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(54) Title: VEGF-MODULATED GENES AND METHODS EMPLOYING THEM

(57) Abstract: The present invention provides methods for modulating angiogenesis and/or apoptosis comprising modulating the activity of at least one VEGF-modulated gene polypeptide. The invention also provides pharmaceutical compositions for modulating angiogenesis and apoptosis for the prevention or treatment of diseases associated with VEGF-modulated genes expression. The invention also provides diagnostic assays that use VEGF-modulated gene polynucleotides that hybridize with naturally occurring sequences encoding VEGF-modulated genes and antibodies that specifically bind to the protein. The invention also provides novel human and mouse arginine-rich proteins (ARPs) and nucleotide sequences. The invention provides for genetically engineered expression vectors and host cells comprising the nucleic acid sequence encoding ARPs and for a method for producing the protein.

VEGF-MODULATED GENES AND METHODS EMPLOYING THEM

RELATED APPLICATIONS

This application claims priority to U.S. provisional application Serial No. 60/191,201 filed 03/21/2000.

BACKGROUND

Cities have roads and alleys, plants have xylem and phloem, and people have arteries, veins and lymphatics. Without these byways, the vertebrate animal cells would starve or drown in their metabolic refuse. Not only do blood vessels deliver food and oxygen and carry away metabolic wastes, but they also transport signaling substances that apprise cells of situations remote to them but to which they need to respond. Hormonal messages are a common signal.

All blood vessels are ensheathed by a basal lamina and a delicate monolayer of remarkably plastic endothelial cells lining the luminal walls. Depending on location and function, smooth muscle and connective tissue may also be present.

Not only do healthy cells depend on the blood resources transported by the circulatory system, but so, too, unwanted cells: tumorigenic and malignant cells. These cells colonize and proliferate if they are able to divert bood resources to themselves. Angiogenesis, the type of blood vessel formation where new vessels emerge from the proliferation of preexisting vessels (Risau, 1995; Risau and Flamme, 1995), is exploited not only by usual processes, such as in wound healing or myocardial infarction repair, but also by tumors themselves and in cancers, diabetic retinopathy, macular degeneration, psoriasis, and rheumatoid arthritis. Regardless of the process, whether pathological or usual physiological, endothelial cells mediate angiogenesis in a multi-step fashion: (1) endothelia receive an extracellular cue, (2) the signaled cells breach the basal lamina sheath, abetted by proteases they secrete, (3) the cells then migrate to the signal and proliferate, and finally, (4) the cells form a tube, a morphogenic event (Alberts *et al.*, 1994). The complexity of this process indicates complex changes in cellular physiology and morphology, gene expression, and signaling. Angiogenic accomplices that are cues include basic fibroblast growth

factors (bFGF), angiopoietins (such as ANG1) and various forms of vascular endothelial growth factor (VEGF).

VEGF is a multifunctional mitogen that is secreted by many cells, including tumor cells (Ferrara, 1999b). Vascular endothelial cells (VECs) are responsive to VEGF, using two receptors: (1) kinase insert domain-containing receptor/fetal liver kinase 1 (KDR/Flk-1; VEGFR1), and (2) Fms-like tyrosine kinase 1 (FLT-1; VEGFR-2) (Warren *et al.*, 1995). These receptors have different affinities for VEGF and appear to have different cellular responses (Athanassiades and Lala, 1998; Li *et al.*, 1999). VEGFR1 and VEGFR-2 null mice die early during embryogenesis (Fong *et al.*, 1995; Shalaby *et al.*, 1995). From these knockout studies, VEGFR1 is necessary for blood island formation and the development of haematopoietic progenitors (Shalaby *et al.*, 1995), while VEGFR-2 is required for organizing embryonic vasculature (Fong *et al.*, 1995). Of these two receptors, VEGFR1 mediates the full spectrum of VEGF's biological effects, including mitogenesis, vasodilation, and tumor vascularization (Ferrara, 1999a), while VEGFR-2 promotes endothelial survival (Carmeliet *et al.*, 1999).

The molecular events and the order in which they occur and the pathways that are required for this process are of fundamental importance to understand angiogenesis. *In vitro* models are useful for identifying alterations in gene expression that occur during angiogenesis. A particularly fruitful model systems involves the supspension in a three-dimensional type I collagen gel and various stimuli, such as phorbol myristate acetate (PMA), basic fibroblast growth factor (bFGF), and VEGF. The combination of the stimuli and the collagen gel results in the formation of a three-dimensional tubular network of endothelial cells with incerconnecting lumenl structures. In this model, endothelial differentiation into tubelike structures is completely blocked by inhibitors of new mRNA or protein synthesis. Furthermore, the cells progress through differentiation in a coordinated and synchronized manner, thus optimizing the profile of gene expression (Kahn *et al.*, 2000; Yang *et al.*, 1999).

VEGF and VEGFR-2 ensure endothelial cell survival. In the developing retina, capillaries disappear in response to hyperoxia (increase in oxygen/oxygen tension), correlating with an inhibition of VEGF secretion by neighboring cells. These vessels disappear by selective apoptosis of endothelial cells (Alon *et al.*, 1995).

Removing VEGF by using function-blocking anti-VEGF antibodies also causes blood vessels to regress, even tumor vasculature (Yuan *et al.*, 1996). The mechanisms that mediate VEGF's ability to promote cell survival involve VEGFR-2. Ligation of VEGFR-2 induces a complex of vascular endothelial (VE)-cadherin, ß-catenin, phosphoinositide-3-OH kinase (PI3-K), and VEGFR1. PI3-K phosphorylates and activates the serine/threonine protein kinase Akt (protein kinase B) (Carmeliet *et al.*, 1999). Activated Akt is necessary and sufficient to mediate VEGF-dependent survival signal (Gerber *et al.*, 1998).

Programmed cell death, apoptosis, and cell survival play crucial roles in development, homeostasis, stress, and various pathologies. Apoptosis (as opposed to necrosis) is mediated by caspases. Caspases reside in healthy cells as inactive proenzymes, which are activated in response to pro-apoptotic stimuli. Mitochondria activate caspases by releasing cytochrome c into the cytosol, binding the adaptor molecule Apaf-1 (apoptotic protease activating factor 1). Apaf1 oligomerizes and recruits and activates pro-caspase-9. Activated caspase-9 activates downstream caspases, and apoptosis has been initiated. Cytochrome c release may be released through mitochondrial permeability transition (PT) pores. Bcl-2, an anti-apoptosis inhibitor, prevents cytochrome c release by interacting with PT pores (Marzo *et al.*, 1998). VEGF induces expression of Bcl-2 in VECs, indicating that regulation of the mitochondrial permeability is part of VEGF survival mechanism (Gerber *et al.*, 1998).

Tumor cells exploit angiogenesis to facilitate tumor growth. Hypoxia--decreased levels of oxygen--induces tumor cells to secrete VEGF, promoting neovascularization. In addition to secreting VEGF, tumor cells, including hematopoietic cells (Bellamy *et al.*, 1999), breast cancer cells (Speirs and Atkin, 1999), and Kaposi's sarcoma (Masood *et al.*, 1997), express VEGFR1. VEGF can act both in a paracrine and autocrine fashion to stimulate endothelial proliferation and survival. The molecules that mediate neovascularization, in addition to VEGF and its receptors and that ultimately enable tumors to survive will be useful in diagnosis, characterization and ultimately in treatment of tumors.

Identifying genes that are modulated by VEGF is useful in not only understanding the complex endothelial responses, including cell differentiation, remodeling, *etc.*, but also in a variety of diagnostic and therapeutic applications. For

example, because mitochondrial cytochrome c release initiates apoptosis and the protective effect of VEGF in inhibiting such action, determining those genes that are modulated by VEGF is useful in controlling apoptosis therapeutically. Such genes and their proteins may be modulated, for example, by gene therapy methods, or the discovery of substances that act on the expression of the gene or the protein itself. Evaluating the expression of VEGF-modulated genes can be used to assess the metastatic potential of a tumor cell. Collections of endothelial-specific markers to assay for vascularization can be used to assay tumor growth. Various pathologies may be treated by exploiting VEGF-mediated angiogenesis.

SUMMARY OF THE INVENTION

The present invention relates to several VEC genes that are differentially expressed in response to VEGF or related cytokines. These differentially expressed genes are collectedly referred to as "VEGF-modulated genes" (VEGFmg) and are:

1) glia-derived neurite promoting factor (GDNPF)/nexin
2) tissue factor pathway inhibitor-2 (TFPI2)/placental protein 5 (PP5)
3) heparin-binding EGF-like growth factor (HB-EGF)
4) regulator of G-protein signaling-3 (RGS3)
5) myasthenia gravis (MG) autoantigen/gravin
6) MKP-1 like protein tyrosine phosphatase
7) amyloid precursor-like protein 2 (APLP2)/CEI-box binding protein
8) osteonidogen (nidogen-2 precursor)
9) amyloid precursor protein (APP)
10) Human gene similar to yeast VPS41 (hVPS41p)
11) arginine-rich protein (ARP)
12) Down's syndrome critical region protein-1 (DSCR1)
13) insulin induced gene-1 (INSIG1)
14) decidual protein induced by progesterone (DEPP)
15) cytochrome oxidase subunit I (MTCO1)
16) NADH-ubiquinone oxidoreductase chain 1 (ND1)

17) NADH-ubiquinone oxidoreductase chain 4 (ND4)

18) connective tissue growth factor (CTGF)

In a first aspect, the present invention is an isolated polypeptide having at least 80% sequence identity to the sequence SEQ ID NO:3 or SEQ ID NO:22, polynucleotides encoding the same, and antibodies that specifically bind the same.

In a second aspect, the present invention is an isolated polynucleotide having at least 80% sequence identity to the sequence SEQ ID NO:2 or SEQ ID NO:21, or a complement thereof.

In a third aspect, the present invention is a transgenic non-human animal, having a disrupted *arginine-rich protein* (*ARP*) gene or a transgenic non-human animal expressing an exogenous polynucleotide having at least 80% sequence identity to the sequence SEQ ID NO:2 or SEQ ID NO:21, or a complement of said polynucleotide.

In a fourth aspect, the present invention is a method of screening a sample for an ARP mutation

In a fifth aspect, the present invention is a method of modulating angiogenesis comprising modulating the activity of at least one VEGF-modulated gene polypeptide.

In a sixth aspect, the present invention is a method of increasing, as well as decreasing angiogenesis, comprising modulating the activity of at least one VEGF-modulated gene polypeptide. Activity modulation of VEGF-modulated gene polypeptides may be over-expressing or eliminating expression of the gene, or impairing a VEGF-modulated gene polypeptide's function by contact with specific antagonists or agonists, such as antibodies or aptamers.

In a seventh aspect, the present invention is a method of treating various pathologies, including tumors, cancers, myocardial infarctions and the like.

In an eighth aspect, the present invention is a method of measuring a VEGF-modulated gene transcriptional and translational up-regulation or down-regulation activity of a compound. In some embodiments, the compounds are calcium channel regulators.

In a ninth aspect, the invention is a method of screening a tissue sample for tumorigenic potential.

In a tenth aspect, the invention is a method of modulating cell survival by modulating the activity of at least one VEGF-modulated gene polypeptide.

In an eleventh aspect, the invention is a method of increasing, as well as decreasing cell survival, comprising modulating the activity of at least one VEGF-modulated gene polypeptide. Activity modulation of VEGF-modulated gene polypeptides may be over-expressing or eliminating expression of the gene, or impairing a VEGF-modulated gene polypeptide's function by contact with specific antagonists or agonists, such as antibodies or aptamers.

In a twelfth aspect, the invention is a method of treating tumors and cancers comprising decreasing cell survival by modulating VEGF-modulated genes. In one embodiment, the modulated gene is DSCR1.

In a thirteenth aspect, the invention is a method of determining the clinical stage of tumor which compares the expression of at least one VEGF-modulated gene in a sample with expression of said at least one gene in control samples. In other embodiments, the VEGF-modulated gene is DSCR1 and/or ARP.

In a fourteenth aspect, the invention is a method of determining if a tumor has a potential for metastasis by determining the clinical stage of the tumor.

Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. The materials, methods, and examples are illustrative only and not intended to be limiting.

BRIEF DESCRIPTION OF THE DRAWING

FIG 1 Survival of human umbilical cord endothelial cells after transfection with various genes related to the present invention.

