translation termination codons, and other sequences within the open reading frame. In another preferred embodiment, the present invention provides that the target regions may be within the 3' UTR region, or the target regions may be in any region of the mRNA transcript.

[0075] The term “antisense,” for the purposes of the invention, refers to a nucleic acid comprising a polynucleotide that is sufficiently complementary to all or a portion of a gene, primary transcript, or processed mRNA, so as to interfere with expression of the endogenous gene. “Complementary” polynucleotides are those that are capable of base pairing according to the standard Watson-Crick complementarity

rules. Specifically, purines will base pair with pyrimidines to form a combination of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. In addition to these standard rules, with respect to RNA, guanine also may be paired with uracil (G:U) in some cases. It is understood that two polynucleotides may hybridize to each other even if they are not completely complementary to each other, provided that each has at least one region that is substantially complementary to the other. The term "antisense nucleic acid" includes single stranded RNA as well as double-stranded DNA expression cassettes that can be transcribed to produce an antisense RNA. "Active" antisense nucleic acids are antisense RNA molecules that are capable of selectively hybridizing with a primary transcript or mRNA encoding a polypeptide having at least 80% sequence identity with the polypeptide of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:17.

[0076] The antisense nucleic acid can be complementary to an entire apelin coding strand, or to only a portion thereof. In one embodiment, an antisense nucleic acid molecule is antisense to a "coding region" of the coding strand of a nucleotide sequence encoding an apelin. The term "coding region" refers to the region of the nucleotide sequence comprising codons that are translated into amino acid residues. In another embodiment, the antisense nucleic acid molecule is antisense to a "noncoding region" of the coding strand of a nucleotide sequence encoding an apelin. The term "noncoding region" refers to 5' and 3' sequences that flank the coding region that are not translated into amino acids (i.e., also referred to as 5' and 3' untranslated regions). The antisense nucleic acid molecule can be complementary to the entire coding region of apelin mRNA, but more preferably is an oligonucleotide that is antisense to only a portion of the coding or noncoding region of apelin mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of apelin mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, or more nucleotides in length. Typically, the antisense molecules of the present invention comprise an RNA having 60-100% sequence identity with at least 10 consecutive nucleotides of a polynucleotide encoding a polypeptide of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:17. Preferably, the sequence identity will be at least 70%, more preferably at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%, and most preferably 100%.

[0077] An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (e.g., an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e., RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

[0078] In yet another embodiment, the antisense nucleic acid molecule of the invention is an α -anomeric nucleic acid molecule. An α -anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gaultier et al., 1987, Nucleic Acids. Res. 15:6625-6641). The antisense nucleic acid molecule can also comprise a 2'-o-methylribonucleotide (Inoue et al., 1987, Nucleic Acids Res. 15:6131-6148) or a chimeric RNA-DNA analogue (Inoue et al., 1987, FEBS Lett. 215:327-330).

[0079] The antisense nucleic acid molecules of the invention are typically administered to a cell or generated *in situ* such that they hybridize with or bind to

cellular mRNA and/or genomic DNA encoding an apelin or APJ to thereby inhibit expression of the polypeptide, e.g., by inhibiting transcription, by inhibiting translation, and/or by causing transcript degradation. The hybridization can be by conventional nucleotide complementarity to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule which binds to DNA duplexes, through specific interactions in the major groove of the double helix. The antisense molecule can be modified such that it specifically binds to a receptor or an antigen expressed on a selected cell surface, e.g., by linking the antisense nucleic acid molecule to a peptide or an antibody which binds to a cell surface receptor or antigen. The antisense nucleic acid molecule can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong prokaryotic, viral, or eukaryotic (including plant) promoter are preferred.

[0080] As an alternative to antisense polynucleotides, ribozymes, sense polynucleotides, or double stranded RNA (dsRNA) can be used to reduce expression of an apelin or APJ polypeptide. As used herein, the term "ribozyme" refers to a catalytic RNA-based enzyme with ribonuclease activity that is capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which it has a complementary region. Ribozymes (e.g., hammerhead ribozymes described in Haselhoff and Gerlach, 1988, *Nature* 334:585-591) can be used to catalytically cleave apelin or APJ mRNA transcripts to thereby inhibit translation of apelin or APJ mRNA. A ribozyme having specificity for an apelin-encoding or APJ-encoding nucleic acid can be designed based upon the nucleotide sequence of an apelin or APJ cDNA or on the basis of a heterologous sequence to be isolated according to methods taught in this invention. For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved in an apelin-encoding mRNA. See, e.g., U.S. Patent Nos. 4,987,071 and 5,116,742 to Cech et al. Alternatively, apelin mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, e.g., Bartel, D. and Szostak, J.W., 1993, *Science* 261:1411-1418. In preferred embodiments, the ribozyme will contain a portion having at least 7, 8, 9, 10, 12, 14, 16, 18, or 20 nucleotides, and more preferably 7 or 8 nucleotides, that have 100% complementarity to a portion of the target RNA. Methods for making ribozymes are

known to those skilled in the art. See, e.g., U.S. Patent Nos. 6,025,167; 5,773,260; and 5,496,698.

[0081] Alternatively, apelin or APJ gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of an apelin or APJ nucleotide sequence (e.g., an apelin or APJ promoter and/or enhancer) to form triple helical structures that prevent transcription of an apelin gene in target cells. See generally, Helene, C., 1991, *Anticancer Drug Des.* 6(6):569-84; Helene, C. et al., 1992, *Ann. N.Y. Acad. Sci.* 660:27-36; and Maher, L.J., 1992, *Bioassays* 14(12):807-15.

[0082] In addition to the use of apelin and APJ nucleic acids and polypeptides described above, the present invention encompasses the use of these nucleic acids and polypeptides attached to a moiety. These moieties include, but are not limited to, detection moieties, hybridization moieties, purification moieties, delivery moieties, reaction moieties, binding moieties, and the like. A typical group of nucleic acids having moieties attached are probes and primers. Probes and primers typically comprise a substantially isolated oligonucleotide that hybridizes under stringent conditions to the desired nucleic acid. In preferred embodiments, the probe further comprises a label group attached thereto, e.g. the label group can be a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor.

[0083] In certain preferred embodiments of the present invention, the methods further comprise administering to the patient a therapeutically effective amount of an anti-cancer agent, wherein the anti-cancer agent is selected from the group consisting of a chemotherapeutic agent, a radiotherapeutic agent, an anti-angiogenic agent, an apoptosis-inducing agent. In one embodiment, the anti-cancer agent is an anti-angiogenic agent that inhibits the expression or activity of an angiogenic factor selected from the group consisting of VEGFs, FGFs, PDGFB, EGF, LPA, HGF, PD-ECF, IL-8, angiogenin, TNF-alpha, TGF-beta, TGF-alpha, proliferin, and PLGF.

[0084] Another aspect of the invention pertains to the use of isolated apelin polypeptides, and biologically active portions thereof. An "isolated" or "purified" polypeptide or biologically active portion thereof is free of some of the cellular material when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of apelin in which the polypeptide is separated from some of the cellular components of the cells in which it is naturally or recombinantly produced. In one embodiment, the language "substantially free of cellular material"

includes preparations of an apelin having less than about 30% (by dry weight) of non-apelin material (also referred to herein as a “contaminating polypeptide”), more preferably less than about 20% of non-apelin material, still more preferably less than about 10% of non-apelin material, and most preferably less than about 5% non-apelin material.

[0085] When the apelin or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, i.e., culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the polypeptide preparation. The language “substantially free of chemical precursors or other chemicals” includes preparations of apelin polypeptide in which the polypeptide is separated from chemical precursors or other chemicals that are involved in the synthesis of the polypeptide. In one embodiment, the language “substantially free of chemical precursors or other chemicals” includes preparations of an apelin polypeptide having less than about 30% (by dry weight) of chemical precursors or other chemicals, more preferably less than about 20% chemical precursors or other chemicals, still more preferably less than about 10% chemical precursors or other chemicals, and most preferably less than about 5% chemical precursors or other chemicals. In preferred embodiments, isolated polypeptides, or biologically active portions thereof, lack contaminating polypeptides from the same organism from which the apelin is derived.

[0086] The present invention also provides the use of antibodies that specifically bind to an apelin polypeptide, or a portion thereof. Antibodies can be made by many well-known methods (See, e.g., Harlow and Lane, “Antibodies; A Laboratory Manual,” Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1988)). Briefly, purified antigen can be injected into an animal in an amount and in intervals sufficient to elicit an immune response. Antibodies can either be purified directly, or spleen cells can be obtained from the animal. The cells can then fused with an immortal cell line and screened for antibody secretion. The antibodies can be used to screen nucleic acid clone libraries for cells secreting the antigen. Those positive clones can then be sequenced. (See, for example, Kelly et al., 1992, Bio/Technology 10:163-167; Bebbington et al., 1992, Bio/Technology 10:169-175).

[0087] The phrases “selectively binds” and “specifically binds” with the polypeptide refer to a binding reaction that is determinative of the presence of the polypeptide in a heterogeneous population of polypeptides and other biologics. Thus,

under designated immunoassay conditions, the specified antibodies bound to a particular polypeptide do not bind in a significant amount to other polypeptides present in the sample. Selective binding of an antibody under such conditions may require an antibody that is selected for its specificity for a particular polypeptide. A variety of immunoassay formats may be used to select antibodies that selectively bind with a particular polypeptide. For example, solid-phase ELISA immunoassays are routinely used to select antibodies selectively immunoreactive with a polypeptide. See Harlow and Lane, "Antibodies, A Laboratory Manual" Cold Spring Harbor Publications, New York, (1988), for a description of immunoassay formats and conditions that could be used to determine selective binding.

[0088] In some instances, it is desirable to prepare monoclonal antibodies from various hosts. A description of techniques for preparing such monoclonal antibodies may be found in Stites et al., eds., "Basic and Clinical Immunology," (Lange Medical Publications, Los Altos, Calif., Fourth Edition) and references cited therein, and in Harlow and Lane "Antibodies, A Laboratory Manual" Cold Spring Harbor Publications, New York, 1988.

[0089] The compositions of this invention further comprises a pharmaceutically acceptable carrier. The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic, or other untoward reaction when administered to an animal, or a human, as appropriate. Veterinary uses are equally included within the invention and "pharmaceutically acceptable" formulations include formulations for both clinical and/or veterinary use. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial, and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. For human administration, preparations should meet sterility, pyrogenicity, and general safety and purity standards as required by FDA Office of Biologics standards. Supplementary active ingredients can also be incorporated into the compositions.

[0090] As used herein with respect to these methods, the term "administering" refers to various means of introducing a composition into a cell or into a patient. These means are well known in the art and may include, for example, injection; tablets, pills,

capsules, or other solids for oral administration; nasal solutions or sprays; aerosols, inhalants; topical formulations; liposomal forms; and the like. As used herein, the term "effective amount" refers to an amount that will result in the desired result and may readily be determined by one of ordinary skill in the art.

[0091] The compositions of the present invention (e.g. angiogenesis-inhibiting, angiogenesis-promoting, and tumorigenesis-inhibiting) may be formulated for various means of administration. As used herein, the term "route" of administration is intended to include, but is not limited to subcutaneous injection, intravenous injection, intraocular injection, intradermal injection, intramuscular injection, intraperitoneal injection, intratracheal administration, epidural administration, inhalation, intranasal administration, oral administration, sublingual administration, buccal administration, rectal administration, vaginal administration, and topical administration. The preparation of an aqueous composition that contains such an apelin peptide, anti-apelin antibody or antibody fragment, anti-APJ antibody or antibody fragment, apelin or APJ antisense nucleic acid, apelin receptor decoy, ribozyme, sense polynucleotide, double stranded RNA, RNAi, aptamer, or small molecule agonist, as an active ingredient will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and the preparations can also be emulsified.

[0092] The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form should be sterile and fluid to the extent that syringability exists. It should be stable under the conditions of manufacture and storage and should be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

[0093] The compositions of the present invention (e.g. angiogenesis-inhibiting, angiogenesis-promoting, tumorigenesis-inhibiting, apoptosis-promoting, or apoptosis-inhibiting) can be formulated into a sterile aqueous composition in a neutral or salt form. Solutions as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free

amino groups of the protein), and those that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, trifluoroacetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine, and the like.

[0094] Suitable carriers include solvents and dispersion media containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. In many cases, it will be preferable to include isotonic agents, for example, sugars, or sodium chloride. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and/or by the use of surfactants.

[0095] Under ordinary conditions of storage and use, all such preparations should contain a preservative to prevent the growth of microorganisms. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate, and gelatin.

[0096] Prior to or upon formulation, the compositions of the present invention (e.g. angiogenesis-inhibiting, angiogenesis-promoting, tumorigenesis-inhibiting, apoptosis-promoting, or apoptosis-inhibiting) should be extensively dialyzed to remove undesired small molecular weight molecules, and/or lyophilized for more ready formulation into a desired vehicle, where appropriate. Sterile injectable solutions are prepared by incorporating the active agents in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as desired, followed by filter sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle that contains the basic dispersion medium and the required other ingredients from those enumerated above.

[0097] In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques that yield a powder of the active ingredient, plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0098] Suitable pharmaceutical compositions in accordance with the invention will generally include an amount of the active ingredient (e.g. the apelin peptide, anti-apelin antibody or antibody fragment, anti-APJ antibody or antibody fragment, apelin or APJ antisense nucleic acid, apelin receptor decoy, ribozyme, sense polynucleotide, double stranded RNA, RNAi, aptamer, or small molecule agonist) admixed with an acceptable pharmaceutical diluent or excipient, such as a sterile aqueous solution, to give a range of final concentrations, depending on the intended use. The techniques of preparation are generally well known in the art as exemplified by Remington's Pharmaceutical Sciences, 16th Ed. Mack Publishing Company, 1980, incorporated herein by reference. It should be appreciated that for human administration, preparations should meet sterility, pyrogenicity, and general safety and purity standards as required by FDA Office of Biological Standards.

[0099] In one embodiment, the present invention provides methods of screening for a modulator of angiogenesis or apoptosis, comprising providing an angiogenesis promoting or apoptosis inhibiting composition comprising apelin; combining a putative modulator of angiogenesis or apoptosis with the composition; introducing the composition or the combination of the putative modulator and the composition to an angiogenesis or apoptosis predictive model; and comparing the amount of vascular branching or intact cells in the model in the presence and absence of the putative modulator. In a preferred embodiment, the composition comprises a polypeptide or peptide selected from the group consisting of: a polypeptide as defined in SEQ ID NO:1; a polypeptide as defined in SEQ ID NO:2; a polypeptide as defined in SEQ ID NO:3; a polypeptide as defined in SEQ ID NO:4; a polypeptide as defined in SEQ ID NO:5; and a polypeptide having at least 80% sequence identity with a polypeptide or peptide of a) through c) above. In another preferred embodiment of the present invention, the angiogenesis predictive model is a chicken chorioallantoic membrane (CAM) assay.

[00100] In other embodiments, the present invention provides methods of screening therapeutic candidate molecules for an ability to modulate APJ-mediated angiogenesis or apoptosis, comprising combining a therapeutic candidate molecule with an APJ activation response indicator system and comparing the APJ response in the absence of the therapeutic candidate. The response can also be compared in the presence and absence of apelin or APJ as defined above. For example, in a high throughput response indicator system known in the art as Fluorometric Imaging Plate

Reader (FLIPR; Molecular Devices Corp.), the identification of functional agonists and antagonists for APJ can be observed by monitoring intracellular calcium mobilization. FLIPR allows APJ-induced calcium responses to be accurately and reliably quantitated in an entire 96 or greater well plate. With a CCD camera, FLIPR collects data at rate sufficient to follow the magnitude and time course of APJ activation in each well. Such methods can also be performed with the assistance of automated combinatorial chemistry techniques well known in the art for the identification of optimally effective therapeutic candidate molecules having similar chemical structures.

[00101] The invention further provides methods of screening therapeutic candidate molecules for an ability to modulate angiogenesis or apoptosis, comprising combining a therapeutic candidate molecule with an angiogenesis or apoptosis predictive model; and comparing the amount of vascular branching or intact cells in the model in the presence and absence of apelin or APJ as defined above.

[00102] In other embodiments, the present invention provides methods of determining a prognosis or a diagnosis of a patient by detecting a modulation of apelin expression in a diseased tissue sample of a patient, such as a tumor biopsy, as compared to apelin expression in a healthy tissue sample of the patient. For example, upregulation of apelin in the diseased tissue sample as compared to normal tissue sample indicates a diagnosis that apelin inhibition of the diseased tissue can provide a therapeutic effect for the patient.

[00103] In other embodiments, the present invention provides methods of determining a prognosis or a diagnosis of a patient by combining a diseased living tissue sample of the patient, such as a tumor biopsy, with a modulator of apelin activity or APJ activity; and comparing the amount of vascular branching or intact cells in the sample with a healthy living tissue sample from another patient of known prognosis or diagnosis. Such methods can also be performed in comparison to samples in the presence or absence of more than one type of modulator of apelin activity or APJ activity, or other therapeutic agents generally.

[00104] Throughout this application, various publications are referenced. The disclosures of all of these publications and those references cited within those publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

[00105] It should also be understood that the foregoing relates to preferred embodiments of the present invention and that numerous changes may be made therein

without departing from the scope of the invention. The invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof, which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

EXAMPLES

EXAMPLE 1

PCR Characterization of Apelin Expression and APJ Expression Distribution

[00106] Total RNA was isolated from mouse yolk sac tissue (positive control), mouse thoracic aorta (aorta), and a mouse brain endothelial cell line (bend.3) using a guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987, Anal. Biochem. 162:156-159). cDNA was prepared from these tissues using standard methods, and RT-PCR amplification of APJ sequences using APJ specific primers was performed for 35 cycles.

[00107] As shown on the left hand panel of Figure 3A, APJ transcripts are present in aorta tissue of the adult mouse. The PCR product was isolated and the presence of APJ sequences were confirmed by DNA sequencing using standard methods. The right hand panel of Figure 3A shows the results of an RT-PCR detection of APJ sequences and shows the presence of APJ transcripts in bend.3 cells (together with aorta and positive control samples).

[00108] The same cDNA samples used for detection of APJ sequences (above) were used as template for detection of apelin sequences. Four different primer pairs were used in this experiment, and all amplifications were carried out for 35 cycles. Note that the mouse aorta sample contained no detectable apelin sequences. In contrast, the bend.3 mouse endothelial cell line is positive for apelin sequences (indicated by asterisks). This result indicates that the bend.3 cell line expresses both apelin and the apelin receptor, APJ.

EXAMPLE 2

In situ Hybridization Analysis of Apelin Expression and APJ Expression Distribution

[00109] *In situ* hybridization was performed on frog embryos, using an antisense probe directed against APJ transcripts.

Whole Mount *In Situ* Hybridization

[00110] *In situ* hybridization was performed by a modified version of the procedure described in Harland, R.M., 1991, Methods in Cell Biology 36:685-695 (modified version designed by Rob Garriock). In all steps of this protocol, embryos were stored in 4 ml glass screw capped tubes.

[00111] Embryos were fixed in a 4% PFA solution (25 ml of 8% PFA stock solution (filtered) in 1x PBS). Embryos or embryo bits were placed in an abundant volume of fixative and rocked gently for 2 hours at room temperature or overnight at 4°C. The fixative was replaced with methanol which had been cooled to -20°C. The embryos can then be stored for several months at -20°C.

[00112] Frozen embryos were rehydrated for staining through a series of washes. First, the embryos are washed for 2 minutes in 2 ml of a 75% Methanol:25% H₂O solution followed by a 2 minute wash in 2 ml of a 50% Methanol:50% H₂O solution. The embryos were then washed two times for 2 minutes each with TTW (200 mM NaCl, 50 mM Tris, pH. 7.4, 0.1% Tween-20). During the washes, the tubes were kept upright and rocked gently.

[00113] The embryos were placed in a vial with 5 µg/ml Proteinase K (1µl of 25 mg/ml stock solution in 5 ml of TTW) for 5-30 minutes on a shaker or nutator. For embryos at Stages 10-20, this incubation was for 5 minutes. For embryos at Stages 20-34, this incubation was for 10 minutes; and for embryos at Stages 35-41, this incubation was for 15-30 minutes. Following the Proteinase K treatment, the embryos were washed once in TTW for 5 minutes. The embryos were then re-fixed in 1-2 ml PFA-fix (4% PFA in PBS) for 20 minutes at room temperature (or longer at 4°C). Then the embryos were washed three times in TTW for 5 minutes each time to remove the PFA-fix.

[00114] The embryos were prehybridized by incubating them in 2 ml of RNA Hybridization buffer (50% formamide, 5X SSC, 1 mg/ml Yeast RNA, 1X Denhart's, 0.1% Tween-20, 5 mM EDTA) at 65°C for 1 hour. After prehybridization, 500µl - 1ml of probe was added, and the embryos were incubated for 4 hours to overnight. Excess probe was removed through a series of washes. First, the embryos were washed twice for 20 minutes each time in approximately 2 ml 2X SSC at 37°C. Then, the embryos were washed once for 30 minutes in 2 ml 2X SSC with 1µl/ 5ml of RNase cocktail (Ambion Cat#2286= 1mg/ml RNase A, 20,000 U/ml RNase T1). Finally, the

embryos were washed 2-3 times for 1 hour each time in a preheated 0.2X SSC + 0.01% Tween-20 solution at 65°C.

[00115] The embryos were then incubated once for 20 minutes in 1-2 ml Maleic acid buffer (MAB buffer-5.8 g Maleic acid and 4.4 g NaCl in water for a total volume of 500 ml, pH 7.5) with 1-2% B&M Blocking reagent. At the same time, 0.5-1 µl of anti-Dig antibody was preblocked by adding the antibody to 5 ml of MAB-block and incubating for 20 minutes. Then the embryos were incubated 3 hours (at room temperature) or overnight (at 4°C) in alkaline phosphatase-conjugated anti-digoxigenin antibody (1/5,000-10,000 dilution in MAB block (1 ml)). The antibody solution was removed, and the tubes were filled completely with MAB and rocked on a nutator on their sides. Alternatively, all washing steps could be performed without rocking. Two short washes were performed at room temperature before an overnight wash at 4°C, followed by 6 more washes (30 minutes each). In some instances, the embryos were washed 10 times (30 minutes each) at room temperature. An additional overnight wash in MAB will produce cleaner *in situ* results.

[00116] The embryos were then incubated for 10 minutes in fresh alkaline phosphatase buffer (APB)(100 mM Tris, pH 9.5, 50 mM MgCl₂, 100 mM NaCl, 0.1% Tween-20) at room temperature. Alternatively, Levamisol was added to buffer just before use to a final concentration of 2 mM. The APB was exchanged with 1-2 ml alkaline phosphatase reaction buffer containing APB and nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP). For this reaction buffer, 2-4µl NBT (75 mg/ml stock in 70% dimethyl formamide) and 2-4µl BCIP (75 mg/ml stock in 100% dimethyl formamide) were added per 1 ml APB.

[00117] When the staining appears optimal, the following procedure was used for stopping the reaction, which tended to reduce background, especially ectodermal background, without any adverse effects on the staining intensity. By comparison to the procedure described below, the direct addition of Bouin's fixative (1 g Picric acid in 70 ml water or 70 ml of a 1.3% saturated solution, 25 ml of 37% formaldehyde, 5 ml of glacial acetic acid) caused a precipitate to form around the embryos which is hard to remove. The stained embryos were incubated for 1 hour in 100% Methanol to remove nonspecific staining. Then the embryos were washed for 2 minutes in 75% Methanol, followed by 2 minutes in 50% Methanol, and finally two washes for 5 minutes each in TTW. The embryos were fixed overnight in MEMPFA, and optionally with Bouin's fixative for yellow counterstain. MEMPFA fixed embryos were then washed in TTW,

and viewed and stored in 50% glycerol. Alternatively, glycerol stored embryos could be paraffin sectioned by washing a few times in TTW and dehydrating in ethanol. Bouin's fixative stops the reaction completely and gave a nice yellow background stain which contrasts well with the blue color reaction, but these embryos did not section well.

[00118] Before viewing and storing, the embryos were washed for 5 minutes in 25% Methanol, followed by 5 minutes in 50% Methanol, 5 minutes in 75% Methanol, before placing into 100% Methanol. If pigmented embryos were used, the pigmentation was reduced by exposing embryos to sunlight or other strong light source in a peroxide/formamide solution in methanol (1 ml of 30% H₂O₂, 10% Formamide, 70% Methanol). These embryos were bleached in the black screw tops placed on a tinfoil wrapped petri dish in order that the sunlight reflected back through the embryo and caused bleaching of both sides. Indoor lighting sources could also be used, however, it would take longer to bleach the embryo.

[00119] To clear embryos, embryos were transferred to a glass vial, and the solution was replaced with methanol with one part benzyl alcohol:two parts benzyl benzoate (BABB). The staining appears to fade in BABB especially when the embryos are left on a lit microscope so transfer back to methanol after viewing. This is ideal for deep staining but not very good for surface staining. Embryos were partially cleared in a 50-70% glycerol solution and easily positioned. Embryos must first be in buffer before transferring them into glycerol and must sit in glycerol for sometime until they equilibrated into the solution. Embryos could also be stored permanently in glycerol at 4°C or -20°C. Uncleared embryos were photographed in a Methanol/Ethanol solution or straight TTW or PBS buffer and returned to 100% Methanol.

[00120] From glycerol, BABB, or Methanol, embryos were transferred into a 100% Ethanol solution. If the embryos were stored in glycerol, they were first washed a few times in TTW to remove the glycerol before transferring to the 100% Ethanol solution. If the embryos were in BABB, they were first washed a few times in Ethanol to remove the BABB before transferring to the 100% Ethanol solution. If the embryos were in Methanol, they were transferred directly to the 100% Ethanol solution. The Ethanol was decanted from the dehydrated embryos and exchanged for Xylene. The embryos were incubated twice for 5 minutes each time in Xylene. Then the Xylene was removed and replaced with molten paraplast. The embryos were incubated twice

for 30-60 minutes each time in the molten paraplast. Finally, the embryos were mounted in fresh paraplast.

[00121] Figures 4, 5, and 13 show the results of *in situ* hybridization experiments staining for apelin and APJ in the frog embryo. *In situ* hybridization experiments with E11.5 day mouse embryos (11.5 days after fertilization) also were conducted, and the results are shown in Figures 14 and 15. These data demonstrate that apelin and APJ are both expressed in the developing vasculature, that apelin and APJ are coexpressed to some extent in the same tissues, and that apelin appears to be expressed at the tips of angiogenic blood vessels.

EXAMPLE 3

Analysis of the Effect of Apelin on Vascular Growth

Effect of Apelin-Soaked Beads

[00122] The apelin 13-mer C-terminal peptide (SEQ ID NO:4), which shows 100% sequence identity between human and frog, was synthesized to greater than 90% purity by Sigma-Genosys. The N-terminal glutamine was modified to a pyroglutamic acid residue to avoid cyclization and poor yield during the manufacturing process. A mutant control apelin peptide was also prepared in which the last four residues were substituted with alanine residues.

[00123] Affi-gel blue beads (Bio-Rad, 50-75 μ m diameter) were soaked in a solution of apelin peptide (0.1 mg/ml), mutated apelin peptide (0.25 mg/ml), BSA (Sigma, 1 mg/ml), or rm VEGF-164 (R&D Systems, 0.25 mg/ml) for 1 hour on ice. Beads were microsurgically implanted in an avascular region, the posterior lateral mesoderm, of stage 24-26 embryos. Embryos were cultured in 0.2X MMR until stage 35-37, at which time they were prepared for whole mount *in situ* hybridization analysis as described in Example 2. The embryonic vascular system was visualized using an antisense probe directed against transcripts encoding the *Xenopus* vascular marker, *erg* (Baltzinger et al., 1999, Dev. Dyn. 216:420-33).

[00124] The photomicrographs of the *in situ* hybridization show that there is outgrowth of developing blood vessels toward the apelin-soaked beads (Figures 6 and 16). The photomicrographs of the *in situ* hybridization using VEGF-soaked beads show that there is a nearly identical outgrowth of developing blood vessels as was shown with the apelin-soaked beads (data not shown). Beads containing only the

mutated apelin peptide or BSA produced no visible effects of BSA or the mutated apelin peptide on vascular outgrowth (data not shown).

[00125] Experiments also were performed in which the frog embryo was stained for VEGF mRNA expression (Figure 16, Panel B). These data show no upregulation of VEGF expression in the vicinity of the bead, suggesting that the stimulation of angiogenic growth by apelin does not proceed via VEGF expression.

Chick Chorioallantoic Membrane (CAM) Assay

[00126] Chicken eggs were incubated at 37°C in a humidified chamber. On day 10 of development, a small window was made in the outer shell of the egg, and the CAM was released from its attachment to the inner shell membrane. A larger window was then made in the egg to allow for addition of the filter paper containing growth factor. Filter discs (3MM Whatman) of 7-8 mm diameter were pretreated with 10 µl of 0.1% cortisone acetate (Sigma) solution to avoid inflammation of the CAM. The filter discs were air dried prior to application of growth factors. Then 50 ng of apelin-13 or VEGF in 10 µl volume was absorbed into the filter discs; 10 µl of PBS absorbed to the discs was used as a negative control. After air-drying, the filter discs were placed on the CAM, and eggs were returned to the incubator for three days. Filter discs and the attached CAM were then excised, washed with PBS, trimmed to the size of the disc, and photographed for quantitative analysis (Figure 7A and 7B). The number of vessel branch points was determined using a blind protocol. The results are shown as the mean of three independent experiments +/- s.e.m. (Figure 7C).

[00127] These data show that blood vessel formation and leakage of blood vessels both increased in the CAM exposed to apelin-13 (Figure 7B), as compared to the CAM exposed to the control PBS (Figure 7A), demonstrating the angiogenic effect of apelin-13 in this assay. It is notable that apelin demonstrated a very similar effect to VEGF, as an angiogenic factor and/or as a vascular permeability factor, based on the results of this CAM assay.

EXAMPLE 4

Effect of Apelin on Vascular Proliferation and Migration

[00128] Bovine aortic endothelial cells (BAE) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. For generation of stable cell lines expressing APJ, the mouse APJ coding region was cloned into the pcDNA 3.1 vector downstream of the CMV promoter, in frame with a

myc epitope at the C-terminal end of the protein, and upstream of the neomycin gene. Bovine aortic endothelial (BAE) cells were transfected with this construction using the Superfect transfection kit (Qiagen) and selected for resistance to G418 (600 µg/ml). Colonies were isolated and screened using RT-PCR for expression of mouse APJ and also by immunocytochemistry for expression of the myc epitope. A total of 14 clones were found to express mouse APJ, and one of these (BAE/APJ#2) was used for both the proliferation and migration assays.

[00129] BAE/APJ#2 cells plated at 70% confluence in 8 well culture slides (VWR) were serum starved for 48 hours and then stimulated with apelin (10 ng/ml), VEGF (10 ng/ml) or FGF-2 (10 ng/ml) in DMEM, or DMEM alone, for 24 hours. Proliferation inhibition experiments were carried out using the VEGF pathway inhibitor SU1498 (Sigma) which blocks phosphorylation of the VEGFR2 receptor. SU1498 was included in proliferation cultures at a concentration of 10 ng/ml and results were assayed using BrdU incorporation. After growth factor incubation, BrdU was added to each well at a final concentration of 10 µM for 2 hours, cells were washed, and fixed for BrdU immunocytochemistry.

[00130] Cells were fixed in 100% ice cold methanol at 4°C for 2 hours to overnight. Prior to staining, cells were air dried and rehydrated for 3 minutes with PBS buffer. DNA was denatured at 37°C with 2 M HCl, and neutralized with 2 washes for 5 minutes in 0.1 M borate buffer (pH 8.5). Cells were washed 3 times for 5 minutes each with PBS and blocked for 30 minutes in 1% normal goat serum/2% BSA. FITC-labeled anti-BrdU antibody (5 µg/ml) in block was added for 1 hour at room temperature. Cells were counterstained with propidium iodide following several washes in PBS buffer. For each experiment, 4 random fields were photographed for determination of percentage BrdU labeling. The results presented in Figure 8B are the mean of 3 independent experiments, +/- S.E.M. These data demonstrate that apelin promotes endothelial cell proliferation to a similar extent as VEGF, and suggest that VEGF and apelin initiate this proliferation through a different pathway, as apelin was still able to promote proliferation in the presence of the VEGF inhibitor.