DETAILED DESCRIPTION OF THE INVENTION

Using amplification and an imaging approach called GeneCalling (Shimkets *et al.*, 1999), genes that are differentially expressed in endothelial cells stimulated by VEGF were identified. This method provides a comprehensive sampling of cDNA populations in conjunction with the sensitive detection of quantitative differences in mRNA abundance for both known and novel genes (Shimkets *et al.*, 1999). In the instant invention, 18 differentially expressed genes are disclosed. Identification and differential expression of these genes is confirmed by a second independent method employing real-time quantitative polymerase chain reaction (RT-PCR). In general, the present invention relates VEGF-modulated genes to angiogenesis and cell survival.

Definitions

Unless defined otherwise, all technical and scientific terms have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. The definitions below are presented for clarity.

The recommendations of (Demerec *et al.*, 1966) where these are relevant to genetics are adapted herein. To distinguish between genes (and related nucleic acids) and the proteins that they encode, the abbreviations for genes are indicated by *italicized* (or underlined) text while abbreviations for the proteins start with a capital letter and are not italicized. Thus, *arginine rich protein* (*ARP*) or arginine rich protein (ARP) refers to the nucleotide sequence that encodes ARP. Likewise, *VEGFmg* represents the VEGF modulate genes nucleotide sequences and fragments, while VEGFmg refers to the encoded polypeptides and fragments.

"Isolated," when referred to a molecule, refers to a molecule that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that interfere with diagnostic or therapeutic use.

"Survival" is a cell remaining alive and maintaining all or most of its morphology and physiological activity, even under conditions of cellular stress, including serum starvation and hypoxia.

Roles of VEGF-modulated genes in cells

1. Apoptosis

Cell survival is impinged under stress, including oxidative stress and serum deprivation. VEGF stimulation appears to evoke a response similar to that of sub-lethal oxidative stress induced by reactive oxygen species (ROS). An important component of cell survival is mitochondrial respiration. Several VEGF-modulated genes of the instant invention, *e.g.* DSCR1, gravin, and HB-EGF, are also associated with ROS responses (Kayanoki *et al.*, 1999). In addition, VEGF administration down-regulates several mitochondrial genes (*e.g.* cytochrome c oxidase subunits and NADH-ubiquinone reductase chains 1, 4 and 5; Examples) and inhibits respiration.

Several observations support the cell survival role of VEGF-modulated genes of the instant invention and their link to mitochondrial respiration. Oxidative stress causes a general, calcium-dependent degradation of mitochondrial polynucleotides in HA-1 fibroblasts (Crawford *et al.*, 1998). When exposed to the anti-prostate cancer compound BMD188 apoptosis induction depends on the mitochondrial respiratory chain (Joshi *et al.*, 1999). Finally, mitochondrial Raf-1 is activated in response to Akt, which counteracts apoptosis (Majewski *et al.*, 1999).

All the genes whose differential expression was confirmed in the present disclosure and that potentially localize in the mitochondria are important components in cell survival based on the experiments disclosed herein. These genes include DSCR1, ARP, INSIG1 and DEPP represent important therapeutic targets. Over expression of DSCR1 was able to hasten apoptosis in human umbilical vascular endothelial cells (HUVECs), while antisense DSCR1 expression promoted cell survival to similar levels as that of activated AKT expression (see Fig. 1).

Adherent cells that become detached from their substrates undergo apoptosis. If the substrate to which they bind, such as the medial and adventitial extracellular matrix layers of arterioles and venules, is defective or eliminated, cells die. These matrices are secreted in part by mesenchymal cells that are recruited by the endothelial cells during the course of angiogenesis. The growth factor, HB-EGF stimulates mesenchymal cell proliferation and migration, and, for example, promotes renal epithelial cell survival (Takemura *et al.*, 1997).

Serpin activity may prevent cell death in endothelia. During angiogenesis when endothelial cells are invading new unvascularized tissues and stroma, serine proteases having thrombin-like activity will be present. Nexin, a serpin, promotes neurite outgrowth and survival by blocking thrombin activity, a multifunctional serine protease that is produced at sites of tissue injury. Thrombin acts via a cell surface protease-activated receptor (PAR-1) and increass in intracellular free calcium levels ([Ca2+]i) (Smith-Swintosky *et al.*, 1995). The present invention demonstrates that serine protease inhibitors (serpins) nexin and placental protein 5 (PP5)/TFPI2 (TFPI2) are induced in response to VEGF. APP and APLP2 appear to play serpin-like roles since these membrane bound proteins can be processed endoproteolytically, yielding secreted forms with serpin-like properties.

Description of genes differentially expressed and identified in the present invention

Several genes are differentially expressed in VECs when contacted with VEGF or related cytokines, and can be divided into four general classes (Table 1). These genes are collectively referred to as "VEGF-modulated genes" (VEGFmgs), "the set of VEGF-modulated genes" or "genes responsive to VEGF". Furthermore, among the VEGF-modulated genes, a novel form of ARP is disclosed. The classes of Serpins, Regulators of G-protein-linked receptors and selected Mitochondrial proteins are especially preferred.

Table 1 VEGF modulated genes

Class	Members
Serpins (serine protease inhibitors)	1) nexin/glia-derived neurite promoting factor (GDNPF) 2) placental protein 5 (PP5)/tissue factor pathway inhibitor-2 (TFPI2) 3) amyloid precursor-like protein 2 (APLP2)/CEI-box binding protein 4) amyloid precursor protein (APP)
Regulators of G-protein-linked receptors	5) regulator of G-protein signaling-3 (RGS3) 6) gravin/myasthenia gravis (MG) autoantigen
Mitochondrial proteins (selected group)	7) arginine-rich protein (ARP) 8) Down's syndrome critical region protein-1 (DSCR1)
Others	9) Human gene similar to yeast VPS41 (hVPS41p) 10) insulin induced gene-1 (INSIG1) 11) decidual protein induced by progesterone (DEPP) 12) cytochrome oxidase subunit I (MTCO1) 13) NADH-ubiquinone oxidoreductase chain 1 (ND1) 14) NADH-ubiquinone oxidoreductase chain 4 (ND4) 15) heparin-binding EGF-like growth factor (HB-EGF) 16) MKP-1 like protein tyrosine phosphatase 17) osteonidogen (nidogen-2 precursor) 18) connective tissue growth factor (CTGF)

1. Serpins

Serpins are serine protease inhibitors; they may be secreted or membrane bound. VEGF-modulated serpins comprise nexin, PP5, APLP2, and APP.

(a) nexin/glia-derived neurite promoting factor (GDNPF)

Protease nexin I (PNI or PN1; GenBank A03911; SEQ ID NOS:4 and 5; (Monard *et al.*, EP 233838, 1990)) promotes neurite outgrowth and survival *in vitro* from neurons and astrocytes by eliminating thrombin's neurite-inhibitory activity. Nexin regulates thrombin's proteolytic activity by forming post-translational, covalent complexes with thrombin (Smith-Swintosky *et al.*, 1995). Thrombin, a multifunctional serine protease, is rapidly produced at sites of tissue injury and catalyzes the final steps in blood coagulation.

In the present invention GeneCalling™ analysis reveals that nexin is up-regulated in VEGF-stimulated endothelial cells at 24 hours (Example 1).

(b) Placental protein 5 (PP5)/TFPI2 (TFPI2)

PP5/TFPI2 (SEQ ID NOS:6 and 7; GenBank NM_006528, D29992) inhibits a number of blood coagulation and fibrinolysis serine proteases. In embryogenesis, PP5 is involved in trophoblast differentiation and helps maintain intervillous blood flow. PP5 is also frequently expressed in ovarian adenocarcinomas (Inaba *et al.*, 1982). As PN1, PP5 acts by blocking thrombin's activity.

GeneCalling™ analysis found PP5 to be up-regulated in VEGF-stimulated endothelial cells at 24 hours (Example 1).

(c) Amyloid precursor-like protein 2 (APLP2)

The human amyloid precursor-like protein APLP2 (SEQ ID NOS:8 and 9; GenBank L27631) belongs to the Alzheimer peptide precursor (APP) family. While structurally related to amyloid precursor protein (APP), APLP2 functions differently. Like APP, APLP2 contains a transmembrane domain and a Kunitz type protease inhibitor domain; however, unlike APP, APLP2 binds DNA, recognizing the centromere DNA sequence element I (CDEI) motif (5'-GTCACATG-3'; SEQ ID NO:10) (Yang *et al.*, 1996).

APLP2 is likely an important component of the cell survival pathway. APLP2 expression is increased in PC12 neuronal cells that undergo apoptosis (Araki and Wurtman, 1998) and is predicted to be a protease inhibitor.

APLP2 was up-regulated in VEGF-stimulated endothelial cells at 24 hours. This result was confirmed by Taqman™ analysis (See Examples).

(d) Amyloid precursor protein (APP)

Amyloid precursor protein (SEQ ID NOS:11 and 12; GenBank D87675) is a ubiquitously expressed, membrane spanning glycoprotein that is endoproteolytically processed yielding a secreted protein identical to protease nexin II (PN2) and an internalized 11.5 kDa, 100 residue C-terminal derivative (CTD). PN2 is an inhibitor of proteinases such as trypsin. APP is the source of the β-amyloid (Aβ), a 39-43 amino acid peptide that is the main component deposited in amyloid plaques in Alzheimer's Disease (AD). Neurons that express APP are protected from apoptosis (Xu *et al.*, 1999), although over-expression of APP in endothelia is toxic (Jahroudi *et al.*, 1998).

APP is down-regulated in VEGF-stimulated endothelial cells at 6 and 24 hours (Example 1).

2. *Regulator of G-protein signaling receptors*

Two regulators of G-protein signaling receptors are VEGF-modulated, comprising RGS3 and gravin.

(a) Regulator of G-protein signaling 3 (RGS3, RGP3)

Prolonged stimulation of signal transduction pathways decreases responsiveness. This desensitization occurs because MAP kinase activation by G-protein-linked receptors becomes impaired. RGS3 (SEQ ID NOS:13 and 14; GenBank U27655) encodes a homologue of Sst2p, a yeast gene that mediates desensitization (Druey *et al.*, 1996). RGS3 inhibits signal transduction by increasing the GTPase activity of G-protein α subunits, driving them to the inactive GDP-bound form.

GeneCalling™ analysis (Example 1) reveals that RGS3 is up-regulated in VEGF-stimulated endothelial cells at 24 hours. *In situ* hybridization analysis reveals high expression in tumors and sarcomas, as well as in adult muscle cells (See Examples). RGS3 expression correlates with VEGF and VEGFR1 expression in ovarian cancer, suggesting that signal transduction pathways are similar between endothelial and tumor cells.

(b) Gravin/myasthenia gravis (MG) autoantigen/A kinase-anchoring proteins (AKAP 250)

Gravin (SEQ ID NOS:15 and 16; GenBank U81607) belongs to the anchoring protein family and anchors both protein kinase A and C to their subcellular sites (Nauert *et al.*, 1997). Gravin is induced by oxidative response (Sato *et al.*, 1998), and mediates recovery from agonist-induced desensitization (Shih *et al.*, 1999), as does RGS3.

GeneCalling analysis (Example 1) reveals that gravin is up-regulated in VEGF-stimulated endothelial cells at 6 and 24 hours. *In situ* hybridization analysis also demonstrates high expression in fetal tissues and non-vascular tumor components, and lower expression in adult tissue and tumor vascular components. Gravin expression correlates with VEGF expression in ovarian cancer (Examples).

3. *Mitochondrial proteins*

(a) Arginine-rich protein (ARP)

The instant invention discloses novel arginine-rich protein nucleic acid and polypeptide sequences (SEQ ID NOS:2, 3, 21 and 22; Tables 2 and 3).

Previously described human ARP (SEQ ID NOS:1 (amino acid) and 17 (nucleotide); GenBank NM_006010, M83751) maps to human chromosomal band 3p21, encoding a basic, 234 amino acid residue polypeptide. Highly conserved, ARP is found in all species examined, including hamster, rat, mouse, cow and yeast (Shridhar *et al.*, 1996a; Shridhar *et al.*, 1996b). ARP polymorphisms have been sometimes observed to correlate with neoplasia (Evron *et al.*, 1997; Shridhar *et al.*, 1996a; Shridhar *et al.*, 1996b; Shridhar *et al.*, 1997).