[00131] Cell migration assays were performed using Transwell cell culture migration chambers (Becton Dickinson) with 8 micron pore size. BAE/APJ#2 cells were plated in serum free conditions in 100 µl at a concentration of 1×10^5 ml in the upper chamber of the migration chamber. Following cell attachment to the membrane,

10 ng/ml of growth factor was added to the bottom chamber, in a total volume of 0.5 ml. Cells were stimulated with growth factor in DMEM for 16 hours, or DMEM alone, at which time the cells remaining in the upper chamber were removed using a cotton tip applicator. Following a brief wash with PBS the remaining cells were fixed in 3.7% formaldehyde at 4°C for 1 hour. Cells attached to the membrane in the lower chamber were stained with DAPI, and 3 random fields were counted for each experimental condition. The results presented in Figure 8A are the mean of 3 independent experiments, +/- S.E.M. These data demonstrate that apelin promotes endothelial cell migration.

[00132] Similar cellular proliferation assays were performed using mouse endothelial cells (bEnd3) that constitutively express APJ. The cells were stimulated with apelin (10 ng/ml) or VEGF (50 ng/ml) in serum, or serum alone. The results are shown in Figure 17, demonstrating that apelin induces the cellular proliferation of endothelial cells. Proliferation inhibition experiments with the bEnd3 cells also were carried out using the VEGF pathway inhibitor SU1498 (Sigma) which blocks phosphorylation of the VEGFR2 receptor (Figure 18). SU1498 was included in proliferation cultures at a 25 μ M concentration, and results were assayed using BrdU incorporation. The graph shows the mean of three separate experiments that were performed in triplicate. Interestingly, these data suggest that apelin-stimulated endothelial cell proliferation is independent of VEGF activity.

[00133] Proliferation inhibition experiments with the bEnd3 cells also were carried out using FGF (50 ng/ml) or the FGF pathway inhibitor SU5402 (Figure 19). SU5402 was included in proliferation cultures at a 20 μ M concentration, and results were assayed using BrdU incorporation. The graph shows the mean of two separate experiments that were performed in triplicate. The FGF pathway inhibitor SU5402 partially inhibited the proliferative effect of FGF, but did not have a significant effect on apelin-stimulated cellular proliferation. These data suggest that apelin-stimulated endothelial cell proliferation is independent of FGF activity as well.

[00134] Cell migration assays were performed using the bEnd3 cells in Transwell cell culture migration chambers (Figure 20). The cells were plated in serum free medium in the upper chamber of the migration chamber. Following cell attachment to the membrane, control PBS (Panel A), 10 ng/ml of apelin (Panel B), or 50 ng/ml of VEGF (Panel C) was added to the bottom chamber. The cells that migrated to the bottom chamber were visualized by DAPI stain of the nuclei of the cells

(Panels A, B, and C). The graph in Panel D summarizes these results, indicating that apelin acts as chemotactic agent for the endothelial cells to the same extent as VEGF.

EXAMPLE 5

Effect of Antisense Nucleic Acids on Vascular Growth

[00135] Loss-of-function experiments were performed using antisense morpholino oligonucleotides (MOs) (Gene Tools, Philomath, OR), and the results are shown in Figure 9. All morpholino oligonucleotides were designed based on recommendations from Gene Tools. Two distinct antisense oligonucleotides overlapping the initiation ATG were designed to block translation from both *Xenopus laevis* pseudoallelic copies of the apelin transcript.

[00136] The primers used were as follows:

ap1 5'-GTGCCCAAAGTCTGAGATTCATGTT-3' (SEQ ID NO:6) and

ap2 5'-GATTCATGTTTCTTGTGGCTGAGTG-3' (SEQ ID NO:7).

[00137] A 5 base pair mismatch control morpholino oligonucleotide (ap2mm) was designed for the ap2 morpholino oligonucleotide:

ap2mm 5'-GATTgATcTTTgTTGTGcCTcAGTG-3' (SEQ ID NO:8); the mismatch bases are represented in lowercase type.

[00138] One antisense morpholino oligonucleotide, designed to block translation from both copies of the APJ transcript, was targeted immediately upstream of the initiation ATG:

apj 5'-AAGGCTGTGTGGAAGCAATAGAAAG-3' (SEQ ID NO:9).

[00139] The 5 base pair mismatch control sequence for the apj morpholino oligonucleotide (apjmm) is:

apjmm 5'-AAGcCTcTGTGcAAcCAATAcAAAG-3' (SEQ ID NO:10); the mismatch bases indicated in lowercase.

[00140] Morpholino oligonucleotides were reconstituted in 50mM HEPES buffer (pH 8.0) and injected into the frog embryo. The ap1 MO was injected at 15 ng per embryo, and the ap2 and apj MOs were injected at 7.5 ng. Mismatch controls, ap2mm and apjmm, were used at twice the experimental dose (15 ng). The MOs were injected into one cell of a 2 cell embryo together with Texas Red Dextran (10 ng; Molecular Probes) as a lineage tracer. Embryos were grown to stage 35 when they were assayed by *in situ* hybridization using the vascular marker, *erg*.

[00141] The ap1 morpholino injections resulted in an inhibition of angiogenic growth of embryonic blood vessels in 67% of the embryos (N=76)(Figure 9B). This decrease in vascularization was clearly evidenced by the lack of the intersomitic vessels which develop by an angiogenic mechanism. The ap2 morpholino, which overlaps the sequence of the ap1 morpholino, was used to confirm the effect of apelin on angiogenic growth in the embryos. Using half the concentration (7.5 ng), the ap2 morpholino still resulted in the loss of intersomitic vessel formation in 63% of the embryos (N=64)(data not shown). Similarly, 7.5 ng of an antisense morpholino to the APJ receptor resulted in a decrease in angiogenic growth of blood vessels in 71% of the embryos (N=48)(data not shown). By contrast, 15 ng of either the ap2mm or apjmm mismatch control morpholinos did not produce a detectable alteration in the vascular growth of the embryos (data not shown). These data clearly demonstrate that inhibition of apelin results in an inhibition of angiogenic growth of embryonic blood vessels.

EXAMPLE 6

Apelin Expression in Human Tumors

[00142] A dot blot membrane carrying cDNA prepared from 154 human tumors was obtained from BD Biosciences (San Jose, CA). Each tumor sample was accompanied by an adjacent non-tumor tissue from the same individual. An approximately 2 kb fragment of the human apelin sequence was labeled with ^{32}P by the Random Priming method, using a standard protocol (Feinberg and Vogelstein, 1984). The ^{32}P -labeled probe was hybridized overnight with the dot blot membrane in a hybridization solution provided by BD with the membrane (BD ExpressHybTM Hybridization solution). After hybridization, the membrane was washed with prewarmed Wash Solution I (2X SSC, 0.5% SDS) for 30 minutes at 68°C, followed by two additional washes for 30 minutes each in Wash Solution I at 68°C. The membrane was then washed two times in prewarmed Wash Solution II (0.2X SSC, 0.5% SDS) for 30 minutes each at 68°C. The membrane was then wrapped in plastic wrap and exposed to X-ray film, in the presence of an intensifying screen at -80°C for 17 hours. Apelin expression was increased in approximately one third of the 154 tumor samples, relative to adjacent non-tumor tissue (60 out of 154 samples)(Figure 10A).

[00143] The apelin probe was stripped from the membrane by washing in a boiling 0.5% SDS solution for 30-40 minutes. Then the membrane was screened with a ^{32}P -labeled probe for human VEGF-A (approximately 700 base fragment), a positive

control for expression of a known angiogenic agent. Hybridization and washing conditions were the same as with the apelin probe. VEGF mRNA expression also was upregulated in approximately one third of the human tumor samples, relative to the non-tumor tissue sample controls (59 out of 154 samples)(Figure 10B).

[00144] Similar results were obtained with a second membrane obtained from BD Biosciences (carrying the cDNA prepared from the 154 human tumors and non-tumor tissue controls) that was probed first with the ³²P-labeled apelin probe, then stripped and probed with the ³²P-labeled VEGF-A probe (data not shown). Both apelin and VEGF were up-regulated in approximately 29 of the tumor samples. These data demonstrate that the expression of apelin is upregulated in a significant portion of human tumors, relative to non-tumor tissue, consistent with a role for apelin in promotion of tumorigenesis.

EXAMPLE 7

Hypoxia-Induced Regulation of Apelin Expression

[00145] Under hypoxic conditions, the expression of various genes is regulated by a transcription factor denoted Hypoxia-Induced Factor-1 alpha (HIF-1 α). During tumor growth, it is likely that there are periods of hypoxia which act to stimulate new blood vessel growth. Because apelin has been shown here to be involved in promoting angiogenesis, the sequence of the human, mouse, and zebrafish apelin genes and their surrounding sequences were analyzed to determine if apelin expression may be regulated by HIF-1 α . The consensus sequence for an HIF-1 α recognition site is BACGTGK (SEQ ID NO:11). In this consensus sequence the B denotes a C, G, or T nucleotide, but not an A nucleotide; the K denotes a G or T nucleotide.

[00146] Figure 11 shows that two to four HIF-1 α recognition sites were present in the apelin regulatory regions. All sites mapped had a confidence value of 0.88 or greater, according to VISTA transcription factor binding site analysis software. The human apelin regions contained an HIF-1 α site (GAGACGTGGA (SEQ ID NO:12); VISTA confidence value = 0.899) located at -4.5 kb (4.5 kb upstream from the transcription initiation site) and three sites within intron 1 (located at approximately +713 bp, +2.4 kb, and +5.4 kb downstream of the transcription initiation site). The relative sequences and confidence values of these three sites are CAGACGTGACA (SEQ ID NO:13; VISTA confidence value = 0.964), TGTACGTGG (SEQ ID NO:14;

VISTA confidence value = 0.964), and AATGACGTGATG (SEQ ID NO:15; VISTA confidence value = 0.916), respectively.

[00147] Similarly, the mouse apelin regulatory region contains two HIF-1 α recognition sites at -4.3 kb and +916 bp with respect to the transcription initiation site. The zebrafish regulatory region contains four HIF-1 α recognition sites at approximately -3.4 kb, +1 kb, +1.4 kb, and +2.3 kb with respect to the transcription initiation site.

[00148] The VEGF-A gene also has been shown to contain two HIF-1 α consensus sites, however, only the site at approximately -975 bp (TACGTGGG (SEQ ID NO:16) is required for the hypoxia response. Therefore, it is possible that one or more of the consensus sequences in the apelin regulatory region may not be necessary for a hypoxia response.

[00149] Because HIF-1 α consensus sites are present in the apelin regulatory region, experiments were conducted to determine whether apelin expression is hypoxia responsive and regulated by HIF-1 α . Upregulation of apelin expression under hypoxic conditions would be consistent with a role for apelin in promotion of tumor angiogenesis. Preliminary experiments were carried out using primary rat cardiomyocyte cells in culture and treatment with cobalt chloride. This procedure is accepted as an equivalent to culturing cells under hypoxic conditions because HIF-1 α is a metal responsive transcription protein (Ladoux and Frelin, 1994; Piret et al., 2002; Maxwell and Salnikow, 2004). Fetal rat cardiac myocytes were plated and cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum and penicillin/streptomycin at 37°C. After four days of culture, medium was removed, and cells were treated with fresh media containing 150 μ M Cobalt Chloride (Piret et al., 2002). Control cells were treated with fresh media that did not contain Cobalt Chloride. After 4 hours of culture, cells were harvested, and total RNA was extracted using the Trizol procedure according to the manufacturers recommendations (Invitrogen, Carlsbad, CA).

[00150] Following preparation of cDNA using standard procedures, the Cobalt Chloride treated or untreated samples were assayed for transcript levels using polymerase chain reaction (PCR) and sequence specific primers for GAPDH as a normalization control, vascular endothelial growth factor (VEGF) as a positive control, or apelin. Following PCR, amplification products were fractionated on an agarose gel

and visualized using ethidium bromide staining under ultraviolet light (Figure 12). GAPDH expression was assayed as a standardization control for template concentration, and VEGF expression was assayed as a positive control known to respond to hypoxic conditions. As shown in Figure 12C, apelin expression was significantly increased in response to these conditions that mimic hypoxia. This upregulation of apelin under these conditions strongly suggest that apelin plays a role in tumor angiogenesis.

[00151] To determine if apelin expression is in fact induced under hypoxic conditions, further experiments were conducted with rat cardiac myocytes. The myocytes were grown in the presence of Cobalt chloride (30 $\mu\text{g/ml}$). A control known to be non-responsive to hypoxic conditions was used (β -actin), as well as two positive controls which are known to be responsive to hypoxic conditions (VEGF and GLUT1). For apelin and each of the controls, three separate experiments were conducted, in triplicate, and the results are shown in Figure 23. These data indicate that apelin expression is induced under hypoxic conditions, correlating with the presence of the HIF1 α sites seen in the gene sequence.

EXAMPLE 8

Effect of Apelin Expression on Apoptosis

[00152] To determine what effect, if any, apelin expression has on apoptosis, TUNEL assays were performed. The results are shown in Figures 21 and 22. Endothelial cells were serum starved for 48 hours, at which time serum free media was added as a control (Figure 21, Panel A) or with VEGF (50 ng/ml) (Figure 21, Panel B) or apelin (10 ng/ml) (Figure 21, Panel C). Cells were stained with a DAPI stain (Figure 21, Panels A, B, and C) or in the TUNEL assay which stains for broken DNA, indicating cells undergoing apoptosis (Figure 21, Panels D, E, and F). Figure 22 shows a graph, quantitating the results of the data in Figure 21. These data demonstrate that apelin has an anti-apoptotic effect on endothelial cells, to the same extent as VEGF, a known inhibitor of apoptosis.

EXAMPLE 9

Effect of Apelin Expression on Tumorigenesis

[00153] To directly test the hypothesis that apelin expression by tumor cells contributes to tumorigenicity, the well established mouse xenograft model system was used to assay for tumor growth.

Preparation of an apelin-expressing human tumor cell line

[00154] A tumor cell line was engineered to express apelin and EGFP sequences or the EGFP sequence alone. First, PCR analysis was used to establish that the human colon cancer cell line, HT-29 (ATTC number HTB-38), expresses low levels of VEGF and undetectable levels of apelin mRNA. Furthermore, the HT-29 cells are known to perform reliably in xenograft studies. Therefore, HT-29 cells were transfected with a vector construct designed to express human apelin. The pIRES2-EGFP vector from BD Clontech (cat #6029-1) was used for these experiments. This vector uses IRES (internal ribosome entry site) technology so that a single transcript encodes both apelin and the EGFP reporter protein. Transcription is under control of the ubiquitous CMV promoter. The full length human apelin coding region (derived from GenBank Accession No. BC021104, encoding the complete apelin preproprotein) was PCR amplified using high fidelity Pfu Turbo DNA polymerase (Stratagene) and was inserted between the BamHI and XhoI sites of the pIRES2-EGFP vector.

[00155] The primers used for the PCR amplification were: N-terminal 5'-AAGCTTAAGGCCACCATGAATCTGCGGCTCTGCG-3' (SEQ ID NO:19) and C-terminal 5'-AACGGATCCTCAGAAAGGCATGGGTCCCT-3' (SEQ ID NO:20). Sequence of the insert was confirmed by DNA sequencing.

[00156] The apelin and empty vector constructs (linearized at the BsaXI restriction cleavage site) were transfected into HT-29 cells using Lipofectamine reagent (Invitrogen) and the manufacturer's recommended conditions. After transfection, cells containing the construct underwent a primary selection for neomycin (G418) resistance at 700 µg/ml in DMEM (Becton Dickinson) under standard culture conditions. Because the pIRES2 vector produces a single transcript that encodes both apelin and the EGFP reporter protein, EGFP fluorescence acts as a direct indicator for the expression of the apelin encoding transcript. Therefore, a fluorescence activated cell sorter (FACS) was used to isolate transfected cells that were specifically expressing the apelin or empty vector constructs. After FACS selection, UV fluorescence showed that approximately 70% of cells were stably transfected for each of the expression constructs.