While Shridar (Shridhar *et al.*, 1996a) was able to define a 1 kb mRNA clone for ARP, as well as a smaller form of about 850bp. Genomic sequence anaylsis and 5' RACE were used to establish the 5' region of this clone. Contrary, the instant invention defines (CuraGen assembly No. 78893638) only a C-terminal fragment of 185 amino acid residues of the sequence deposited in GenBank. The novel nucleotide sequence (SEQ ID NO:2) and the translation of the encoded polypeptide (SEQ ID NO:3) are shown in Tables 2 and 3. Although SEQ ID NO:1 is a hydrophobic polypeptide, predicted by PSORT (Nakai and Horton, 1999) to enter the nucleus (see Fig. 2A), SEQ ID NO:3 is more hydrophilic and predicted to be nuclear localized (see Fig. 2B). Other ARP sequences include a Drosophila ARP-like protein (SEQ ID NOS:18 and 19; Genbank AF132912).

Table 2 Nucleotide sequence sequence of novel human ARP (SEQ ID NO:2)

```
atgaggagga tgaggaggat gtgggccacg caggggctgg cggtgcgcgt ggctctgagc   60
gtgctgccgg gcagccgggc gctgcggccg ggcgactgcg aagtttgtat ttcttatctg  120
ggaagatttt accaggacct caaagacaga gatgtcacat tctcaccagc cactattgaa  180
aacgaactta taaagttctg ccgggaagca agaggcaaag agaatcggtt gtgctactat  240
atcgggggca cagatgatgc agccaccaaa atcatcaatg aggtatcaaa gcctctggcc  300
caccacatcc ctgtggagaa gatctgtgag aagcttaaga agaaggacag ccagatatgt  360
gagcttaagt atgacaagca gatcgacctg agcacagtgg acctgaagaa gctccgagtt  420
aaagagctga agaagattct ggatgactgg ggggagacat gcaaaggctg tgcagaaaag  480
tctgactaca tccggaagat aaatgaactg atgcctaaat atgcccccaa ggcagccagt  540
gcaccgaccg atttgtagtc tgctcaatct ctgttgcacc tgaggggggaa aaaacagttc  600
aactgcttac tcccaaaaca gccttttttgt aatttatttt ttaagtgggc tcctgacaat  660
actgtatcag atgtgaagcc tggagctttc ctgatgatgc tggccctaca gtacccccat  720
gaggggattc ccttccttct gttgctggtg tactctagga cttcaaagtg t            771
```

Table 3 Amino acid sequence of novel human ARP (SEQ ID NO:3)

```
Met Arg Arg Met Arg Arg Met Trp Ala Thr Gln Gly Leu Ala Val Ala
1               5                  10                  15

Leu Ala Leu Ser Val Leu Pro Gly Ser Arg Ala Leu Arg Pro Gly Asp
            20                  25                  30

Cys Glu Val Cys Ile Ser Tyr Leu Gly Arg Phe Tyr Gln Asp Leu Val
        35                  40                  45

Glu Gly Phe Arg Asp Val Thr Phe Ser Pro Ala Thr Ile Glu Asn Glu
    50                  55                  60

Leu Ile Lys Phe Cys Arg Glu Ala Arg Gly Lys Glu Asn Arg Leu Cys
65                  70                  75                  80

Tyr Tyr Ile Gly Ala Thr Asp Asp Ala Ala Thr Lys Ile Ile Asn Glu
                85                  90                  95

Val Ser Lys Pro Leu Ala His His Ile Pro Val Glu Lys Ile Cys Glu
            100                 105                 110

Lys Leu Lys Lys Lys Asp Ser Gln Ile Cys Glu Leu Lys Tyr Asp Lys
        115                 120                 125

Gln Ile Asp Leu Ser Thr Val Asp Leu Lys Lys Leu Arg Val Lys Glu
    130                 135                 140

Leu Lys Lys Ile Leu Asp Asp Trp Gly Glu Thr Cys Lys Gly Cys Ala
145                 150                 155                 160

Glu Lys Ser Asp Tyr Ile Arg Lys Ile Asn Glu Leu Met Pro Lys Tyr
                165                 170                 175

Ala Pro Lys Ala Ala Ser Ala Arg Thr Asp Leu
            180                 185
```

The present invention discloses a novel gene for murine ARP, assembled from EST sequences (SEQ ID NO:20; GenBank AI595930). The murine nucleotide sequence (SEQ ID NO:21) is shown in Table 4, and the translated polypeptide sequence it encodes (SEQ ID NO:22) is shown in Table 5.

Table 4 Nucleotide sequence of novel murine ARP (SEQ ID NO:21)

```
ccgggtgcgg ttcattcgcg cggcatccgg cggtggtgga gacggctgag gaggatgtgg   60

gctacgcgcg ggctggcggt acgctggccc tgagcgtgct gcctgacagc cgggcgctgc  120

ggccaggaga ctgtgaagtt tgtatttctt atctgggacg attttaccag gacctcaaag  180

acagagatgt cacattttca ccagccacta ttgaagaaga acttataaag ttttgccgtg  240
```

```
aagcaagagg caaagagaat cggttgtgct actacattgg agccacagat gatgctgcca  300

ccaagatcat caatgaggtg tcgaagcccc tggcccacca tatccctgtg gaaaagatct  360

gtgagaagct gaagaagaaa gacagccaga tctgtgaact aaaatacgac aagcagattg  420

acctgagcac agtggacctg aagaagctcc gggtgaaaga gctgaagaag atcctggacg  480

actgggggga gatgtgcaaa ggctgtgcag aaaagtctga ctatatccgg aagataaatg  540

aactgatgcc taaatacgcc cccaaggcag ccagcgcacg gactgatctg tagtctgccc  600

aattcctgct gcacctgaag gggaaaaagc agtttatctg tctcttcccc aaataaccat  660

tttgtaattt atttttaag cgggctcctg acaatgagat gtgaacctag agctttccta  720

gtgatgctgg ttttgcagtt ccctcttgcc catccccgag tggggacaat ttccccatcc  780

ccaagtgggg acaatttact tccttctttg ctggtttact ctaggacttc aaagtttgtc  840

tgggattttt ttattaaaaa aaattgtctt tggagagtta aaaaaaaaaa             890
```

Table 5 Amino acid sequence novel murine ARP (SEQ ID NO:22)

```
Gly Cys Gly Ser Phe Ala Arg His Pro Ala Val Val Glu Thr Ala Glu
1               5                  10                  15

Glu Asp Val Gly Tyr Ala Arg Ala Gly Gly Thr Leu Ala Leu Ser Val
            20                  25                  30

Leu Pro Asp Ser Arg Ala Leu Arg Pro Gly Asp Cys Glu Val Cys Ile
        35                  40                  45

Ser Tyr Leu Gly Arg Phe Tyr Gln Asp Leu Val Glu Gly Phe Arg Asp
    50                  55                  60

Val Thr Phe Ser Pro Ala Thr Ile Glu Glu Glu Leu Ile Lys Phe Cys
65                  70                  75                  80

Arg Glu Ala Arg Gly Lys Glu Asn Arg Leu Cys Tyr Tyr Ile Gly Ala
                85                  90                  95

Thr Asp Asp Ala Ala Thr Lys Ile Ile Asn Glu Val Ser Lys Pro Leu
            100                 105                 110

Ala His His Ile Pro Val Glu Lys Ile Cys Glu Lys Leu Lys Lys Lys
        115                 120                 125

Asp Ser Gln Ile Cys Glu Leu Lys Tyr Asp Lys Gln Ile Asp Leu Ser
    130                 135                 140

Thr Val Asp Leu Lys Lys Leu Arg Val Lys Glu Leu Lys Lys Ile Leu
145                 150                 155                 160
```

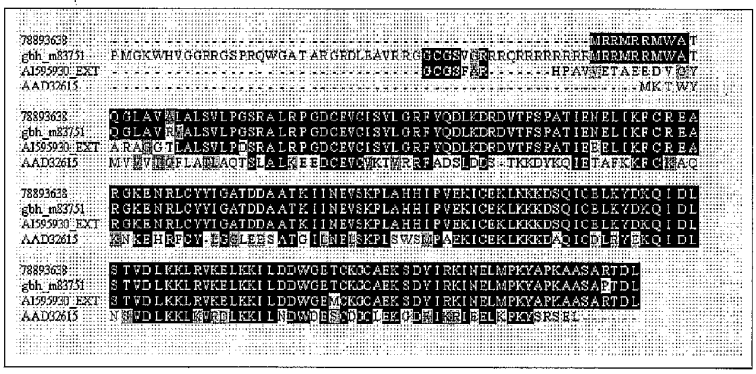
```
Asp Asp Trp Gly Glu Met Cys Lys Gly Cys Ala Glu Lys Ser Asp Tyr
                 165                 170                 175

Ile Arg Lys Ile Asn Glu Leu Met Pro Lys Tyr Ala Pro Lys Ala Ala
            180                 185                 190

Ser Ala Arg Thr Asp Leu
        195
```

Table 6 shows the alignment of the novel human ARP of the instant invention (Curagen assembly 78893608; SEQ ID NO:3), a published human sequence (Shridar *et al.* (1996b); gbh_m83751), mouse (AI595930_EXT), and *Drosophila melanogaster* (AAD32615) using ClustalW alignment. Only the protein described by Shridar et al. (1996b) has the longer N-terminal sequence; while that of the instant invention is truncated at the N-terminus.

Table 6 Alignment of human and mouse ARP



GeneCalling™ analysis (Example 1) reveals that ARP is up-regulated in VEGF-stimulated endothelial cells during the first 6 hours. *In situ* hybridization analysis reveals high expression in fetal and non-vascularized tumor components. Over-expression of ARP correlates with ovarian cancer.

(b) Down's syndrome critical region protein 1 (DSCR1)

DSCR1 (SEQ ID NOS:23 and 24; GenBank NM_004414, U28833) is a member of the minimal candidate region for the Down syndrome phenotype. DSCR1

has an acidic domain, a serine-proline motif, a putative DNA binding domain and a proline-rich region, much like SH3 domain ligands (Fuentes *et al.*, 1995). The hamster homologue, adapt78, is related to Gpr78, a glucose-regulated protein (Leahy *et al.*, 1999) and is oxidant- and calcium-inducible. PSORT (Nakai and Horton, 1999) predicts mitochondrial localization. DSCR1's structural and functional features suggest roles in transcriptional regulation and/or signal transduction.

GeneCalling™ analysis (Example 1) demonstrated that DSCR1 is up-regulated in VEGF-stimulated endothelial cells during the first 6 hours. Taqman™ analysis revealed that DSCR1 is up-regulated in an *in vitro* model of endothelial tube formation. *In situ* hybridization analysis reveals high expression in fetal tissues, but lower levels in adult and tumor non-vascular tissues. Over-expression of DSCR1 correlates with clinical stage of ovarian cancer. Elimination of DSCR1 by antisense experiments increases endothelial cell survival.

4. *Other VEGF-modulated genes*

(a) Human gene similar to yeast VPS41 (hVSP41p)

hVSP41p (SEQ ID NOS:25 and 26; GenBank U87309) in yeast n(*VSP41*) is required for vacuolar traffic (Radisky *et al.*, 1997) and is involved in endocytosis (Singer-Kruger and Ferro-Novick, 1997).

In the present invention, GeneCalling analysis (Example 1) reveals that hVPS41 is down-regulated in VEGF-stimulated endothelial cells at 24 hours. *In situ* hybridization analysis localised expression to non-vascularized regions of tumors. Expression of hVSP41 correlates with ovarian cancer (Examples).

(b) insulin induced gene 1 (INSIG1)

INSIG1 (SEQ ID NOS:27 and 28; GenBank 5031800, U96876) expression is transcriptionally up-regulated in rat regenerating livers, and is induced in murine adipocyte differentiation, suggesting that INSIG1 may play a role in growth and differentiation of tissues involved in metabolic control (Peng *et al.*, 1997). INSIG1 is also expressed by monocytes in a model of atherogenesis, as are oxidized lipoprotein HB-EGF and gravin (Falb, WO9730065, 1997). Hydrophobicity analysis predicts a transmembrane localization. The protein is homologous to sodium channels and to G-

protein coupled receptors. PSORT (Nakai and Horton, 1999) predicts localization to the mitochondrial inner membrane.

GeneCalling analysis (Example 1) demonstrated that INSIG1 was up-regulated in VEGF-stimulated endothelial cells at 24 hours and in an *in vitro* model of endothelial tube formation.