[00157] Secretion of bioactive apelin was confirmed by using conditioned medium from transfected HT-29 cells and assaying for stimulation of bEnd.3 endothelial cell proliferation. A positive control of conditioned medium from VEGF(165) expressing cells was included in this experiment. Results indicate that

medium from apelin transfected cells stimulated proliferation of bEnd.3 cells approximately 2.5 fold over medium from the empty vector control (Figure 24). These results indicate that the transfected HT-29 cells express and secrete bioactive apelin.

Xenograft experiments

[00158] Severe combined immune deficient (scid) mice were used for xenograft studies, and all manipulations and measurements were carried out in the Experimental Mouse Shared Service (EMSS) core at the University of Arizona Cancer Center. HT-29 cells transfected either with the apelin-expressing construct or the negative control construct were injected subcutaneously into the flanks of nude mice (10 million cells per injection). Six animals were used for each construct. These experiments used populations of antibiotic resistant cells, rather than clonal cell lines, to avoid the possibility that deleterious mutations may have occurred in a cell line during the selection and clone isolation procedure. After cell injection, the mice were checked for tumor formation, and tumor volume was measured twice per week, in 3 dimensions, using calipers. Mice were sacrificed when the tumor volume reached 2 cubic centimeters, and the tumors were excised for histological examination. The results of the xenograft experiments are shown in Figure 25. It is clear from this experiment that expression of apelin by HT-29 cells increases the rate of tumor growth relative to cells containing the empty vector. We conclude from this study that apelin promotes tumor growth in the live animal model.

APPENDIX

Amino Acid Sequence of Preproapelin from Homo sapiens (SEQ ID NO:1)

MNLRLCVQALLLWLSLTAVCGGSLMPLPDGNGLEDGNVRHLVQPRGSRNGP
GPWQGGRRKFRRQRPRLSHKGPMMPF

Amino Acid Sequence of Apelin-36 from Homo sapiens (SEQ ID NO:2)

LVQPRGSRNGPGPWQGGRRKFRRQRPRLSHKGPMMPF

Amino Acid Sequence of Apelin-17 from Homo sapiens (SEQ ID NO:3)

KFRRQRPRLSHKGPMMPF

Amino Acid Sequence of Apelin-13 (SEQ ID NO:4)

QRPRLSHKGPMMPF

Amino Acid Sequence of Apelin-13 from Zebrafish (SEQ ID NO:5)

PRPRLSHKGPMMPF

Amino Acid Sequence of APJ receptor from Homo sapiens (SEQ ID NO:17)

MEEGGDFDNYYGADNQSECEYTDWKSSGALIPAIYMLVFLLGTTGNGLVLWT
VFRSSREKRRSADIFIASLAVADLTFVVTLPWATYTYRDYDWPFGTFFCKLSS
YLIFVNMYASVFCLTGLSFDRYLAIVRPVANARLRLRVSGAVATAVLWVLAAL
LAMPVMVLRTTGDLENTTKVQCYMDYSMVATVSSEWAWEVGLGVSSTTVGF
VVPFTIMLTCYFFIAQTIAGHFRKERIEGLRKRRRLLSIIVVLVVTFALCWMPYH
LVKTLYMLGSLHWPCLDFLMNIFPYCTCISYVNSCLNPFLYAFFDPRFRQA
CTSMMLCCGQSRCAGTSHSSSGEKSASYSSGHSQGPGPNMGKGGEQMHEKSIPY
SQETLVVD

Nucleotide Sequence of Apelin from Homo sapiens (SEQ ID NO:18)

ATGAATCTGCGGCTCTGCGTGCAGGCGCTCCTGCTGCTCTGGCTCTCCTTGA
CCGCGGTGTGTGGAGGGTCCCTGATGCCGCTTCCCGATGGGAATGGGCTGG
AAGACGGCAATGTCCGCCACCTGGTGCAGCCCAGAGGGTCAAGGAATGGG
CCAGGGCCCTGGCAGGGAGGTCGGAGGAAATTCCGCCGCCAGCGGCCCCG
CCTCTCCCATAGGGACCCATGCCTTTCTGA

CLAIMS**WE CLAIM:**

1. A method of promoting apoptosis in a biological sample, comprising:
 - a. providing a biological sample; and
 - b. combining the sample with an apoptosis-promoting amount of a composition comprising an inhibitor of apelin activity.
2. The method of Claim 1, wherein the composition inhibits tumorigenesis.
3. The method of Claim 1, wherein the composition interferes with the interaction of an apelin polypeptide or apelin peptide with a receptor polypeptide.
4. The method of Claim 1, wherein the composition interferes with the interaction of an apelin polypeptide or apelin peptide with APJ.
5. The method of Claim 1, wherein the composition comprises an anti-apelin antibody or fragment thereof.
6. The method of Claim 5, wherein the antibody or fragment thereof binds a polypeptide that is selected from the group consisting of:
 - a. a polypeptide as defined in SEQ ID NO:1;
 - b. a polypeptide as defined in SEQ ID NO:2;
 - c. a polypeptide as defined in SEQ ID NO:3;
 - d. a polypeptide as defined in SEQ ID NO:4;
 - e. a polypeptide as defined in SEQ ID NO:5; and
 - f. a polypeptide that has at least 80% sequence identity with the polypeptide of a) through e) above and that interacts with APJ.
7. The method of Claim 5, wherein the antibody or fragment thereof binds the polypeptide of SEQ ID NO:4.
8. The method of Claim 5, wherein the antibody or fragment thereof binds a polypeptide that has at least 90% sequence identity with the polypeptide or peptide of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5 and that interacts with APJ.

9. The method of Claim 1, wherein the inhibitor of apelin activity is an anti-APJ antibody or fragment thereof.
10. The method of Claim 9, wherein the antibody or fragment thereof binds a polypeptide as defined in SEQ ID NO:17.
11. The method of Claim 9, wherein the antibody or fragment thereof binds a polypeptide having at least 90% sequence identity with the polypeptide as defined in SEQ ID NO:17.
12. The method of Claim 1, wherein the inhibitor of apelin activity is selected from the group consisting of an apelin antisense nucleic acid, receptor decoy, ribozyme, sense polynucleotide, double stranded RNA, RNAi, aptamer, and small molecule antagonist.
13. The method of Claim 1, wherein the inhibitor of apelin activity is selected from the group consisting of an APJ antisense nucleic acid, receptor decoy, ribozyme, sense polynucleotide, double stranded RNA, RNAi, aptamer, and small molecule antagonist.
14. The method of Claim 1, wherein the inhibitor of apelin activity is an inhibitor of a serine protease that cleaves a polypeptide specifically after an arginine residue.
15. The method of Claim 1, wherein the composition comprises a pharmaceutically acceptable carrier.
16. The method of Claim 1, wherein the biological sample is from a mammal.
17. The method of Claim 1, wherein the biological sample is a human biological sample.
18. The method of Claim 17, wherein the biological sample is in a patient.
19. The method of Claim 18, wherein the composition is introduced by a route selected from the group consisting of subcutaneous injection, intravenous injection, intraocular injection, intradermal injection, intramuscular injection, intraperitoneal injection, intratracheal administration, epidural administration, inhalation, intranasal

administration, oral administration, sublingual administration, buccal administration, rectal administration, vaginal administration, and topical administration.

20. The method of Claim 18, wherein the patient has a disease or condition involving reduced apoptosis.

21. The method of Claim 18, wherein the composition inhibits tumor growth.

22. The method of Claim 20, wherein the disease or condition is selected from the group consisting of stroke, hemangioma, solid tumors, leukemias, lymphomas, myelomas, metastasis, telangiectasia psoriasis scleroderma, pyogenic granuloma, Myocardial angiogenesis, plaque neovascularization, coronary collaterals, ischemic limb angiogenesis, corneal diseases, rubeosis, neovascular glaucoma, diabetic retinopathy, retrolental fibroplasia, arthritis, diabetic neovascularization, macular degeneration, wound healing, peptic ulcer, fractures, keloids, vasculogenesis, hematopoiesis, ovulation, menstruation, placentation, polycystic ovary syndrome, dysfunctional uterine bleeding, endometrial hyperplasia and carcinoma, endometriosis, failed implantation and subnormal foetal growth, myometrial fibroids (uterine leiomyomas) and adenomyosis, ovarian hyperstimulation syndrome, ovarian carcinoma, obesity, and obesity-associated disorders.

23. The method of Claim 18, further comprising

- c. administering to the patient a therapeutically effective amount of an anti-cancer agent,
wherein the anti-cancer agent is selected from the group consisting of a chemotherapeutic agent, a radiotherapeutic agent, an anti-angiogenic agent, and an apoptosis-inducing agent.

24. The method of Claim 23, wherein the anti-cancer agent is an anti-angiogenic agent.

25. The method of Claim 23, wherein the anti-angiogenic agent is an inhibitor of an angiogenic factor selected from the group consisting of VEGFs, FGFs, PDGFB, EGF, LPA, HGF, PD-ECF, IL-8, angiogenin, TNF-alpha, TGF-beta, TGF-alpha, proliferin, and PLGF.

26. A method of inhibiting apoptosis in a biological sample, comprising:
- a. providing a biological sample; and
 - b. combining the sample with a biologically effective amount of an apoptosis inhibiting composition comprising apelin activity.
27. The method of Claim 26, wherein the apoptosis inhibiting composition comprises a serine protease that cleaves a polypeptide specifically after an arginine residue.
28. The method of Claim 26, wherein the composition comprises a polypeptide selected from the group consisting of:
- a. a polypeptide as defined in SEQ ID NO:1;
 - b. a polypeptide as defined in SEQ ID NO:2;
 - c. a polypeptide as defined in SEQ ID NO:3;
 - d. a polypeptide as defined in SEQ ID NO:4;
 - e. a polypeptide as defined in SEQ ID NO:5; and
 - f. a polypeptide that has at least 80% sequence identity with the polypeptide of a) through e) above and that interacts with APJ.
29. The method of Claim 26, wherein the composition comprises the polypeptide as defined in SEQ ID NO:4.
30. The method of Claim 26, wherein the composition comprises a small molecule agonist.
31. The method of Claim 26, wherein the apelin composition comprises a polypeptide that has at least 90% sequence identity with the polypeptide or peptide of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5; and that interacts with APJ.
32. The method of Claim 26, wherein the biological sample is from a mammal.
33. The method of Claim 26, wherein the biological sample is a human biological sample.
34. The method of Claim 33, wherein the biological sample is in a patient.

35. The method of Claim 26, wherein the composition comprises a pharmaceutically acceptable carrier.
36. The method of Claim 34, wherein the patient has a disease or condition that is indicated by increased levels of apoptosis.
37. A method for identifying a modulator of apoptosis, comprising
- a. providing an apoptosis inhibiting composition comprising apelin;
 - b. combining a putative modulator of apoptosis with the composition;
 - c. introducing the composition or the combination of the putative modulator and the composition to an apoptosis predictive model; and
 - d. comparing the amount of intact cells in the model in the presence and absence of the putative modulator.
38. The method of Claim 37, wherein the composition comprises a polypeptide selected from the group consisting of:
- a. a polypeptide as defined in SEQ ID NO:1;
 - b. a polypeptide as defined in SEQ ID NO:2;
 - c. a polypeptide as defined in SEQ ID NO:3;
 - d. a polypeptide as defined in SEQ ID NO:4;
 - e. a polypeptide as defined in SEQ ID NO:5; and
 - f. a polypeptide that has at least 80% sequence identity with the polypeptide of a) through e) above and that interacts with APJ.
39. The method of Claim 37, wherein the composition comprises the polypeptide as defined in SEQ ID NO:4.
40. The method of Claim 37, wherein the composition comprises a polypeptide that has at least 90% sequence identity with the polypeptide or peptide of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5, and that interacts with APJ.
41. The method of Claim 37, wherein the apoptosis predictive model is a TUNEL assay.

FIGURE 1

RQRPRLSHKGPMF	Frog
RQRPRLSHKGPMF	Consensus
<u>RQRPRLSHKGPMF</u>	Human

FIGURE 2

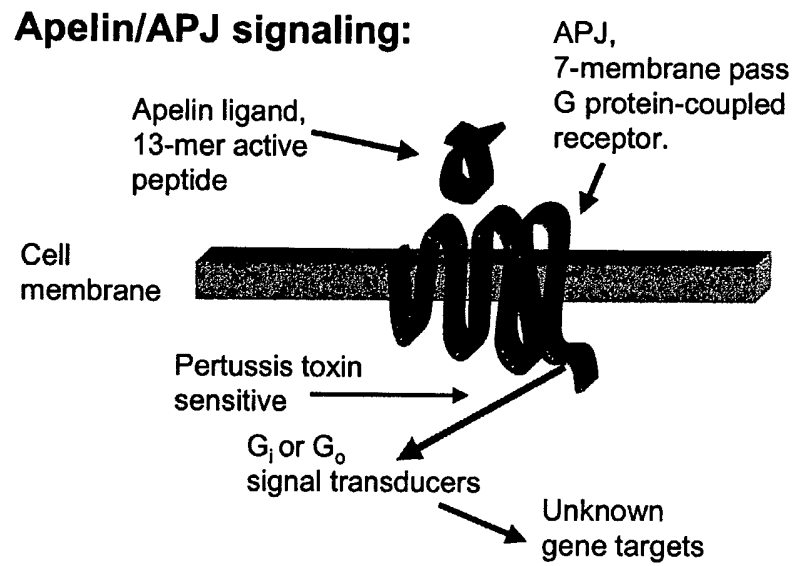
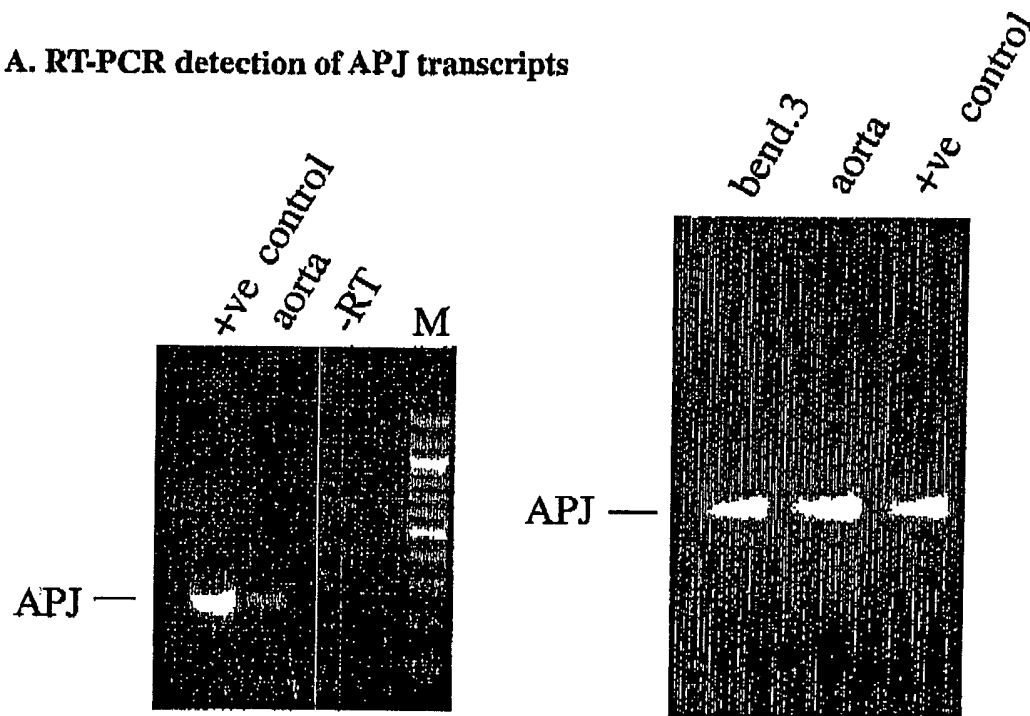


FIGURE 3

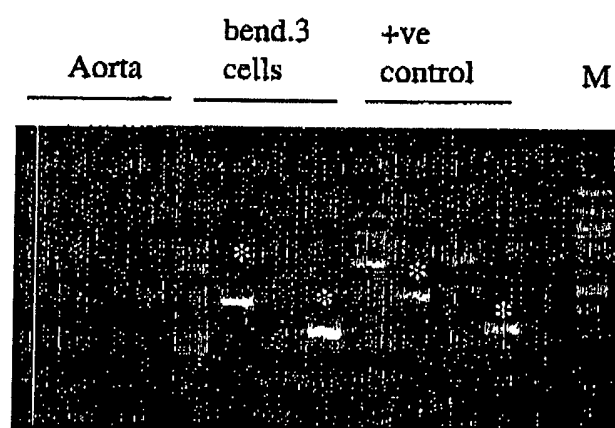
RT-PCR analysis of APJ and apelin expression in adult mouse tissue and in a mouse endothelial cell line.

A. RT-PCR detection of APJ transcripts



PCR product from aorta has been sequenced and confirmed as APJ.

B. RT-PCR detection of Apelin transcripts



Asterisks mark correct size apelin PCR products

FIGURE 4

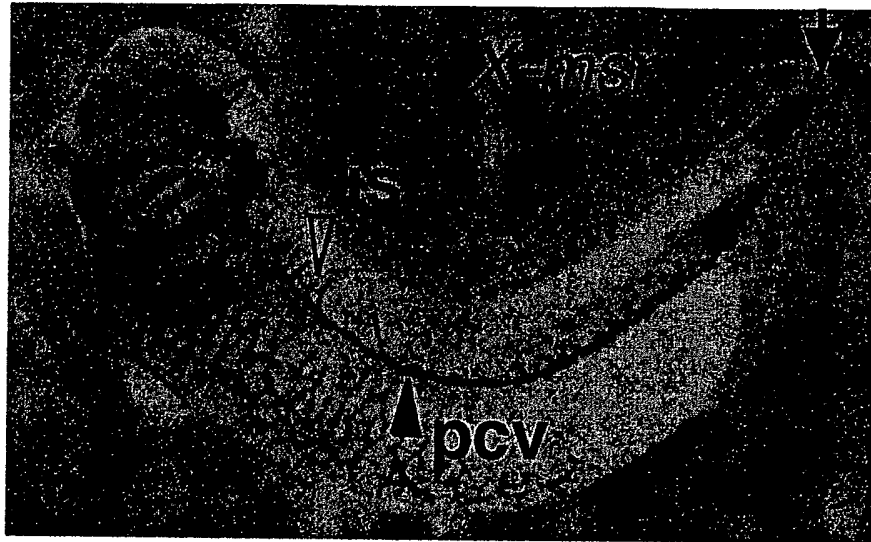
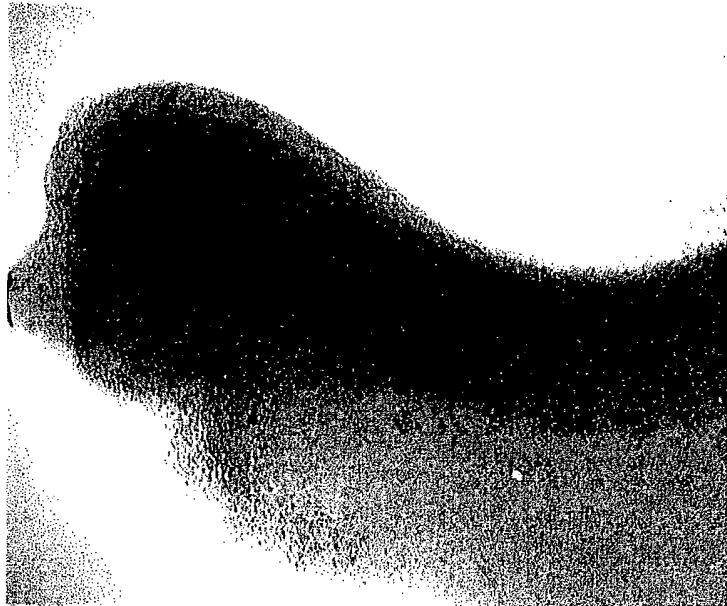


FIGURE 5

A



B



FIGURE 6

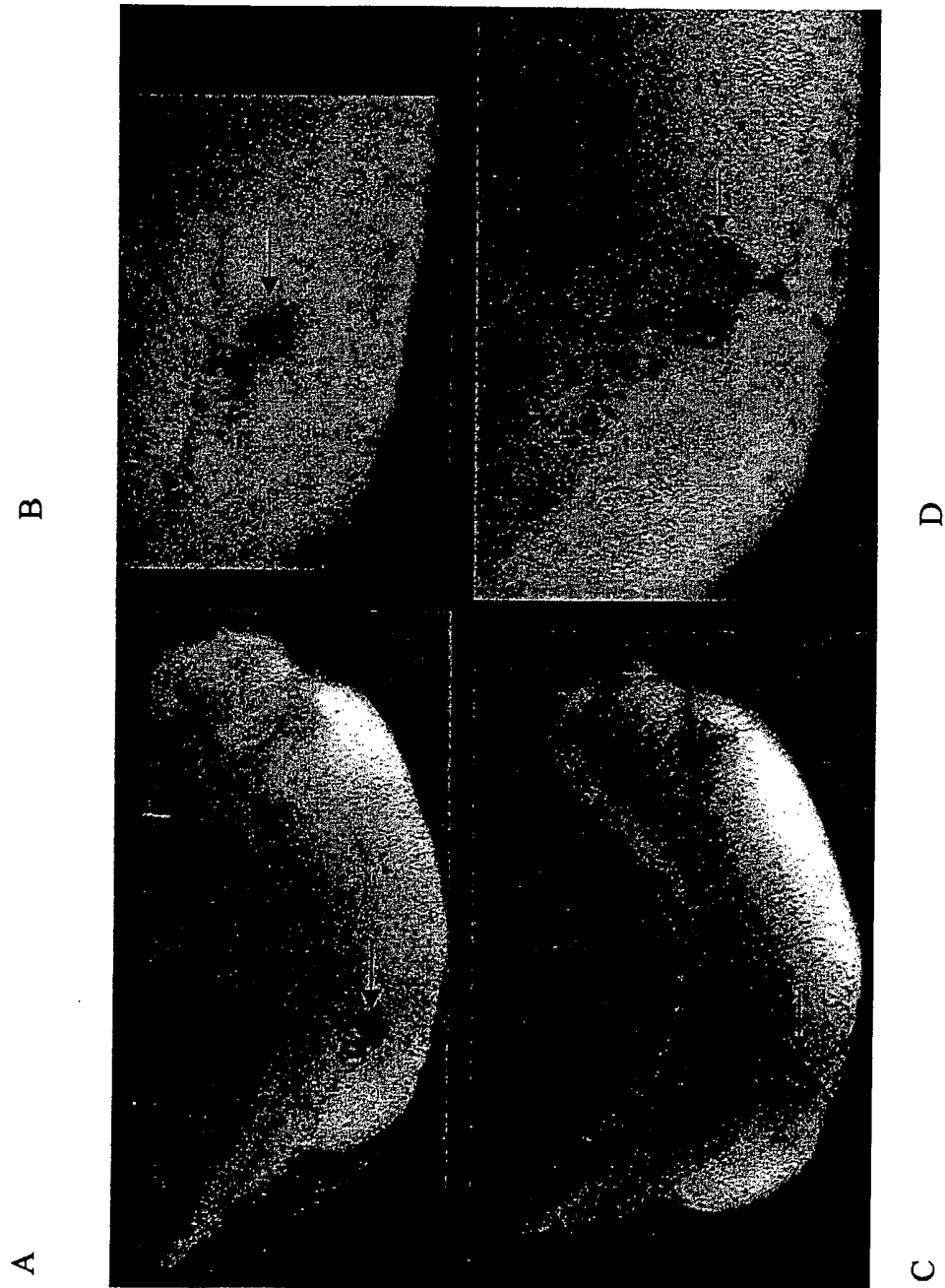


FIGURE 7

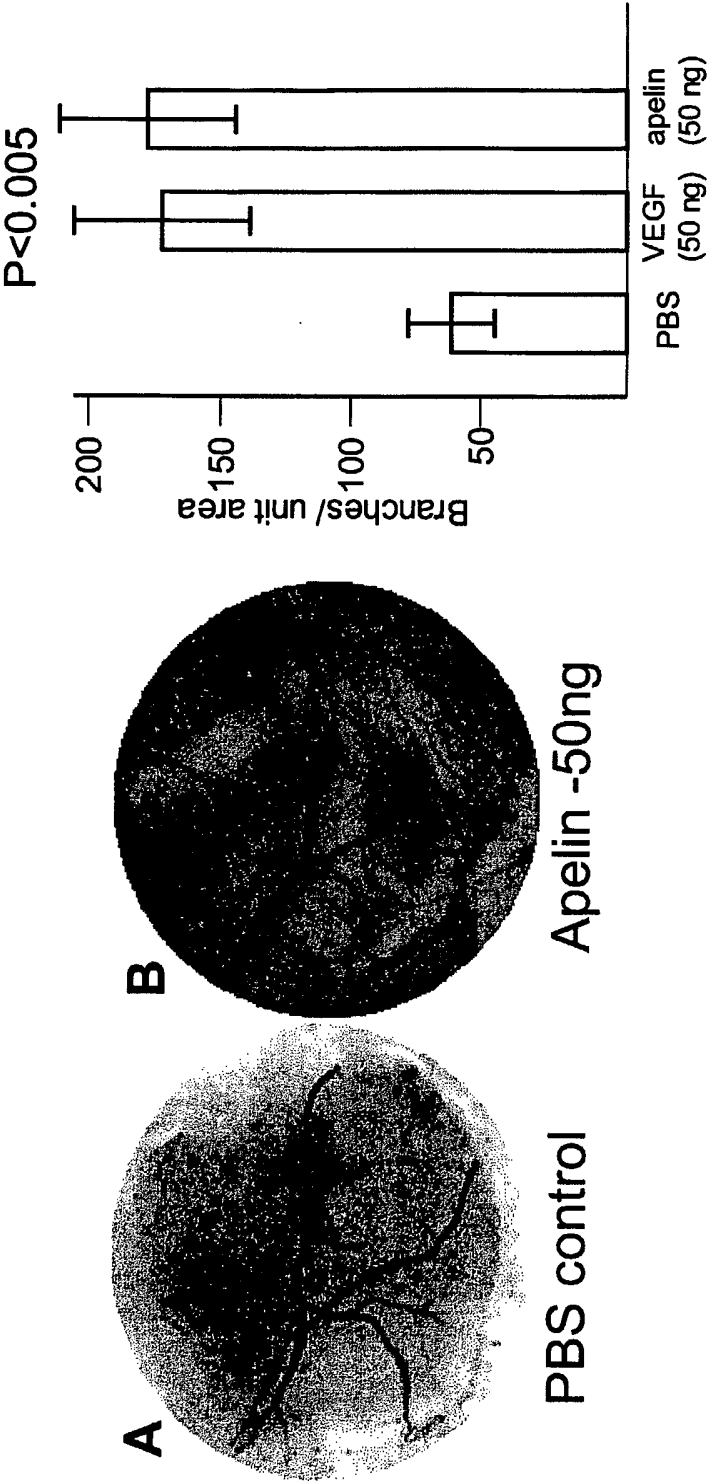


FIGURE 8

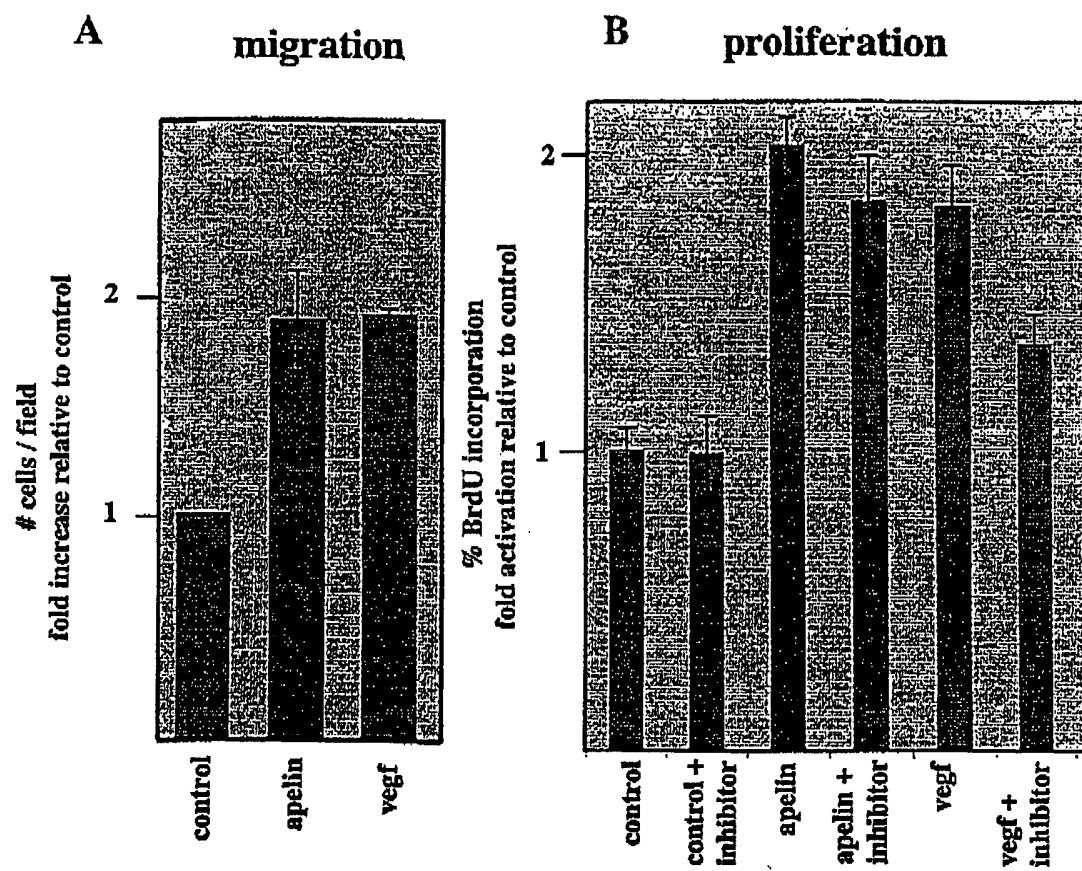


FIGURE 9

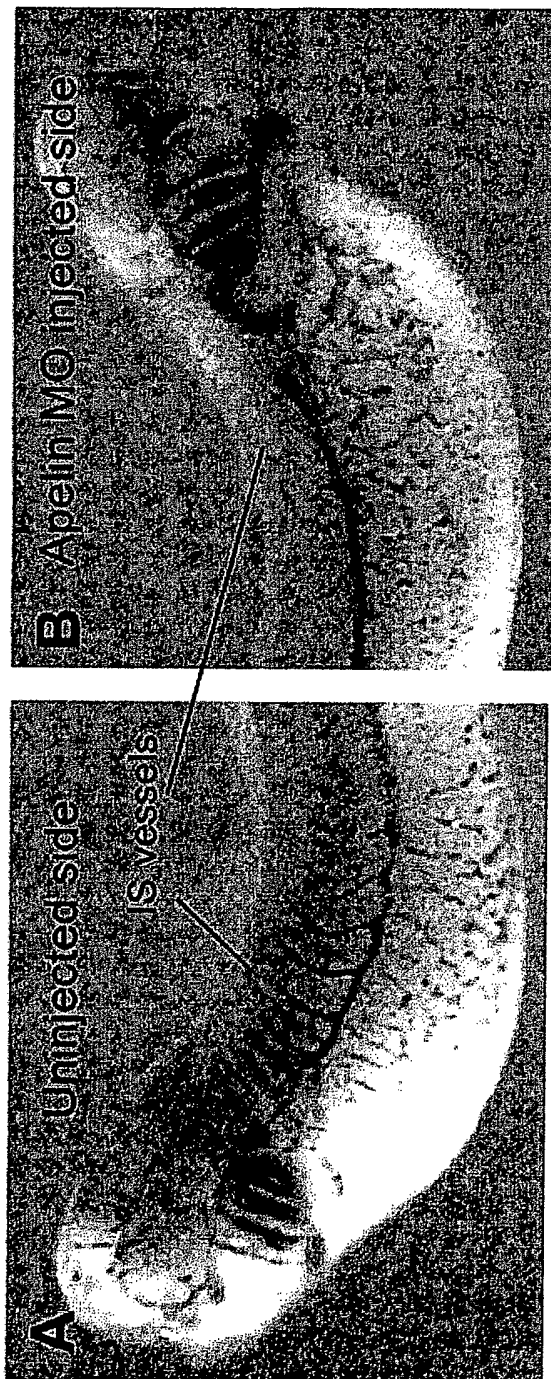
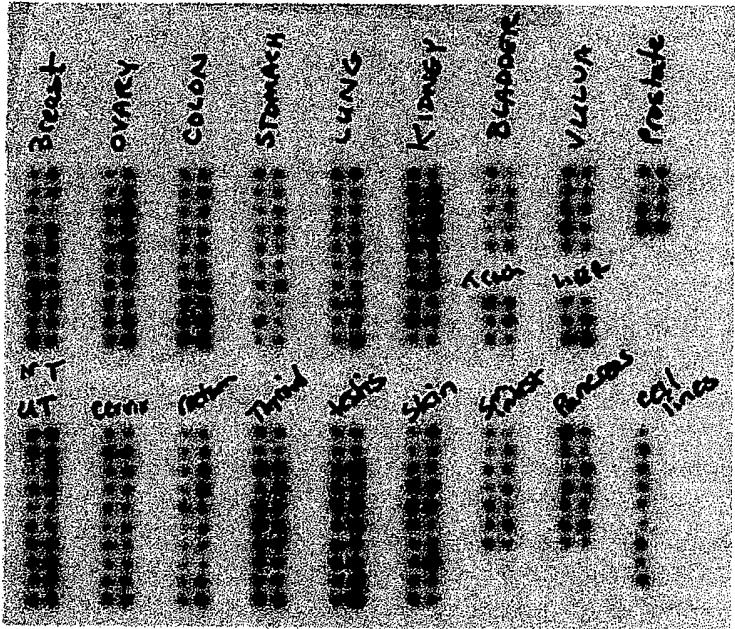