(c) decidual protein induced by progesterone (DEPP)

DEPP (SEQ ID NOS:29 and 30; GenBank AB022718) is published only in the database. SEQ ID NO:29 comprises a 2114 bp transcript encoding a putative 212 amino acid peptide that is induced by the steroid progesterone. Steroid hormones play vital roles in angiogenesis, especially in the female reproductive tract (Hyder and Stancel, 1999).

GeneCalling analysis (Example 1) reveals that DEPP was up-regulated in VEGF-stimulated endothelial cells at 6 hours.

(d) cytochrome oxidase subunit I (MTCO1)

Cytochrome c oxidase subunit I (MTCO1, SEQ ID NO:31 (nucleotide sequence extracted from the complete human mitochondrial genome sequence, GenBank NC_001807) and SEQ ID NO:32 (amino acid; GenBank NP_008344) is 1 of 3 mitochondrial DNA encoded subunits of respiratory Complex IV. Complex IV localizes to the mitochondrial inner membrane and mediates the final step in the electron transport chain of oxidative phosphorylation. Complex IV collects electrons from reduced cytochrome c and transfers them to oxygen, producing energy and water. The released energy is used to transport protons across the mitochondrial inner membrane.

(e) NADH-ubiquinone oxidoreductase chain 1 (ND1 or DNHUN1) and

(f) NADH-ubiquinone oxidoreductase chain 4 (ND4 or DNHUN4)

The proton-translocating NADH:ubiquinone oxidoreductase or complex I chain 1 (SEQ ID NOS:33, GenBank NC_001807and 34; GenBank DUNHUN1) and chain 4 (SEQ ID NOS:35, GenBank NC_001807 and 36; GenBank DUNHUN4) are located in the inner membranes of mitochondria. Complex I is the site for electrons

entering the respiratory chain and important in conserving cell energy. The complex I-catalyzed oxidation of NADH is coupled to proton membrane translocation.

(g) heparin-binding EGF-like growth factor (HB-EGF)

HB-EGF (SEQ ID NOS:37 and 38; GenBank NM_001945) is an EGF family member that ligates EGF receptors 1(HER-1) or 4 (HER-4) to induce mitogenic and/or chemotactic activities. HB-EGF is expressed by numerous cell types, including leukemia cells (Vinante *et al.*, 1999), and does not directly induce endothelial cell mitosis, but does induce these cells to migrate and induces the vascular smooth muscle cells to release factors that induce endothelia mitosis (Morita *et al.*, 1993). While previously observed to be induced by VEGF (Arkonac *et al.*, 1998), no specific role in endothelial cell survival has been proposed.

In addition to VEGF, reactive oxygen species and calcium induce HB-EGF expression (Kayanoki *et al.*, 1999) as they do for DSCR1. Membrane-bound HB-EGF retains growth activity, adhesion capabilities and promotes renal epithelial cellssurvival (Takemura *et al.*, 1997). ProHB- EGF forms a complex in the plasma membrane with the tetraspanin CD9 that also increases the survival activity of HB-EGF expression (Takemura *et al.*, 1999).

In the instant invention (Examples), HB-EGF was found to be up-regulated in VEGF-stimulated endothelial cells at 24 hours. *In situ* hybridization analysis reveals expression in non vascular component in tumors, fetal and adult tissue, and high expression in endothelial cells of the appendix.

(h) MKP-1 like protein tyrosine phosphatase (SEQ ID NOS:39 and 40; GenBank AF038844)

The protein sequence is 58% similar to Mitogen-activated protein (MAP) kinase phosphatase-1 (MKP-1), a dual-specificity protein tyrosine phosphatase. Homology for the catalytic domain is very high, although no specific substrate has yet been described for MKP-1 like protein tyrosine phosphatase. MAP kinase cascades play critical roles in inhibiting apoptosis, phosphorylating Bcl-2 (Deng *et al.*, 2000). MAP kinases are activated by tyrosine and threonine phosphorylation and inactivated by dephosphorylation (Wilkinson and Millar, 2000). MKP-1 increases cell survival

(Winter *et al.*, 1998), and is induced by elevated calcium (Scimeca *et al.*, 1997). Because of its similarly to MKP-1, the MKP-1-like protein tyrosine phosphatase may regulate one or more MAP kinases involved in cell survival.

(i) osteonidogen (nidogen-2 precursor)

Nidogen-2 (SEQ ID NOS:41 and 42; GenBank D86425) is 46% identical, and has a similar domain structure with the basement membrane (basal lamina) protein nidogen-1/enactin. Nidogens 1 and 2 have similar but distinct binding and adhesive properties for basement membrane components (Lohi *et al.*, 1998). The complex laminin-entactin can stimulate and inhibit angiogenesis in a dose-dependent fashion (Nicosia *et al.*, 1994).

In the present invention, GeneCalling analysis (Example 1) reveals that nidogen-2 is up-regulated in VEGF-stimulated endothelial cells at 6 and 24 hours. *In situ* hybridization analysis demonstrates expression in fetal tissues, inflamed appendix and vascular and non-vascular component of peritumoral stroma. (Oivula *et al.*, 1999) also report expression by the endothelial basal lamina and stroma in carcinomas.

(j) *Connective tissue growth factor (CTGF)*

CTGF (connective tissue growth factor; SEQ ID NOS: 43 and 44, GenBank X78947) is a member of a family of secreted proteins that includes CYR61, Nov, Elm-1, Cop-1/WISP-2, WISP-3 and the mouse CTGF homolog, Fisp12. CTGF stimulates fibroblast migration and promotes adhesion and mitogenesis in both fibroblasts and endothelial cells through the integrin receptor $\alpha v\beta 3$. In addition, the presence of CTGF promotes endothelial cell survival. *In vivo*, CTGF induces neovascularization in rat corneal micropocket implants.

In the instant invention, CTGF is up-regulated in VEGF-stimulated endothelial cells at 6 and 24 hours. *In situ* hybridization analysis reveals that CTGF is expressed in most tested tissues, which the highest expression in fetal tissues. These observations, with the localization of CTGF in angiogenic tissues and in atherosclerotic plaques, suggest a possible role for CTGF in the regulation of vessel growth during development, wound healing, and vascular disease.

VEGFmg polynucleotides

One aspect of the invention pertains to isolated nucleic acid molecules that encode VEGFmg or biologically-active portions thereof. Also included in the invention are nucleic acid fragments sufficient for use as hybridization probes to identify VEGFmg-encoding nucleic acids (*e.g.*, *VEGFmg* mRNAs) and fragments for use as polymerase chain reaction (PCR) primers for the amplification and/or mutation of *VEGFmg* molecules. A "nucleic acid molecule" includes DNA molecules (*e.g.*, cDNA or genomic DNA), RNA molecules (*e.g.*, mRNA), analogs of the DNA or RNA generated using nucleotide analogs, and derivatives, fragments and homologs. The nucleic acid molecule may be single-stranded or double-stranded, but preferably comprises double-stranded DNA.

1. *control sequences*

Control sequence are DNA sequences that enable the expression of an operably-linked coding sequence in a particular host organism. Prokaryotic control sequences include promoters, operator sequences, and ribosome binding sites. Eukaryotic cells utilize promoters, polyadenylation signals, and enhancers.

2. *operably-linked*

Nucleic acid is operably-linked when it is placed into a functional relationship with another nucleic acid sequence. For example, a promoter or enhancer is operably-linked to a coding sequence if it affects the transcription of the sequence, or a ribosome-binding site is operably-linked to a coding sequence if positioned to facilitate translation. Generally, "operably-linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by conventional recombinant DNA methods.

3. *isolated nucleic acids*

An isolated nucleic acid molecule is purified from the setting in which it is found in nature and is separated from at least one contaminant nucleic acid molecule. Isolated *ARP* molecules are distinguished from the specific *ARP* molecules, as they exist in cells. However, an isolated *ARP* molecule includes *ARP* molecules contained in cells that ordinarily express the ARP where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

4. *probes*

Probes are nucleic acid sequences of variable length, preferably between at least about 10 nucleotides (nt), 100 nt, or many (*e.g.*, 6,000 nt) depending on the specific use. Probes are used to detect identical, similar, or complementary nucleic acid sequences. Longer length probes can be obtained from a natural or recombinant source, are highly specific, and much slower to hybridize than shorter-length oligomer probes. Probes may be single- or double-stranded and designed to have specificity in PCR, membrane-based hybridization technologies, or ELISA-like technologies. Probes are substantially purified oligonucleotides that will hybridize under stringent conditions to at least optimally12, 25, 50, 100, 150, 200, 250, 300, 350 or 400 consecutive sense strand nucleotide sequence; or an anti-sense strand nucleotide sequence; or of a naturally occurring mutant of the *VEGFmg* sequence of interest.

The full- or partial length native sequence *VEGFmg* may be used to "pull out" similar (homologous) sequences (Ausubel *et al.*, 1987; Sambrook, 1989), such as: (1) full-length or fragments of *VEGFmg* cDNA from a cDNA library from any species (*e.g.* human, murine, feline, canine, bacterial, viral, retroviral, yeast), (2) from cells or tissues, (3) variants within a species, and (4) homologues and variants from other species. To find related sequences that may encode related genes, the probe may be designed to encode unique sequences or degenerate sequences. Sequences may also be genomic sequences including promoters, enhancer elements and introns of native sequence *VEGFmg*.

For example, *VEGFmg* coding region in another species may be isolated using such probes. A probe of about 40 bases is designed, based on *VEGFmg*, and made. To detect hybridizations, probes are labeled using, for example, radionuclides such as ^{32}P or ^{35}S, or enzymatic labels such as alkaline phosphatase coupled to the probe via avidin-biotin systems. Labeled probes are used to detect nucleic acids having a complementary sequence to that of *VEGFmg* in libraries of cDNA, genomic DNA or mRNA of a desired species.

Such probes can be used as a part of a diagnostic test kit for identifying cells or tissues which mis-express a VEGFmg, such as by measuring a level of a VEGFmg in a sample of cells from a subject *e.g.*, detecting *VEGFmg* mRNA levels or determining whether a genomic *VEGFmg* has been mutated or deleted.

5. *isolated nucleic acid*

An isolated nucleic acid molecule is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. Preferably, an isolated nucleic acid is free of sequences that naturally flank the nucleic acid (*i.e.*, sequences located at the 5'- and 3'-termini of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, isolated *VEGFmg* molecules can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell/tissue from which the nucleic acid is derived (*e.g.*, brain, heart, liver, spleen, *etc.*). Moreover, an isolated nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material or culture medium when produced by recombinant techniques, or of chemical precursors or other chemicals when chemically synthesized.

A nucleic acid molecule of the invention, *e.g.*, a *VEGFmg* nucleic acid molecule, or a complement of this aforementioned nucleotide sequence, can be isolated using standard molecular biology techniques and the provided sequence information. Using all or a portion of a *VEGFmg* nucleic acid sequence of interest as a hybridization probe, *VEGFmg* molecules can be isolated using standard hybridization and cloning techniques (Ausubel *et al.*, 1987; Sambrook, 1989).

PCR amplification techniques can be used to amplify *VEGFmg* using cDNA, mRNA or alternatively, genomic DNA, as a template and appropriate oligonucleotide primers. Such nucleic acids can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to *VEGFmg* sequences can be prepared by standard synthetic techniques, *e.g.*, an automated DNA synthesizer.

6. *oligonucleotide*

An oligonucleotide comprises a series of linked nucleotide residues, which oligonucleotide has a sufficient number of nucleotide bases to be used in a PCR reaction or other application. A short oligonucleotide sequence may be based on, or designed from, a genomic or cDNA sequence and is used to amplify, confirm, or reveal the presence of an identical, similar or complementary DNA or RNA in a particular cell or tissue. Oligonucleotides comprise portions of a nucleic acid

sequence having about 10 nt, 50 nt, or 100 nt in length, preferably about 15 nt to 30 nt in length. In one embodiment of the invention, an oligonucleotide comprising a nucleic acid molecule less than 100 nt in length would further comprise at least 6 contiguous nucleotides of a *VEGFmg* sequence of interest, or a complement thereof. Oligonucleotides may be chemically synthesized and may also be used as probes.

7. *complementary nucleic acid sequences; binding*

In another embodiment, an isolated nucleic acid molecule comprises a nucleic acid molecule that is a complement of a *VEGFmg* nucleotide sequence of the invention, or a portion of this nucleotide sequence (*e.g.*, a fragment that can be used as a probe or primer or a fragment encoding a biologically-active portion of a VEGFmg). A nucleic acid molecule that is complementary to a *VEGFmg* nucleotide sequence of interest, is one that is sufficiently complementary to that nucleotide sequence such that it can hydrogen bond with little or no mismatches, forming a stable duplex.