FIGURE 10

Apelin probe A



VEGF probe B

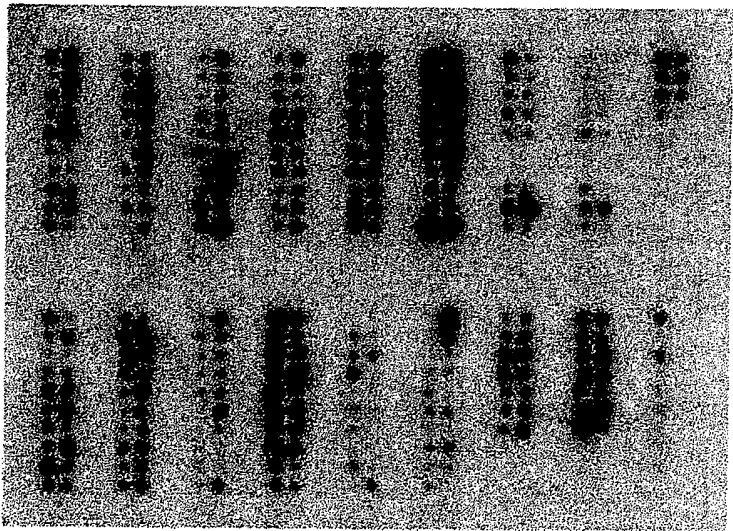


FIGURE 11

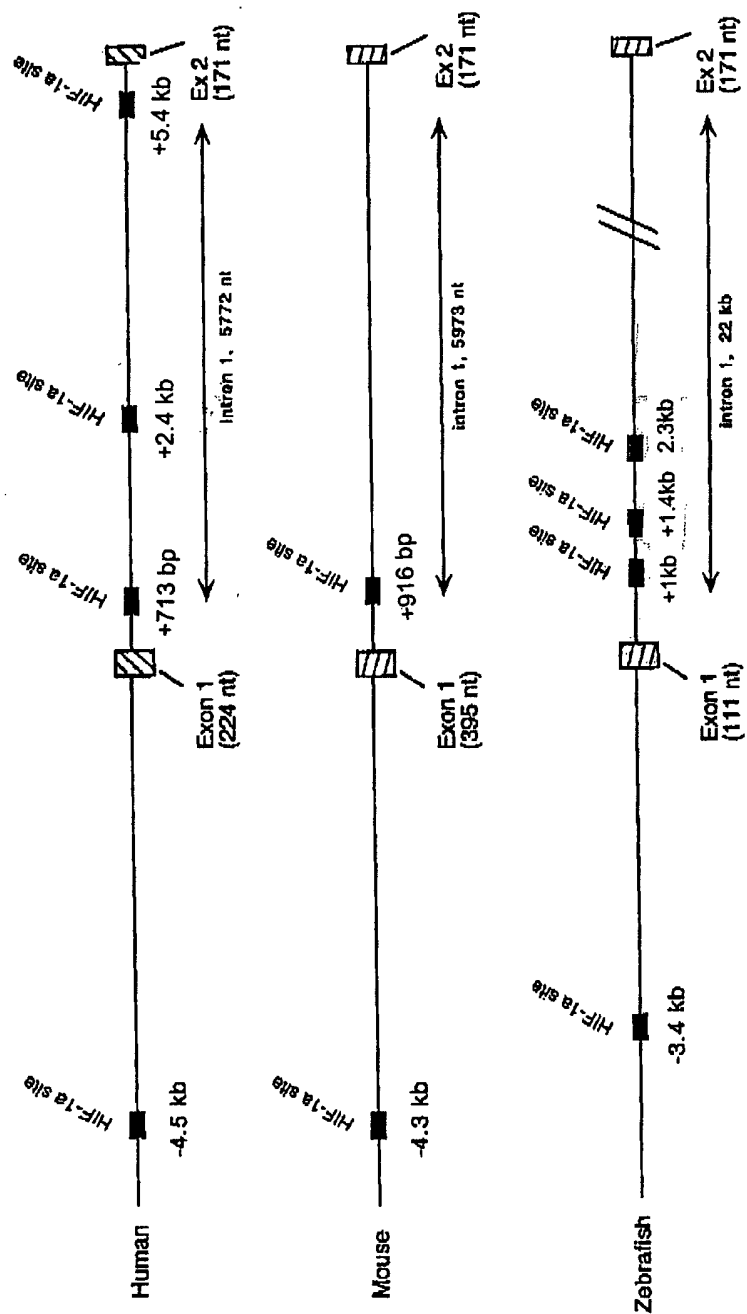


FIGURE 12

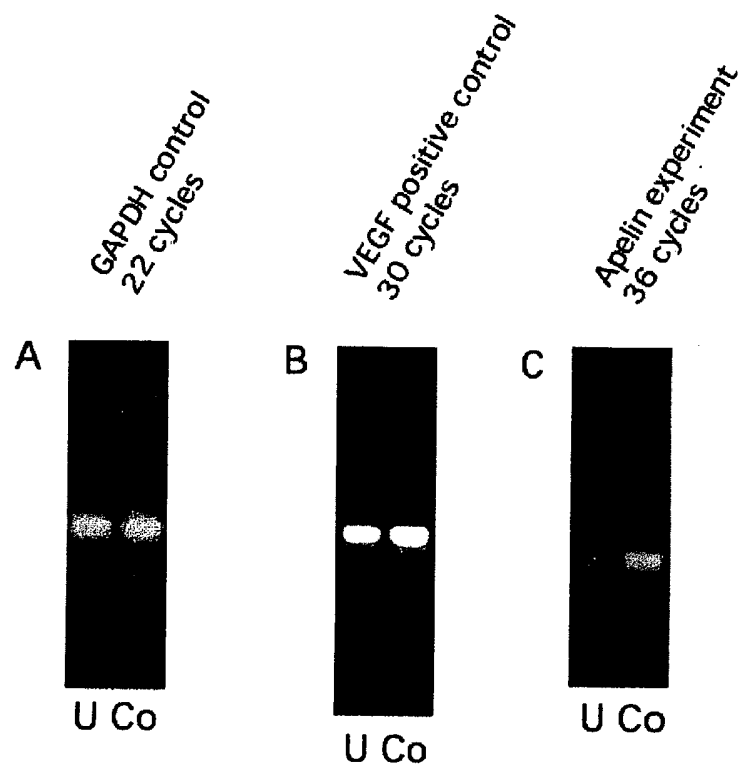


FIGURE 13

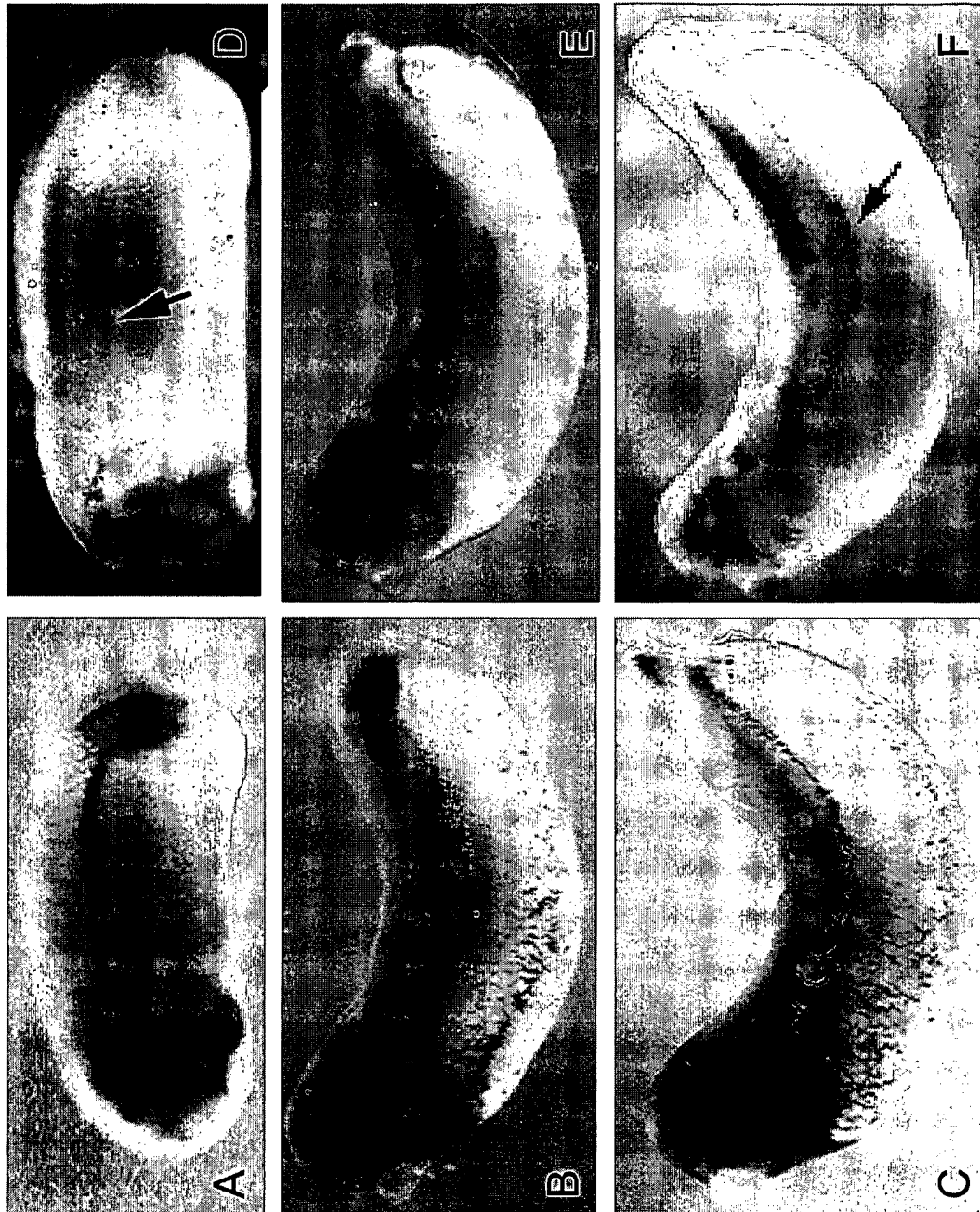


FIGURE 14

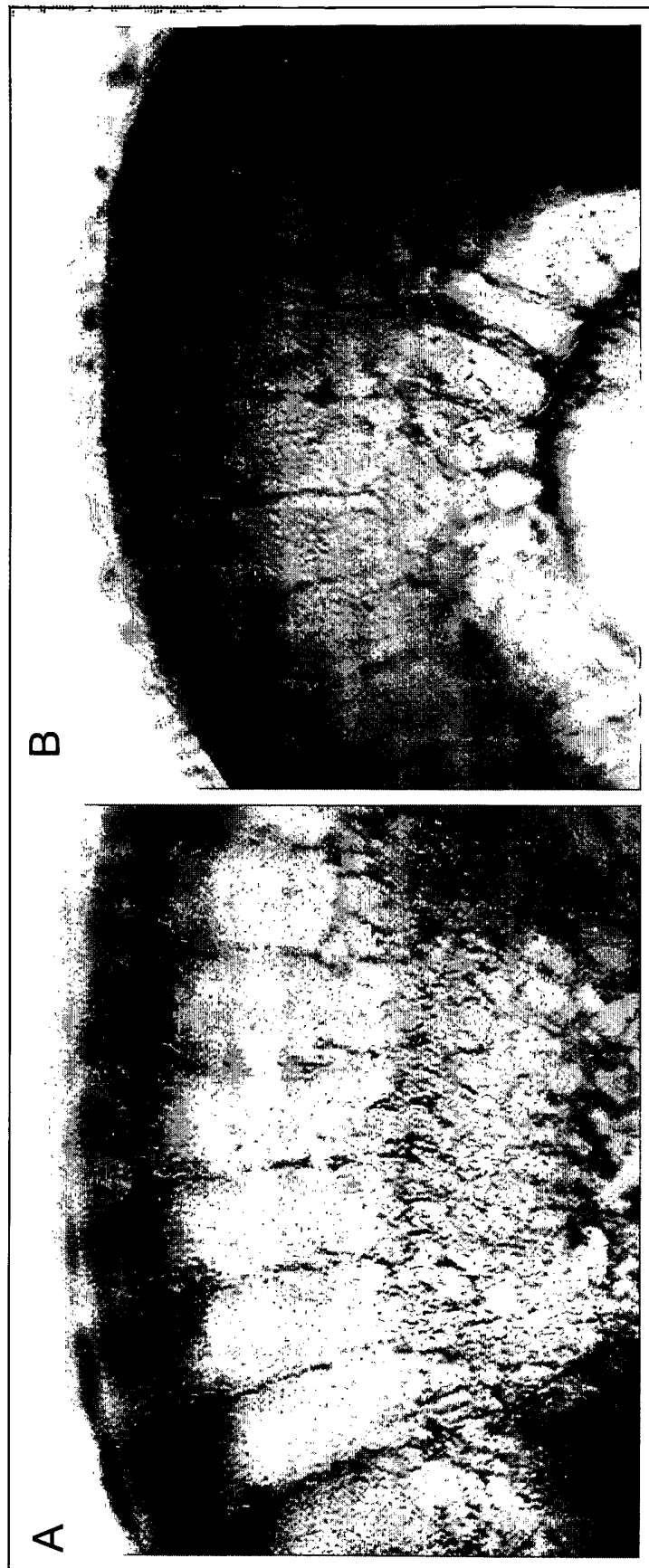