"Complementary" refers to Watson-Crick or Hoogsteen base pairing between nucleotides units of a nucleic acid molecule, and the term "binding" means the physical or chemical interaction between two polypeptides or compounds or associated polypeptides or compounds or combinations thereof. Binding includes ionic, non-ionic, van der Waals, hydrophobic interactions, and the like. A physical interaction can be either direct or indirect. Indirect interactions may be through or due to the effects of another polypeptide or compound. Direct binding refers to interactions that do not take place through, or due to, the effect of another polypeptide or compound, but instead are without other substantial chemical intermediates.

Nucleic acid fragments are at least 6 (contiguous) nucleic acids or at least 4 (contiguous) amino acids, a length sufficient to allow for specific hybridization in the case of nucleic acids or for specific recognition of an epitope in the case of amino acids, respectively, and are at most some portion less than a full-length sequence. Fragments may be derived from any contiguous portion of a nucleic acid or amino acid sequence of choice.

8. *derivatives, and analogs*

Derivatives are nucleic acid sequences or amino acid sequences formed from the native compounds either directly or by modification or partial substitution.

Analogs are nucleic acid sequences or amino acid sequences that have a structure similar to, but not identical to, the native compound but differ from it in respect to certain components or side chains. Analogs may be synthetic or from a different evolutionary origin and may have a similar or opposite metabolic activity compared to wild type. Homologs are nucleic acid sequences or amino acid sequences of a particular gene that are derived from different species.

Derivatives and analogs may be full length or other than full length, if the derivative or analog contains a modified nucleic acid or amino acid, as described below. Derivatives or analogs of the nucleic acids or proteins of the invention include, but are not limited to, molecules comprising regions that are substantially homologous to the nucleic acids or proteins of the invention, in various embodiments, by at least about 70%, 80%, or 95% identity (with a preferred identity of 80-95%) over a nucleic acid or amino acid sequence of identical size or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art, or whose encoding nucleic acid is capable of hybridizing to the complement of a sequence encoding the aforementioned proteins under stringent, moderately stringent, or low stringent conditions (Ausubel *et al.*, 1987).

9. *open reading frames*

The open reading frame (ORF) of a *VEGFmg* gene encodes VEGFmg. An ORF is a nucleotide sequence that has a start codon (ATG) and terminates with one of the three "stop" codons (TAA, TAG, or TGA). In this invention, however, an ORF may be any part of a coding sequence that may or may not comprise a start codon and a stop codon. To achieve a unique sequence, preferable *VEGFmg* ORFs encode at least 50 amino acids.

10. *homology*

A "homologous nucleic acid sequence" or "homologous amino acid sequence," or variations thereof, refer to sequences characterized by a homology at the nucleotide level or amino acid level as discussed above. Homologous nucleotide sequences encode those sequences coding for isoforms of VEGFmg. Isoforms can be expressed in different tissues of the same organism as a result of, for example, alternative splicing of RNA. Alternatively, different genes can encode isoforms. In the invention, homologous nucleotide sequences include nucleotide sequences

encoding for a VEGFmg of species other than humans, including, but not limited to: vertebrates, and thus can include, *e.g.*, frog, mouse, rat, rabbit, dog, cat cow, horse, and other organisms. Homologous nucleotide sequences also include, but are not limited to, naturally occurring allelic variations and mutations of the nucleotide sequences set forth herein. A homologous nucleotide sequence does not, however, include the exact nucleotide sequence encoding a human VEGFmg. Homologous nucleic acid sequences include those nucleic acid sequences that encode conservative amino acid substitutions in a *VEGFmg* sequence of interest, as well as a polypeptide possessing VEGFmg biological activity. Various biological activities of the VEGFmg are described below.

11. *sequence identity*

"Percent (%) nucleic acid sequence identity" with respect to a *VEGFmg* is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in that particular *VEGFmg*, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining % nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

When nucleotide sequences are aligned, the % nucleic acid sequence identity of a given nucleic acid sequence C to, with, or against a given nucleic acid sequence D (which can alternatively be phrased as a given nucleic acid sequence C that has or comprises a certain % nucleic acid sequence identity to, with, or against a given nucleic acid sequence D) can be calculated as follows:

$$\%_{\text{nucleic acid sequence identity}} = W/Z \cdot 100$$

where

W is the number of nucleotides cored as identical matches by the sequence alignment program's or algorithm's alignment of C and D

And

Z is the total number of nucleotides in D.

When the length of nucleic acid sequence C is not equal to the length of nucleic acid sequence D, the % nucleic acid sequence identity of C to D will not equal the % nucleic acid sequence identity of D to C.

12. *Stringency*

Homologs (*i.e.*, nucleic acids encoding VEGF-modulated molecules derived from species other than human) or other related sequences (*e.g.*, paralogs) can be obtained by low, moderate or high stringency hybridization with all or a portion of the particular human sequence as a probe using methods well known in the art for nucleic acid hybridization and cloning.

The specificity of single stranded DNA to hybridize complementary fragments is determined by the "stringency" of the reaction conditions. Hybridization stringency increases as the propensity to form DNA duplexes decreases. In nucleic acid hybridization reactions, the stringency can be chosen to either favor specific hybridizations (high stringency), which can be used to identify, for example, full-length clones from a library. Less-specific hybridizations (low stringency) can be used to identify related, but not exact, DNA molecules (homologous, but not identical) or segments.

DNA duplexes are stabilized by: (1) the number of complementary base pairs, (2) the type of base pairs, (3) salt concentration (ionic strength) of the reaction mixture, (4) the temperature of the reaction, and (5) the presence of certain organic solvents, such as formamide which decreases DNA duplex stability. In general, the longer the probe, the higher the temperature required for proper annealing. A common approach is to vary the temperature: higher relative temperatures result in more stringent reaction conditions. (Ausubel *et al.*, 1987) provide an excellent explanation of stringency of hybridization reactions.

To hybridize under "stringent conditions" describes hybridization protocols in which nucleotide sequences at least 60% homologous to each other remain hybridized. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength

and *p*H. The Tm is the temperature (under defined ionic strength, *p*H and nucleic acid concentration) at which 50% of the probes complementary to the target sequence hybridize to the target sequence at equilibrium. Since the target sequences are generally present at excess, at Tm, 50% of the probes are occupied at equilibrium.

(a) *high stringency*

"Stringent hybridization conditions" conditions enable a probe, primer or oligonucleotide to hybridize only to its target sequence. Stringent conditions are sequence-dependent and will differ. Stringent conditions comprise: (1) low ionic strength and high temperature washes (*e.g.* 15 mM sodium chloride, 1.5 mM sodium citrate, 0.1 % sodium dodecyl sulfate at 50°C); (2) a denaturing agent during hybridization (*e.g.* 50% (v/v) formamide, 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 50mM sodium phosphate buffer (pH 6.5; 750 mM sodium chloride, 75 mM sodium citrate at 42°C); or (3) 50% formamide. Washes typically also comprise 5X SSC (0.75 M NaCl, 75 mM sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1 x SSC containing EDTA at 55°C. Preferably, the conditions are such that sequences at least about 65%, 70%, 75%, 85%, 90%, 95%, 98%, or 99% homologous to each other typically remain hybridized to each other. These conditions are presented as examples and are not meant to be limiting.

(b) *moderate stringency*

"Moderately stringent conditions" use washing solutions and hybridization conditions that are less stringent (Sambrook, 1989), such that a polynucleotide will hybridize to the entire, fragments, derivatives or analogs of a target *VEGFmg* target sequence. One example comprises hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 mg/ml denatured salmon sperm DNA at 55°C, followed by one or more washes in 1X SSC, 0.1% SDS at 37°C. The temperature, ionic strength, *etc.*, can be adjusted to accommodate experimental factors such as probe length. Other moderate stringency conditions are described in (Ausubel *et al.*, 1987; Kriegler, 1990).

(c) *low stringency*

"Low stringent conditions" use washing solutions and hybridization conditions that are less stringent than those for moderate stringency (Sambrook, 1989), such that a polynucleotide will hybridize to the entire, fragments, derivatives or analogs of a target *VEGFmg* target sequence. A non-limiting example of low stringency hybridization conditions are hybridization in 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 mg/ml denatured salmon sperm DNA, 10% (wt/vol) dextran sulfate at 40°C, followed by one or more washes in 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS at 50°C. Other conditions of low stringency, such as those for cross-species hybridizations are described in (Ausubel *et al.*, 1987; Kriegler, 1990; Shilo and Weinberg, 1981).

13. *Conservative mutations*

In addition to naturally-occurring allelic variants of *VEGFmg*, changes can be introduced by mutation into *VEGFmg* sequences that incur alterations in the amino acid sequences of the encoded VEGF-modulated molecules that do not alter VEGF-modulated molecules function. For example, nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues can be made in the sequence of a VEGFmg polypeptide. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequences of VEGFmg without altering their biological activity, whereas an "essential" amino acid residue is required for such biological activity. For example, amino acid residues that are conserved among the VEGFmg molecules of the invention are predicted to be particularly non-amenable to alteration. Amino acids for which conservative substitutions can be made are well-known in the art.

Useful conservative substitutions are shown in Table A, "Preferred substitutions." Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution does not materially alter the biological activity of the compound. If such substitutions result in a change in biological activity, then more substantial changes, indicated in Table B as exemplary are introduced and the products screened for VEGFmg polypeptide biological activity.

Table A Preferred substitutions

Original residue	Exemplary substitutions	Preferred substitutions
Ala (A)	Val, Leu, Ile	Val
Arg (R)	Lys, Gln, Asn	Lys
Asn (N)	Gln, His, Lys, Arg	Gln
Asp (D)	Glu	Glu
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn
Glu (E)	Asp	Asp
Gly (G)	Pro, Ala	Ala
His (H)	Asn, Gln, Lys, Arg	Arg
Ile (I)	Leu, Val, Met, Ala, Phe, Norleucine	Leu
Leu (L)	Norleucine, Ile, Val, Met, Ala, Phe	Ile
Lys (K)	Arg, Gln, Asn	Arg
Met (M)	Leu, Phe, Ile	Leu
Phe (F)	Leu, Val, Ile, Ala, Tyr	Leu
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr, Phe	Tyr
Tyr (Y)	Trp, Phe, Thr, Ser	Phe
Val (V)	Ile, Leu, Met, Phe, Ala, Norleucine	Leu

Non-conservative substitutions that effect (1) the structure of the polypeptide backbone, such as a β-sheet or α-helical conformation, (2) the charge or (3) hydrophobicity, or (4) the bulk of the side chain of the target site can modify VEGFmg function or immunological identity. Residues are divided into groups based on common side-chain properties as denoted in Table B. Non-conservative substitutions entail exchanging a member of one of these classes for another class. Substitutions may be introduced into conservative substitution sites or more preferably into non-conserved sites.

Table B Amino acid classes

Class	Amino acids
hydrophobic	Norleucine, Met, Ala, Val, Leu, Ile
neutral hydrophilic	Cys, Ser, Thr

acidic	Asp, Glu
basic	Asn, Gln, His, Lys, Arg
disrupt chain conformation	Gly, Pro
aromatic	Trp, Tyr, Phe

The variant polypeptides can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis (Carter, 1986; Zoller and Smith, 1987), cassette mutagenesis, restriction selection mutagenesis (Wells *et al.*, 1985) or other known techniques can be performed on the cloned DNA to produce the *VEGFmg* variant DNA (Ausubel *et al.*, 1987; Sambrook, 1989).

In one embodiment, the isolated nucleic acid molecule comprises a nucleotide sequence encoding a protein, wherein the protein comprises an amino acid sequence at least about 45%, preferably 60%, more preferably 70%, 80%, 90%, and most preferably about 95% homologous to that of a *VEGFmg* of interest.

A mutant VEGFmg can be assayed for modulating cell survival and/or angiogenesis *in vitro*.

14. VEGFmg *variant polynucleotides, genes and recombinant genes*

The invention further encompasses nucleic acid molecules that differ from the nucleotide sequences due to degeneracy of the genetic code and thus encode the same VEGFmg as that encoded by, for example, the *ARP* nucleotide sequences shown in SEQ ID NO NOS:2 or 21. An isolated nucleic acid molecule of the invention has a nucleotide sequence encoding, for example, an ARP protein having an amino acid sequence shown in SEQ ID NOS:3 or 22.

In addition sequence polymorphisms that change the amino acid sequences of the VEGFmg may exist within a population. For example, allelic variation among individuals will exhibit genetic polymorphism in *VEGFmg*. The terms "gene" and "recombinant gene" refer to nucleic acid molecules comprising an open reading frame (ORF) encoding VEGFmg, preferably a vertebrate VEGFmg. Such natural allelic variations can typically result in 1-5% variance in *VEGFmg*. Any and all such nucleotide variations and resulting amino acid polymorphisms in the VEGFmg, which are the result of natural allelic variation and that do not alter the functional activity of the VEGFmg are within the scope of the invention.

Moreover, *VEGFmg* from other species that have a nucleotide sequence that differs from the human sequence of *VEGFmgs* are contemplated. Nucleic acid molecules corresponding to natural allelic variants and homologues of *VEGFmg* cDNAs of the invention can be isolated based on their homology to *VEGFmg* using cDNA-derived probes to hybridize to homologous *VEGFmg* sequences under stringent conditions.

"VEGFmg variant polynucleotide" or "VEGFmg variant nucleic acid sequence" means a nucleic acid molecule which encodes an active VEGFmg that (1) has at least about 80% nucleic acid sequence identity with a nucleotide acid sequence encoding a full-length native VEGFmg, (2) a full-length native VEGFmg lacking the signal peptide, (3) an extracellular domain of a VEGFmg, with or without the signal peptide, or (4) any other fragment of a full-length VEGFmg. Ordinarily, a *VEGFmg* variant polynucleotide will have at least about 80% nucleic acid sequence identity, more preferably at least about 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% nucleic acid sequence identity and yet more preferably at least about 99% nucleic acid sequence identity with the nucleic acid sequence encoding a full-length native VEGFmg. A *VEGFmg* variant polynucleotide may encode full-length native VEGFmg lacking the signal peptide, an extracellular domain of a VEGFmg, with or without the signal sequence, or any other fragment of a full-length VEGFmg. Variants do not encompass the native nucleotide sequence.

Ordinarily, *VEGFmg* variant polynucleotides are at least about 30 nucleotides in length, often at least about 60, 90, 120, 150, 180, 210, 240, 270, 300, 450, 600 nucleotides in length, more often at least about 900 nucleotides in length, or more.

VEGFmg polypeptides

1. *mature*

A VEGFmg can encode a mature VEGFmg. A "mature" form of a polypeptide or protein disclosed in the present invention is the product of a naturally occurring polypeptide or precursor form or proprotein. The naturally occurring polypeptide, precursor or proprotein includes, by way of nonlimiting example, the

full-length gene product, encoded by the corresponding gene. Alternatively, it may be defined as the polypeptide, precursor or proprotein encoded by an open reading frame described herein. The product "mature" form arises, again by way of nonlimiting example, as a result of one or more naturally occurring processing steps as they may take place within the cell, or host cell, in which the gene product arises. Examples of such processing steps leading to a "mature" form of a polypeptide or protein include the cleavage of the N-terminal methionine residue encoded by the initiation codon of an open reading frame, or the proteolytic cleavage of a signal peptide or leader sequence. Thus a mature form arising from a precursor polypeptide or protein that has residues 1 to N, where residue 1 is the N-terminal methionine, would have residues 2 through N remaining after removal of the N-terminal methionine. Alternatively, a mature form arising from a precursor polypeptide or protein having residues 1 to N, in which an N-terminal signal sequence from residue 1 to residue M is cleaved, would have the residues from residue M+1 to residue N remaining. Further as used herein, a "mature" form of a polypeptide or protein may arise from a step of post-translational modification other than a proteolytic cleavage event. Such additional processes include, by way of non-limiting example, glycosylation, myristoylation or phosphorylation. In general, a mature polypeptide or protein may result from the operation of only one of these processes, or a combination of any of them.

2. *Isolated VEGFmg polypeptide*

An "isolated" or "purified" polypeptide, protein or biologically active fragment is separated and/or recovered from a component of its natural environment. Contaminant components include materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous materials. Preferably, the polypeptide is purified to a sufficient degree to obtain at least 15 residues of N-terminal or internal amino acid sequence. To be substantially isolated, preparations having less than 30% by dry weight of non-VEGFmg contaminating material (contaminants), more preferably less than 20%, 10% and most preferably less than 5% contaminants. An isolated, recombinantly-produced VEGFmg or biologically active portion is preferably substantially free of culture medium, *i.e.*, culture medium

represents less than 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the VEGFmg preparation. Examples of contaminants include cell debris, culture media, and substances used and produced during *in vitro* synthesis of VEGFmg.

When the molecule is a purified polypeptide, the polypeptide will be purified (1) to obtain at least 15 residues of N-terminal or internal amino acid sequence using a sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or silver stain. Isolated polypeptides include those expressed heterologously in genetically-engineered cells or expressed *in vitro*, since at least one component of the VEGFmg's natural environment will not be present. Ordinarily, isolated polypeptides are prepared by at least one purification step.

3. *Biologically active*

Biologically active portions of VEGFmgs include peptides comprising amino acid sequences sufficiently homologous to or derived from VEGFmg amino acid sequences that include fewer amino acids than the full-length VEGFmg, and exhibit at least one activity of a VEGFmg. Biologically active portions comprise a domain or motif with at least one activity of native VEGFmg. A biologically active portion of a VEGFmg can be a polypeptide that is, for example, 10, 25, 50, 100 or more amino acid residues in length. Other biologically active portions, in which other regions of the protein are deleted, can be prepared by recombinant techniques and evaluated for one or more of the functional activities of a native VEGFmg.

Biologically active portions of VEGFmg may retain the functional activity of the protein, yet differs in amino acid sequence due to natural allelic variation or mutagenesis.

4. *anti-VEGFmg Abs*

Antibody may be single anti-VEGFmg monoclonal Abs (mAbs; including agonist, antagonist, and neutralizing Abs), anti-VEGFmg antibody compositions with polyepitopic specificity, single chain anti-VEGFmg Abs, and fragments of anti-VEGFmg Abs. A "monoclonal antibody" refers to an antibody obtained from a population of substantially homogeneous Abs, *i.e.*, the individual Abs comprising the population are identical except for naturally-occurring mutations that may be present in minor amounts.

5. *epitope tags*

An epitope tagged polypeptide refers to a chimeric polypeptide fused to a "tag polypeptide". Such tags provide epitopes against which Abs can be made or are available, but do not interfere with polypeptide activity. To reduce anti-tag antibody reactivity with endogenous epitopes, the tag polypeptide is preferably unique. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8 and 50 amino acid residues, preferably between 8 and 20 amino acid residues). Examples of epitope tag sequences include HA from *Influenza* A virus and FLAG.

6. *Variant VEGFmg polypeptides*

In general, a VEGFmg variant that preserves VEGFmg-like function and includes any variant in which residues at a particular position in the sequence have been substituted by other amino acids, and further includes the possibility of inserting an additional residue or residues between two residues of the parent protein as well as the possibility of deleting one or more residues from the parent sequence. Any amino acid substitution, insertion, or deletion is encompassed by the invention. In favorable circumstances, the substitution is a conservative substitution as defined above. "VEGFmg polypeptide variant" means an active VEGFmg polypeptide having at least: (1) about 80% amino acid sequence identity with a full-length native sequence VEGFmg polypeptide sequence, (2) a VEGFmg polypeptide sequence lacking the signal peptide, (3) an extracellular domain of a VEGFmg polypeptide, with or without the signal peptide, or (4) any other fragment of a full-length VEGFmg polypeptide sequence. For example, VEGFmg polypeptide variants include VEGFmg polypeptides wherein one or more amino acid residues are added or deleted at the N- or C- terminus of the full-length native amino acid sequence. A VEGFmg polypeptide variant will have at least about 80% amino acid sequence identity, preferably at least about 81% amino acid sequence identity, more preferably at least about 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% amino acid sequence identity and most preferably at least about 99% amino acid sequence identity with a full-length native sequence VEGFmg polypeptide sequence. A VEGFmg polypeptide variant may have a sequence lacking the signal peptide, an extracellular domain of a VEGFmg polypeptide, with or without

the signal peptide, or any other fragment of a full-length VEGFmg polypeptide sequence. Ordinarily, VEGFmg variant polypeptides are at least about 10 amino acids in length, often at least about 20 amino acids in length, more often at least about 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, or 300 amino acids in length, or more.

"Percent (%) amino acid sequence identity" is defined as the percentage of amino acid residues that are identical with amino acid residues in the disclosed VEGFmg polypeptide sequence in a candidate sequence when the two sequences are aligned. To determine % amino acid identity, sequences are aligned and if necessary, gaps are introduced to achieve the maximum % sequence identity; conservative substitutions are not considered as part of the sequence identity. Amino acid sequence alignment procedures to determine percent identity are well known to those of skill in the art. Often publicly available computer software such as BLAST, BLAST2, ALIGN2 or Megalign (DNASTAR) software is used to align peptide sequences. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

When amino acid sequences are aligned, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) can be calculated as:

$$\%_{\text{amino acid sequence identity}} = X/Y \cdot 100$$

where

X is the number of amino acid residues scored as identical matches by the sequence alignment program's or algorithm's alignment of A and B

and

Y is the total number of amino acid residues in B.

If the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A.

7. *Determining homology between two or more sequences*

"VEGFmg variant" means an active VEGFmg having at least: (1) about 80% amino acid sequence identity with a full-length native sequence VEGFmg sequence, (2) a VEGFmg sequence lacking the signal peptide, (3) an extracellular domain of a VEGFmg, with or without the signal peptide, or (4) any other fragment of a full-length VEGFmg sequence. For example, VEGFmg variants include VEGFmg wherein one or more amino acid residues are added or deleted at the N- or C-terminus of the full-length native amino acid sequence. A VEGFmg variant will have at least about 80% amino acid sequence identity, preferably at least about 81% amino acid sequence identity, more preferably at least about 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% amino acid sequence identity and most preferably at least about 99% amino acid sequence identity with a full-length native sequence VEGFmg sequence. A VEGFmg variant may have a sequence lacking the signal peptide, an extracellular domain of a VEGFmg, with or without the signal peptide, or any other fragment of a full-length VEGFmg sequence. Ordinarily, VEGFmg variant polypeptides are at least about 10 amino acids in length, often at least about 20 amino acids in length, more often at least about 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, or 300 amino acids in length, or more.

"Percent (%) amino acid sequence identity" is defined as the percentage of amino acid residues that are identical with amino acid residues in the disclosed VEGFmg sequence in a candidate sequence when the two sequences are aligned. To determine % amino acid identity, sequences are aligned and if necessary, gaps are introduced to achieve the maximum % sequence identity; conservative substitutions are not considered as part of the sequence identity. Amino acid sequence alignment procedures to determine percent identity are well known to those of skill in the art. Often publicly available computer software such as BLAST, BLAST2, ALIGN2 or Megalign (DNASTAR) software is used to align peptide sequences. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

When amino acid sequences are aligned, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) can be calculated as:

$$\%_{\text{amino acid sequence identity}} = X/Y \cdot 100$$

where

X is the number of amino acid residues scored as identical matches by the sequence alignment program's or algorithm's alignment of A and B

and

Y is the total number of amino acid residues in B.

If the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A.

8. *Chimeric and fusion proteins*

Fusion polypeptides are useful in expression studies, cell-localization, bioassays, and VEGFmg purification. A VEGFmg "chimeric protein" or "fusion protein" comprises VEGFmg fused to a non-VEGFmg polypeptide. A VEGFmg fusion protein may include any portion to the entire VEGFmg, including any number of the biologically active portions. VEGFmg may be fused to the C-terminus of the GST (glutathione S-transferase) sequences. Such fusion proteins facilitate the purification of recombinant VEGFmg. In certain host cells, (*e.g.* mammalian), heterologous signal sequences fusions may ameliorate VEGFmg expression and/or secretion. Additional exemplary fusions are presented in Table C.

Other fusion partners can adapt VEGFmg therapeutically. Fusions with members of the immunoglobulin (Ig) protein family are useful in therapies that inhibit VEGFmg ligand or substrate interactions, consequently suppressing VEGFmg-mediated signal transduction *in vivo*. Such fusions, incorporated into pharmaceutical compositions, may be used to treat proliferative and differentiation disorders, as well as modulating cell survival. VEGFmg-Ig fusion polypeptides can also be used as

immunogens to produce anti-VEGFmg Abs in a subject, to purify VEGFmg ligands, and to screen for molecules that inhibit interactions of VEGFmg with other molecules.