FIGURE 15

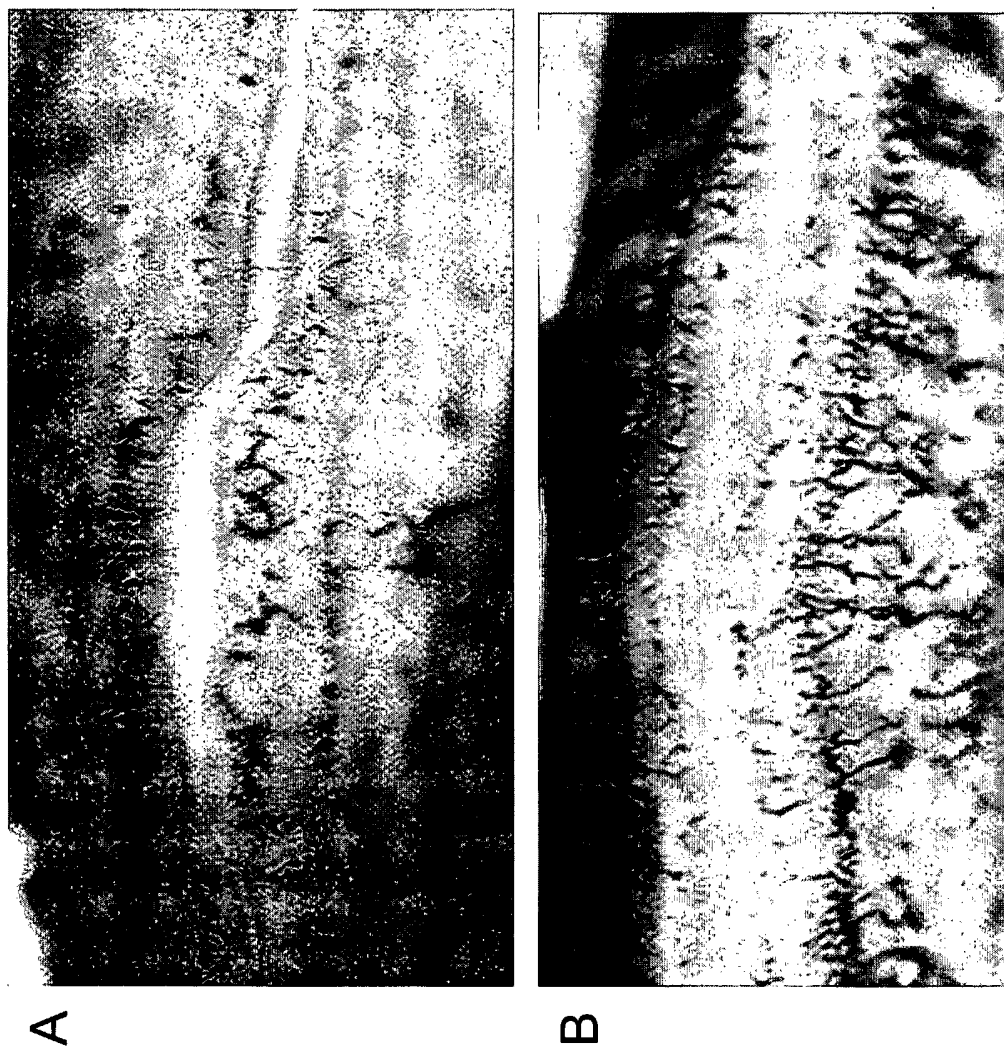


FIGURE 16

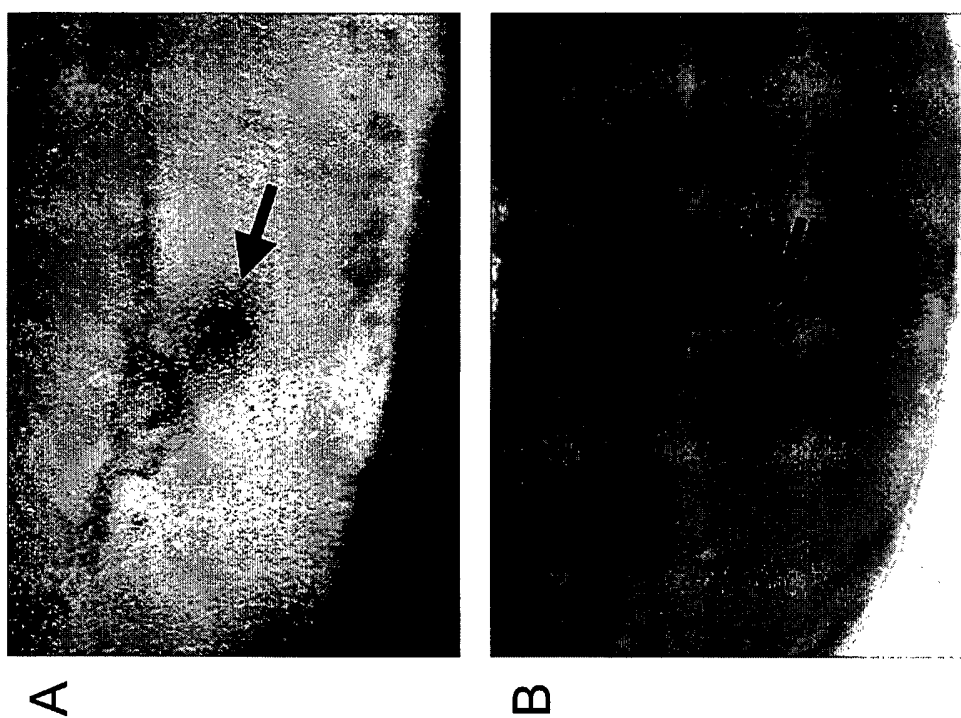


FIGURE 17

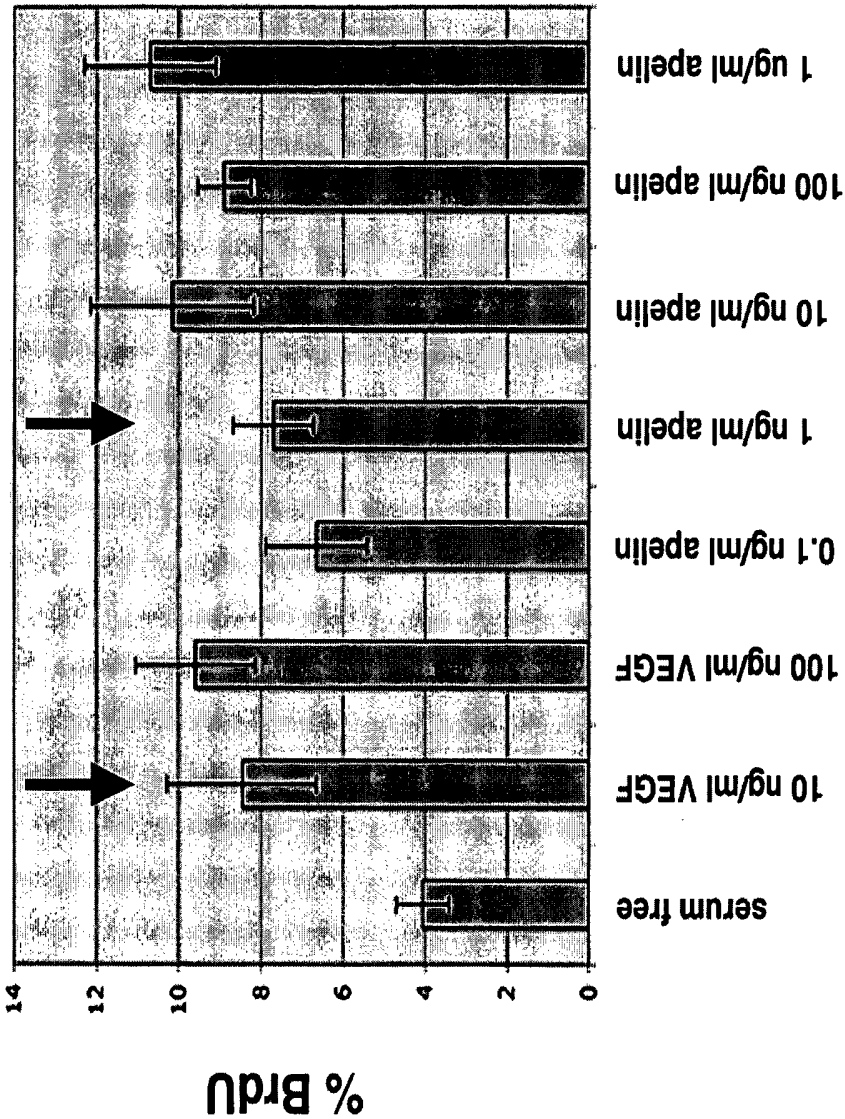


FIGURE 18

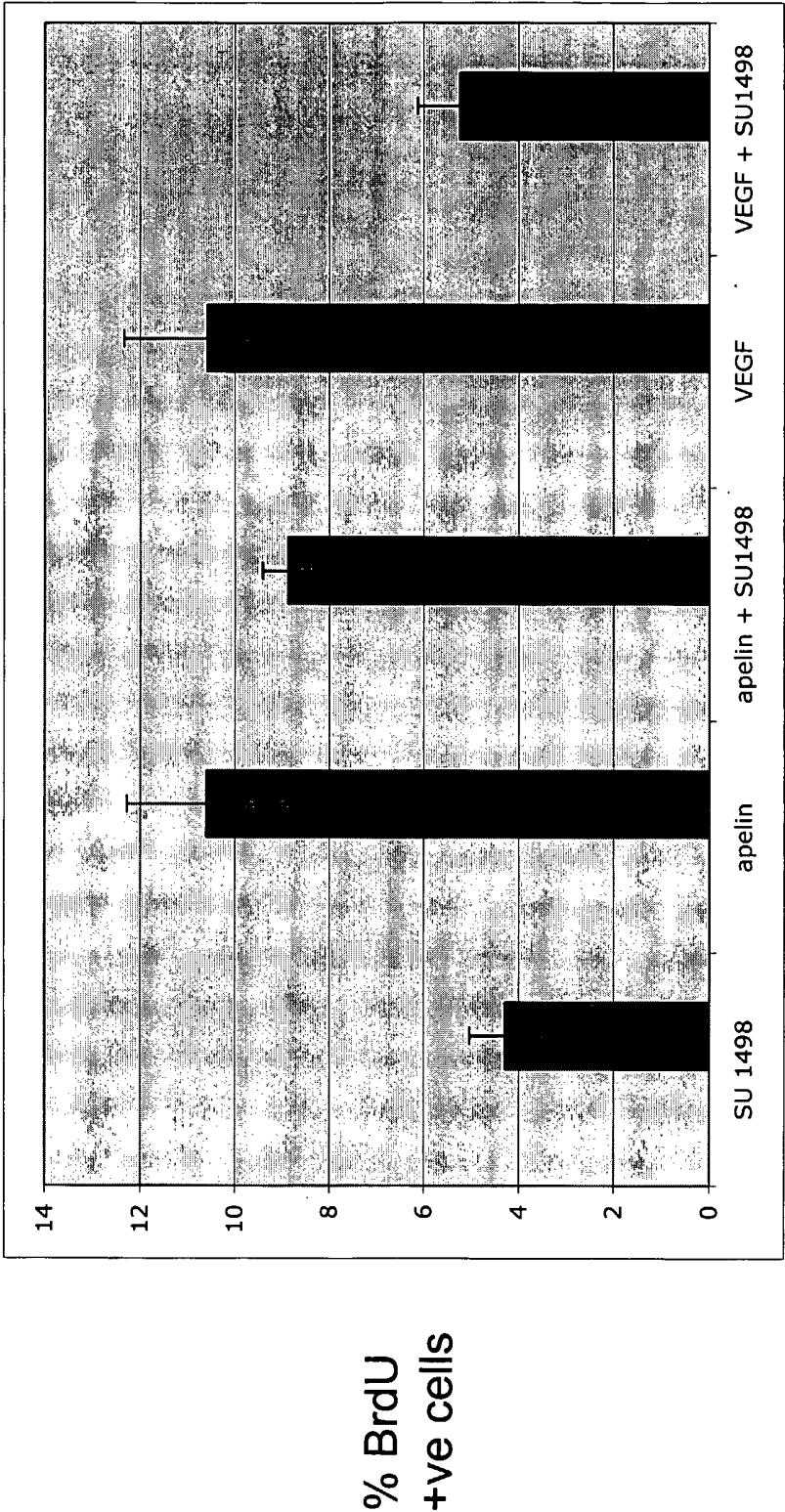


FIGURE 19

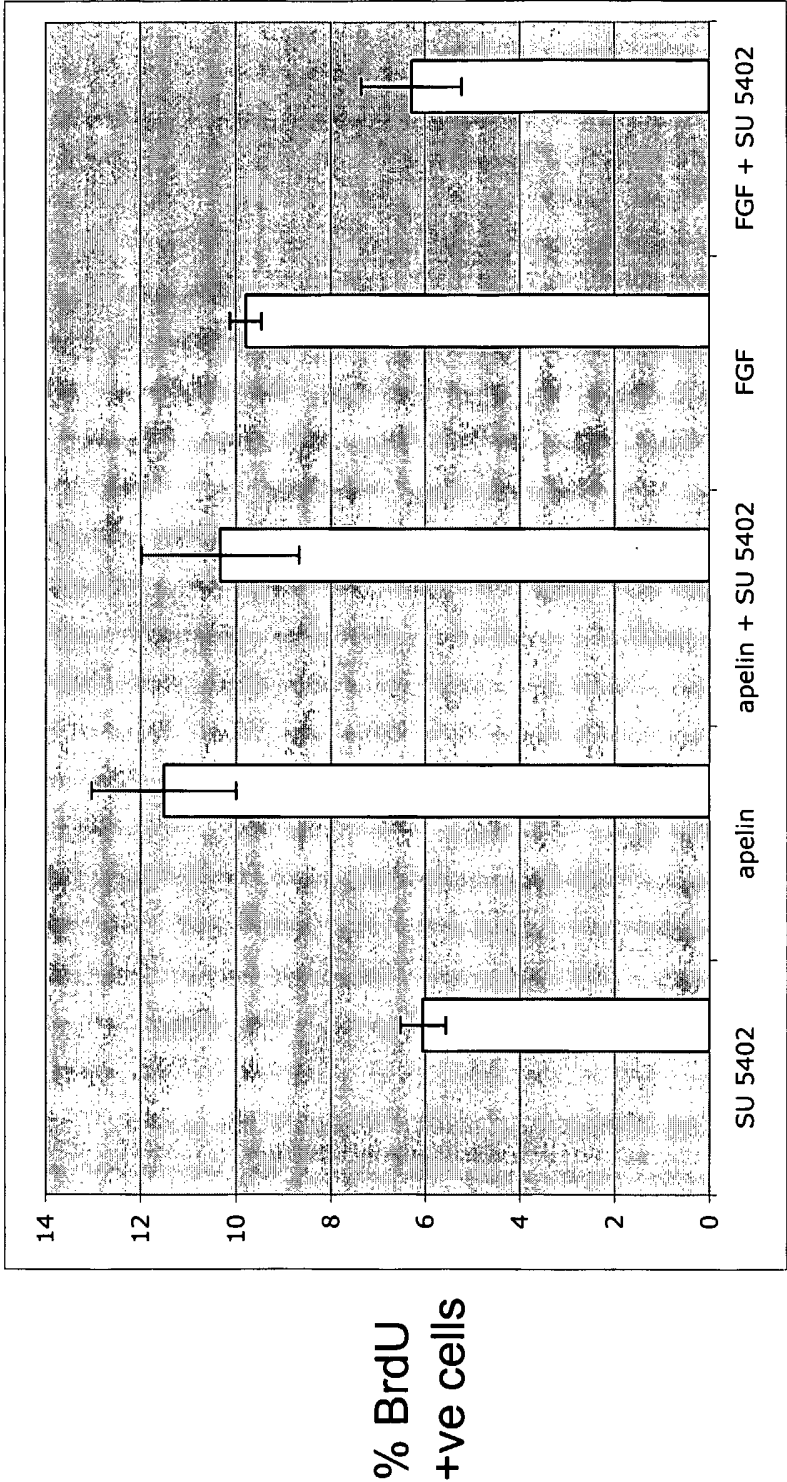