Fusion proteins can be easily created using recombinant methods. A nucleic acid encoding VEGFmg can be fused in-frame with a non-VEGFmg encoding nucleic acid, to the VEGFmg NH_2- or COO- -terminus, or internally. Fusion genes may also be synthesized by conventional techniques, including automated DNA synthesizers. PCR amplification using anchor primers that give rise to complementary overhangs between two consecutive gene fragments that can subsequently be annealed and reamplified to generate a chimeric gene sequence (Ausubel *et al.*, 1987) is also useful. Many vectors are commercially available that facilitate sub-cloning *VEGFmg* in-frame to a fusion moiety.

Table C Useful non-VEGFmg fusion polypeptides

Reporter	*in vitro*	*in vivo*	Notes	Reference
Human growth hormone (hGH)	Radioimmuno-assay	none	Expensive, insensitive, narrow linear range.	(Selden *et al.*, 1986)
β-glucu-ronidase (GUS)	Colorimetric, fluorescent, or chemi-luminescent	colorimetric (histo-chemical staining with X-gluc)	sensitive, broad linear range, non-iostopic.	(Gallagher, 1992)
Green fluorescent protein (GFP) and related molecules (RFP, BFP, VEGFmg, *etc.*)	Fluorescent	fluorescent	can be used in live cells; resists photo-bleaching	(Chalfie *et al.*, 1994)
Luciferase (firefly)	bioluminsecent	Bio-luminescent	protein is unstable, difficult to reproduce, signal is brief	(de Wet *et al.*, 1987)

Chloramphenicol acetyltransferase (CAT)	Chromato-graphy, differential extraction, fluorescent, or immunoassay	none	Expensive radioactive substrates, time-consuming, insensitive, narrow linear range	(Gorman *et al.*, 1982)
β-galacto-sidase	colorimetric, fluorescence, chemi-luminscence	colorimetric (histochemical staining with X-gal), bio-luminescent in live cells	sensitive, broad linear range; some cells have high endogenous activity	(Alam and Cook, 1990)
Secrete alkaline phosphatase (SEAP)	colorimetric, bioluminescent, chemi-luminescent	none	Chem-iluminscence assay is sensitive and broad linear range; some cells have endogenouse alkaline phosphatase activity	(Berger *et al.*, 1988)

9. *VEGFmg recombinant expression vectors and host cells*

Vectors are tools used to shuttle DNA between host cells or as a means to express a nucleotide sequence. Some vectors function only in prokaryotes, while others function in both prokaryotes and eukaryotes, enabling large-scale DNA preparation from prokaryotes for expression in eukaryotes. Inserting the DNA of interest, such as VEGFmg nucleotide sequence or a fragment, is accomplished by ligation techniques and/or mating protocols well-known to the skilled artisan. Such DNA is inserted such that its integration does not disrupt any necessary components of the vector. In the case of vectors that are used to express the inserted DNA protein, the introduced DNA is operably-linked to the vector elements that govern its transcription and translation.

Vectors can be divided into two general classes: Cloning vectors are replicating plasmid or phage with regions that are non-essential for propagation in an appropriate host cell, and into which foreign DNA can be inserted; the foreign DNA

is replicated and propagated as if it were a component of the vector. An expression vector (such as a plasmid, yeast, or animal virus genome) is used to introduce foreign genetic material into a host cell or tissue in order to transcribe and translate the foreign DNA. In expression vectors, the introduced DNA is operably-linked to elements, such as promoters, that signal to the host cell to transcribe the inserted DNA. Some promoters are exceptionally useful, such as inducible promoters that control gene transcription in response to specific factors. Operably-linking *VEGFmg* or anti-sense construct to an inducible promoter can control the expression of *VEGFmg* or fragments, or anti-sense constructs. Examples of classic inducible promoters include those that are responsive to α-interferon, heat-shock, heavy metal ions, and steroids such as glucocorticoids (Kaufman, 1990) and tetracycline. Other desirable inducible promoters include those that are not endogenous to the cells in which the construct is being introduced, but, however, is responsive in those cells when the induction agent is exogenously supplied.

Vectors have many difference manifestations. A "plasmid" is a circular double stranded DNA molecule into which additional DNA segments can be introduced. Viral vectors can accept additional DNA segments into the viral genome. Certain vectors are capable of autonomous replication in a host cell (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. In general, useful expression vectors are often plasmids. However, other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses) are contemplated. Recombinant expression vectors that comprise *VEGFmg* (or fragments) regulate *VEGFmg* transcription by exploiting one or more host cell-responsive (or that can be manipulated *in vitro*) regulatory sequences that is operably-linked to *VEGFmg*. "Operably-linked" indicates that a nucleotide sequence of interest is linked to regulatory sequences such that expression of the nucleotide sequence is achieved.

Vectors can be introduced in a variety of organisms and/or cells (Table D). Alternatively, the vectors can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Table D Examples of hosts for cloning or expression

Organisms	Examples	Sources and References*
Prokaryotes		
Enterobacteriaceae	*E. coli* K 12 strain MM294 X1776 W3110 K5 772	 ATCC 31,446 ATCC 31,537 ATCC 27,325 ATCC 53,635
	Enterobacter	
	Erwinia	
	Klebsiella	
	Proteus	
	Salmonella (*S. tyhpimurium*)	
	Serratia (*S. marcescans*)	
	Shigella	
	Bacilli (B. *subtilis* and *B. licheniformis*)	
	Pseudomonas (P. *aeruginosa*)	
	Streptomyces	
Eukaryotes		
Yeasts	*Saccharomyces cerevisiae*	
	Schizosaccharomyces pombe	
	Kluyveromyces *K. lactis* MW98-8C, CBS683, CBS4574 *K. fragilis* *K. bulgaricus* *K. wickeramii* *K. waltii* *K. drosophilarum* *K. thermotolerans* *K. marxianus; yarrowia*	(Fleer *et al.*, 1991) (de Louvencourt *et al.*, 1983) ATCC 12,424 ATCC 16,045 ATCC 24,178 ATCC 56,500 ATCC 36,906 (EPO 402226, 1990)
	Pichia pastoris	(Sreekrishna *et al.*, 1988)
	Candida	
	Trichoderma reesia	
	Neurospora crassa	(Case *et al.*, 1979)
	Torulopsis	
	Rhodotorula	
	Schwanniomyces (*S. occidentalis*)	
Filamentous Fungi	*Neurospora*	
	Penicillium	

Table D Examples of hosts for cloning or expression

Organisms	Examples	Sources and References*
	Tolypocladium	(WO 91/00357, 1991)
	Aspergillus (*A. nidulans* and *A. niger*)	(Kelly and Hynes, 1985; Tilburn *et al.*, 1983; Yelton *et al.*, 1984)
Invertebrate cells	*Drosophila* S2	
	Spodoptera Sf9	
Vertebrate cells	Chinese Hamster Ovary (CHO)	
	simian COS COS-7	ATCC CRL 1651
	HEK 293	

*Unreferenced cells are generally available from American Type Culture Collection (Manassas, VA).

Vector choice is dictated by the organism or cells being used and the desired fate of the vector. Vectors may replicate once in the target cells, or may be "suicide" vectors. In general, vectors comprise signal sequences, origins of replication, marker genes, enhancer elements, promoters, and transcription termination sequences. The choice of these elements depends on the organisms in which the vector will be used and are easily determined. Some of these elements may be conditional, such as an inducible or conditional promoter that is turned "on" when conditions are appropriate. Examples of inducible promoters include those that are tissue-specific, which relegate expression to certain cell types, steroid-responsive, or heat-shock reactive. Some bacterial repression systems, such as the *lac* operon, have been exploited in mammalian cells and transgenic animals (Fieck *et al.*, 1992; Wyborski *et al.*, 1996; Wyborski and Short, 1991). Vectors often use a selectable marker to facilitate identifying those cells that have incorporated the vector. Many selectable markers are well known in the art for the use with prokaryotes, usually antibiotic-resistance genes or the use of autotrophy and auxotrophy mutants.

Using antisense and sense *VEGFmg* oligonucleotides can prevent VEGFmg polypeptide expression. These oligonucleotides bind to target nucleic acid sequences, forming duplexes that block transcription or translation of the target sequence by enhancing degradation of the duplexes, terminating prematurely transcription or translation, or by other means.

Antisense or sense oligonucleotides are singe-stranded nucleic acids, either RNA or DNA, which can bind target *VEGFmg* mRNA (sense) or *VEGFmg* DNA (antisense) sequences. According to the present invention, antisense or sense oligonucleotides comprise a fragment of the *VEGFmg* DNA coding region of at least about 14 nucleotides, preferably from about 14 to 30 nucleotides. In general, antisense RNA or DNA molecules can comprise at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 bases in length or more. Among others, (Stein and Cohen, 1988; van der Krol *et al.*, 1988a) describe methods to derive antisense or a sense oligonucleotides from a given cDNA sequence.

Modifications of antisense and sense oligonucleotides can augment their effectiveness. Modified sugar-phosphodiester bonds or other sugar linkages (WO 91/06629, 1991), increase *in vivo* stability by conferring resistance to endogenous nucleases without disrupting binding specificity to target sequences. Other modifications can increase the affinities of the oligonucleotides for their targets, such as covalently linked organic moieties (WO 90/10448, 1990) or poly-(L)-lysine. Other attachments modify binding specificities of the oligonucleotides for their targets, including metal complexes or intercalating (*e.g.* ellipticine) and alkylating agents.

To introduce antisense or sense oligonucleotides into target cells (cells containing the target nucleic acid sequence), any gene transfer method may be used and are well known to those of skill in the art. Examples of gene transfer methods include 1) biological, such as gene transfer vectors like Epstein-Barr virus or conjugating the exogenous DNA to a ligand-binding molecule (WO 91/04753, 1991), 2) physical, such as electroporation, and 3) chemical, such as $CaPO_4$ precipitation and oligonucleotide-lipid complexes (WO 90/10448, 1990).

The terms "host cell" and "recombinant host cell" are used interchangeably. Such terms refer not only to a particular subject cell but also to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term.

Methods of eukaryotic cell transfection and prokaryotic cell transformation are well known in the art. The choice of host cell will dictate the preferred technique for

introducing the nucleic acid of interest. Table E which is not meant to be limiting, summarizes many of the known techniques in the art. Introduction of nucleic acids into an organism may also be done with *ex vivo* techniques that use an *in vitro* method of transfection, as well as established genetic techniques, if any, for that particular organism.

Table E Methods to introduce nucleic acid into cells

Cells	Methods	References	Notes
Prokaryotes (bacteria)	Calcium chloride	(Cohen *et al.*, 1972; Hanahan, 1983; Mandel and Higa, 1970)	
	Electroporation	(Shigekawa and Dower, 1988)	
Eukaryotes Mammalian cells	Calcium phosphate transfection	*N*-(2-Hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid (HEPES) buffered saline solution (Chen and Okayama, 1988; Graham and van der Eb, 1973; Wigler *et al.*, 1978) BES (*N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid) buffered solution (Ishiura *et al.*, 1982)	Cells may be "shocked" with glycerol or dimethylsulfoxide (DMSO) to increase transfection efficiency (Ausubel *et al.*, 1987).
	Diethylaminoethyl (DEAE)-Dextran transfection	(Fujita *et al.*, 1986; Lopata *et al.*, 1984; Selden *et al.*, 1986)	Most useful for transient, but not stable, transfections. Chloroquine can be used to increase efficiency.
	Electroporation	(Neumann *et al.*, 1982; Potter, 1988; Potter *et al.*, 1984; Wong and Neumann, 1982)	Especially useful for hard-to-transfect lymphocytes.
	Cationic lipid reagent transfection	(Elroy-Stein and Moss, 1990; Felgner *et al.*, 1987; Rose *et al.*, 1991; Whitt *et al.*, 1990)	Applicable to both *in vivo* and *in vitro* transfection.