FIGURE 20

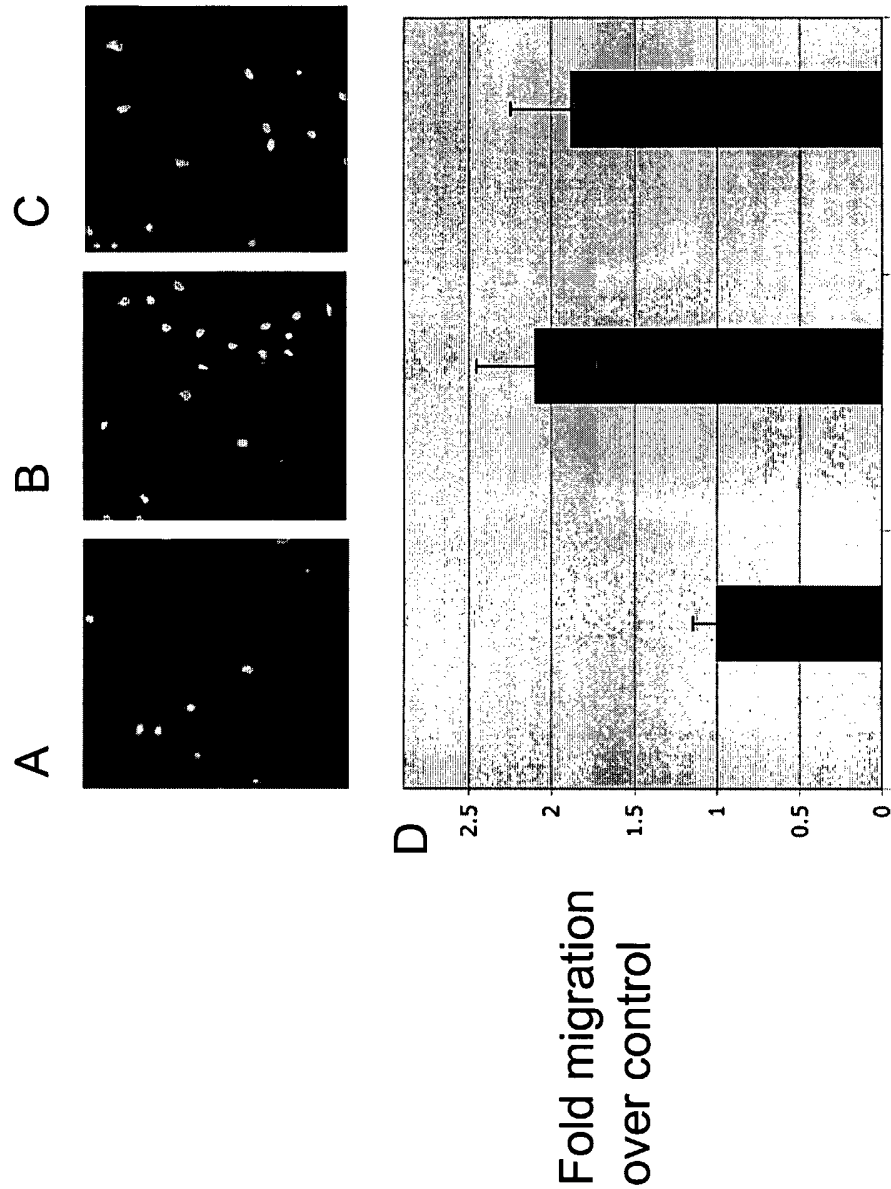


FIGURE 21

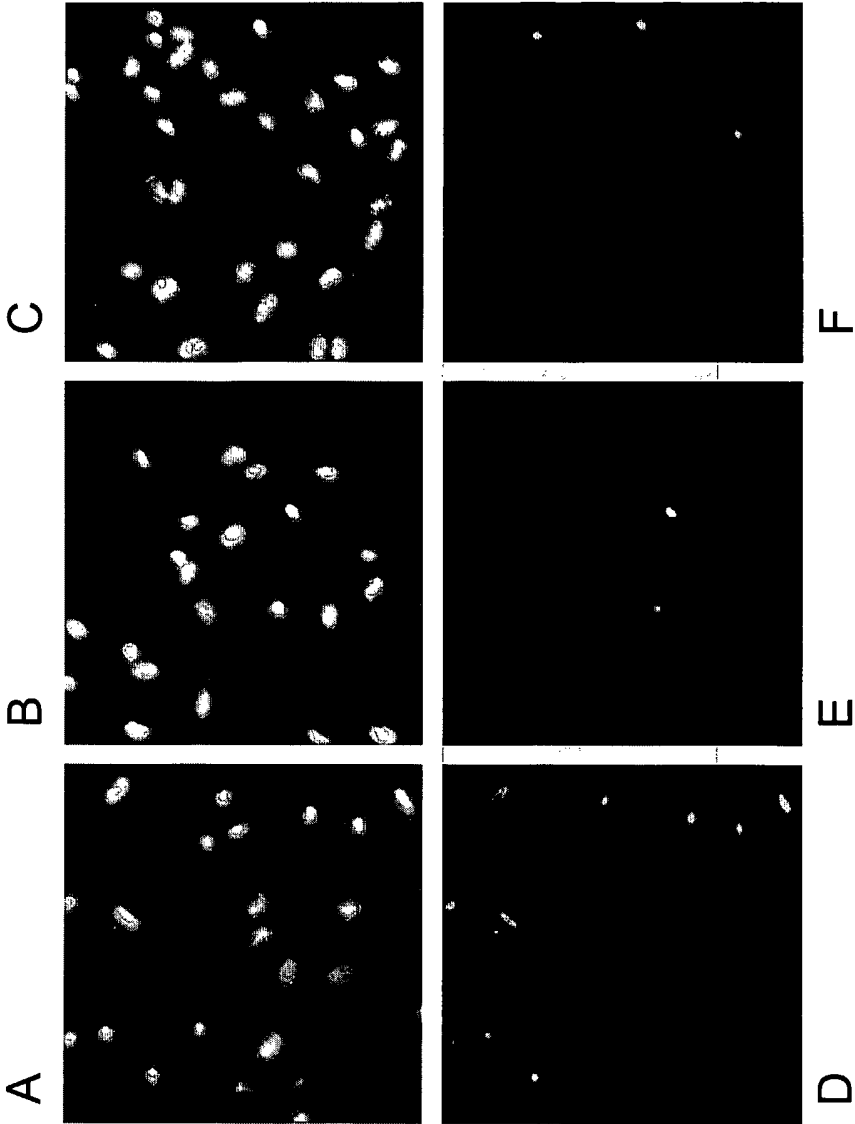


FIGURE 22

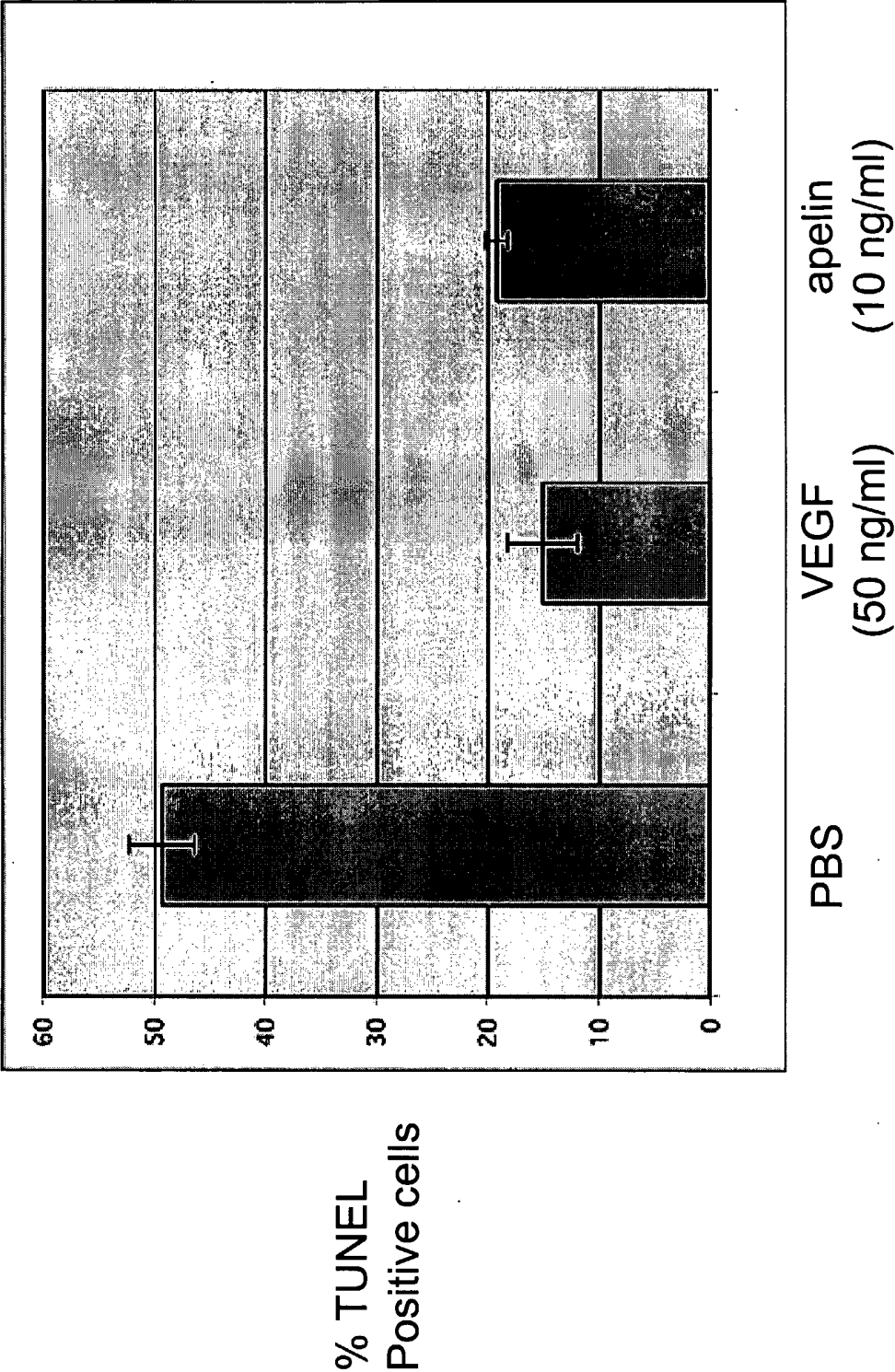


FIGURE 23

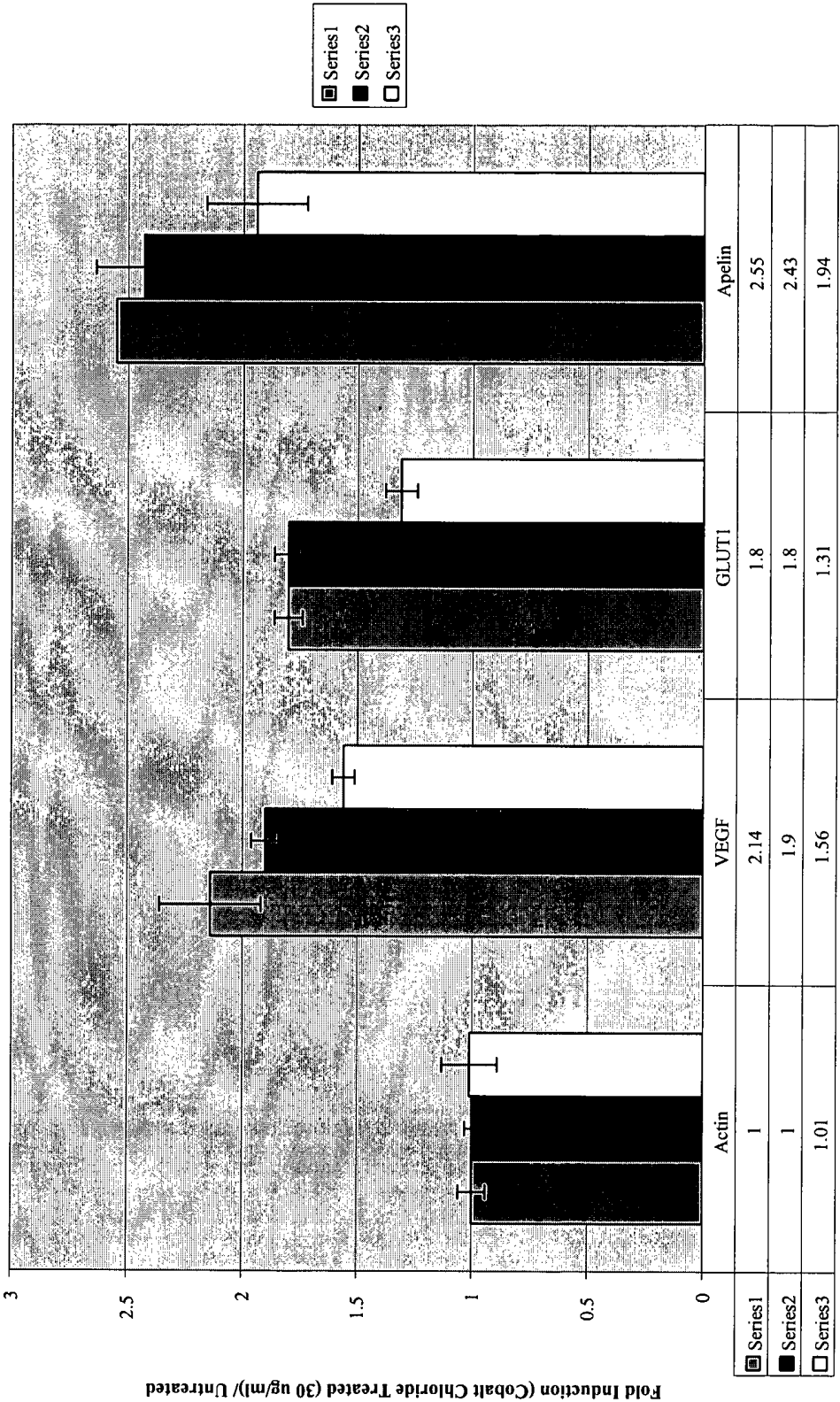


FIGURE 24

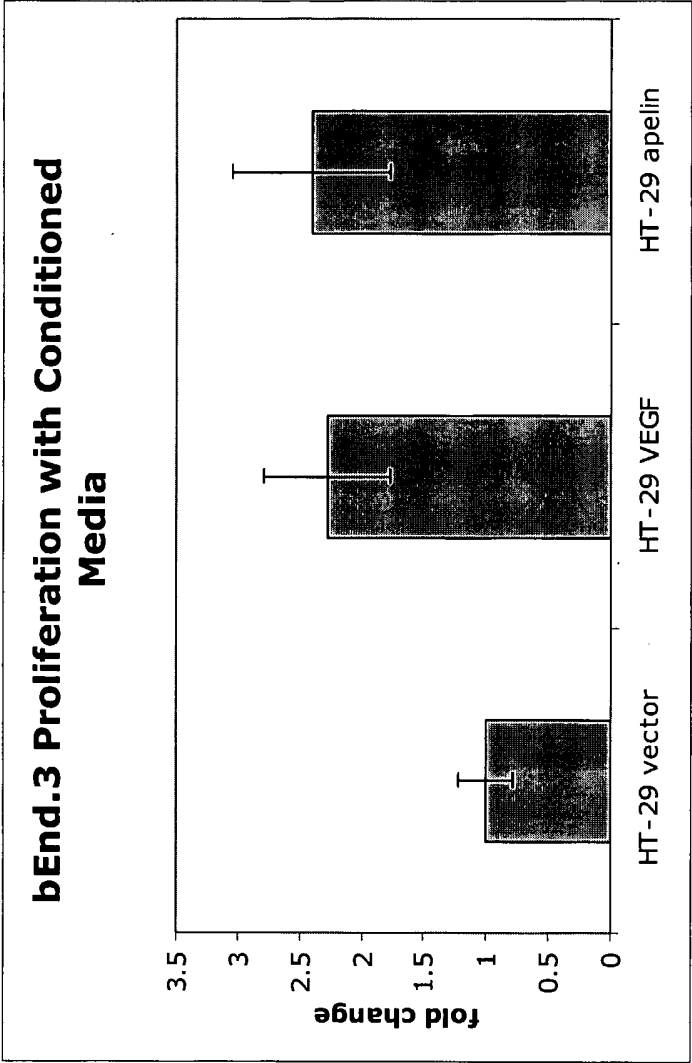


FIGURE 25