Table E Methods to introduce nucleic acid into cells

Cells	Methods	References	Notes
	Retroviral	Production exemplified by (Cepko *et al.*, 1984; Miller and Buttimore, 1986; Pear *et al.*, 1993) Infection *in vitro* and *in vivo*: (Austin and Cepko, 1990; Bodine *et al.*, 1991; Fekete and Cepko, 1993; Lemischka *et al.*, 1986; Turner *et al.*, 1990; Williams *et al.*, 1984)	Lengthy process, many packaging lines available at ATCC. Applicable to both *in vivo* and *in vitro* transfection.
	Polybrene	(Chaney *et al.*, 1986; Kawai and Nishizawa, 1984)	
	Microinjection	(Capecchi, 1980)	Can be used to establish cell lines carrying integrated copies of VEGFmg DNA sequences.
	Protoplast fusion	(Rassoulzadegan *et al.*, 1982; Sandri-Goldin *et al.*, 1981; Schaffner, 1980)	
Insect cells (*in vitro*)	Baculovirus systems	(Luckow, 1991; Miller, 1988; O'Reilly *et al.*, 1992)	Useful for *in vitro* production of proteins with eukaryotic modifications.
Yeast	Electroporation	(Becker and Guarente, 1991)	
	Lithium acetate	(Gietz *et al.*, 1998; Ito *et al.*, 1983)	
	Spheroplast fusion	(Beggs, 1978; Hinnen *et al.*, 1978)	Laborious, can produce aneuploids.
Plant cells (general reference: (Hansen and Wright, 1999))	Agrobacterium transformation	(Bechtold and Pelletier, 1998; Escudero and Hohn, 1997; Hansen and Chilton, 1999; Touraev and al., 1997)	
	Biolistics (microprojectiles)	(Finer *et al.*, 1999; Hansen and Chilton, 1999; Shillito, 1999)	

Table E Methods to introduce nucleic acid into cells

Cells	Methods	References	Notes
	Electroporation (protoplasts)	(Fromm *et al.*, 1985; Ou-Lee *et al.*, 1986; Rhodes *et al.*, 1988; Saunders *et al.*, 1989) May be combined with liposomes (Trick and al., 1997)	
	Polyethylene glycol (PEG) treatment	(Shillito, 1999)	
	Liposomes	May be combined with electroporation (Trick and al., 1997)	
	in planta microinjection	(Leduc and al., 1996; Zhou and al., 1983)	
	Seed imbibition	(Trick and al., 1997)	
	Laser beam	(Hoffman, 1996)	
	Silicon carbide whiskers	(Thompson and al., 1995)	

Vectors often use a selectable marker to facilitate identifying those cells that have incorporated the vector. Many selectable markers are well known in the art for the use with prokaryotes, usually antibiotic-resistance genes or the use of autotrophy and auxotrophy mutants. Table F lists often-used selectable markers for mammalian cell transfection.

Table F Useful selectable markers for eukaryote cell transfection

Selectable Marker	Selection	Action	Reference
Adenosine deaminase (ADA)	Media includes 9-β-D-xylofuranosyl adenine (Xyl-A)	Conversion of Xyl-A to Xyl-ATP, which incorporates into nucleic acids, killing cells. ADA detoxifies	(Kaufman *et al.*, 1986)
Dihydrofolate reductase (DHFR)	Methotrexate (MTX) and dialyzed serum (purine-free media)	MTX competitive inhibitor of DHFR. In absence of exogenous purines, cells require DHFR, a necessary enzyme in purine biosynthesis.	(Simonsen and Levinson, 1983)
Aminoglycoside phosphotransferase	G418	G418, an aminoglycoside	(Southern and Berg,

Table F Useful selectable markers for eukaryote cell transfection

Selectable Marker	Selection	Action	Reference
("APH", "neo", "G418")		detoxified by APH, interferes with ribosomal function and consequently, translation.	1982)
Hygromycin-B-phosphotransferase (HPH)	hygromycin-B	Hygromycin-B, an aminocyclitol detoxified by HPH, disrupts protein translocation and promotes mistranslation.	(Palmer *et al.*, 1987)
Thymidine kinase (TK)	Forward selection (TK+): Media (HAT) incorporates aminopterin. Reverse selection (TK-): Media incorporates 5-bromodeoxyuridine (BrdU).	Forward: Aminopterin forces cells to synthesze d'TTP from thymidine, a pathway requiring TK. Reverse: TK phosphorylates BrdU, which incorporates into nucleic acids, killing cells.	(Littlefield, 1964)

A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce VEGFmg. Accordingly, the invention provides methods for producing VEGFmg using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of the invention (into which a recombinant expression vector encoding VEGFmg has been introduced) in a suitable medium, such that VEGFmg is produced. In another embodiment, the method further comprises isolating VEGFmg from the medium or the host cell.

Transgenic VEGFmg animals

Transgenic animals are useful for studying the function and/or activity of *VEGFmg* and for identifying and/or evaluating modulators of VEGFmg activity. "Transgenic animals" are non-human animals, preferably mammals, more preferably rodents such as rats or mice, in which one or more of the cells include a transgene.

Other transgenic animals include primates, sheep, dogs, cows, goats, chickens, amphibians, *etc.* A "transgene" is exogenous DNA that is integrated into the genome of a cell from which a transgenic animal develops, and that remains in the genome of the mature animal. Transgenes preferably direct the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal with the purpose of preventing expression of a naturally encoded gene product in one or more cell types or tissues (a "knockout" transgenic animal), or serving as a marker or indicator of an integration, chromosomal location, or region of recombination (*e.g. cre/loxP* mice). A "homologous recombinant animal" is a non-human animal, such as a rodent, in which endogenous *VEGFmg* has been altered by an exogenous DNA molecule that recombines homologously with endogenous *VEGFmg* in a (*e.g.* embryonic) cell prior to development the animal. Host cells with exogenous *VEGFmg* can be used to produce non-human transgenic animals, such as fertilized oocytes or embryonic stem cells into which *VEGFmg*-coding sequences have been introduced. Such host cells can then be used to create non-human transgenic animals or homologous recombinant animals.

1. *Approaches to transgenic animal production*

A transgenic animal can be created by introducing *VEGFmg* into the male pronuclei of a fertilized oocyte (*e.g.*, by microinjection, retroviral infection) and allowing the oocyte to develop in a pseudopregnant female foster animal (pffa). The *VEGFmg* cDNA sequences can be introduced as a transgene into the genome of a non-human animal. Alternatively, a homologue of *VEGFmg* can be used as a transgene. Intronic sequences and polyadenylation signals can also be included in the transgene to increase transgene expression. Tissue-specific regulatory sequences can be operably-linked to the *VEGFmg* transgene to direct expression of VEGFmg to particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art, *e.g.* (Evans *et al.*, U.S. Patent No. 4,870,009, 1989; Hogan, 0879693843, 1994; Leder and Stewart, U.S. Patent No. 4,736,866, 1988; Wagner and Hoppe, US Patent No. 4,873,191, 1989). Other non-mice transgenic animals may be made by similar methods. A transgenic founder animal, which can be used to breed additional transgenic animals, can be identified based upon the presence of the transgene in its

genome and/or expression of the transgene mRNA in tissues or cells of the animals. Transgenic (*e.g. VEGFmg*) animals can be bred to other transgenic animals carrying other transgenes.

2. *Vectors for transgenic animal production*

To create a homologous recombinant animal, a vector containing at least a portion of *VEGFmg* into which a deletion, addition or substitution has been introduced to thereby alter, *e.g.*, functionally disrupt, *VEGFmg*. *VEGFmg* can be a murine gene or other *VEGFmg* homologue, such as the naturally occurring variant. In one approach, a knockout vector functionally disrupts the endogenous *VEGFmg* gene upon homologous recombination, and thus a non-functional VEGFmg protein, if any, is expressed.

Alternatively, the vector can be designed such that, upon homologous recombination, the endogenous *VEGFmg* is mutated or otherwise altered but still encodes functional protein (*e.g.*, the upstream regulatory region can be altered to alter the expression of endogenous VEGFmg). In this type of homologous recombination vector, the altered portion of the *VEGFmg* is flanked at its 5'- and 3'-termini by additional nucleic acid of the *VEGFmg* to allow for homologous recombination to occur between the exogenous *VEGFmg* carried by the vector and an endogenous *VEGFmg* in an embryonic stem cell. The additional flanking VEGFmg nucleic acid is sufficient to engender homologous recombination with endogenous *VEGFmg*. Typically, several kilobases of flanking DNA (both at the 5'- and 3'-termini) are included in the vector (Thomas and Capecchi, 1987). The vector is then introduced into an embryonic stem cell line (*e.g.*, by electroporation), and cells in which the introduced *VEGFmg* has homologously-recombined with the endogenous *VEGFmg* are selected (Li *et al.*, 1992).

3. *Introduction of* VEGFmg *transgene cells during development*

Selected cells are then injected into a blastocyst of an animal (*e.g.*, a mouse) to form aggregation chimeras (Bradley, 1987). A chimeric embryo can then be implanted into a suitable pffa and thc cmbryo brought to term. Progeny harboring the homologously-recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously-recombined DNA by germline transmission of the transgene. Methods for constructing homologous recombination

vectors and homologous recombinant animals are described (Berns *et al.*, WO 93/04169, 1993; Bradley, 1991; Kucherlapati *et al.*, WO 91/01140, 1991; Le Mouellic and Brullet, WO 90/11354, 1990).

Alternatively, transgenic animals that contain selected systems that allow for regulated expression of the transgene can be produced. An example of such a system is the *cre/loxP* recombinase system of bacteriophage P1 (Lakso *et al.*, 1992). Another recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman *et al.*, 1991). If a *cre/loxP* recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the *Cre* recombinase and a selected protein are required. Such animals can be produced as "double" transgenic animals, by mating an animal containing a transgene encoding a selected protein to another containing a transgene encoding a recombinase.

Clones of transgenic animals can also be produced (Wilmut *et al.*, 1997). In brief, a cell from a transgenic animal can be isolated and induced to exit the growth cycle and enter G_0 phase. The quiescent cell can then be fused to an enucleated oocyte from an animal of the same species from which the quiescent cell is isolated. The reconstructed oocyte is then cultured to develop to a morula or blastocyte and then transferred to a pffa. The offspring borne of this female foster animal will be a clone of the "parent" transgenic animal.

Anti-VEGFmg Abs

The invention encompasses Abs and antibody fragments, such as F_{ab} or $(F_{ab})_2$, that bind immunospecifically to any VEGFmg epitopes.

"Antibody" (Ab) comprises single Abs directed against VEGFmg (anti-VEGFmg Ab; including agonist, antagonist, and neutralizing Abs), anti-VEGFmg Ab compositions with poly-epitope specificity, single chain anti-VEGFmg Abs, and fragments of anti-VEGFmg Abs. A "monoclonal antibody" is obtained from a population of substantially homogeneous Abs, *i.e.*, the individual Abs comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Exemplary Abs include polyclonal (pAb), monoclonal (mAb), humanized, bi-specific (bsAb), and heteroconjugate Abs.

1. *Polyclonal Abs (pAbs)*

Polyclonal Abs can be raised in a mammalian host, for example, by one or more injections of an immunogen and, if desired, an adjuvant. Typically, the immunogen and/or adjuvant are injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunogen may include VEGFmg or a fusion protein. Examples of adjuvants include Freund's complete and monophosphoryl Lipid A synthetic-trehalose dicorynomycolate (MPL-TDM). To improve the immune response, an immunogen may be conjugated to a protein that is immunogenic in the VEGF host, such as keyhole limpet hemocyanin (KLH), serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. Protocols for antibody production are described by (Ausubel *et al.*, 1987; Harlow and Lane, 1988). Alternatively, pAbs may be made in chickens, producing IgY molecules (Schade ***et al.***, 1996).

2. *Monoclonal Abs (mAbs)*

Anti-VEGFmg mAbs may be prepared using hybridoma methods (Milstein and Cuello, 1983). Hybridoma methods comprise at least four steps: (1) immunizing a host, or lymphocytes from a host; (2) harvesting the mAb secreting (or potentially secreting) lymphocytes, (3) fusing the lymphocytes to immortalized cells, and (4) selecting those cells that secrete the desired (anti-VEGFmg) mAb.

A mouse, rat, guinea pig, hamster, or other appropriate host is immunized to elicit lymphocytes that produce or are capable of producing Abs that will specifically bind to the immunogen. Alternatively, the lymphocytes may be immunized *in vitro*. If human cells are desired, peripheral blood lymphocytes (PBLs) are generally used; however, spleen cells or lymphocytes from other mammalian sources are preferred. The immunogen typically includes VEGFmg or a fusion protein.

The lymphocytes are then fused with an immortalized cell line to form hybridoma cells, facilitated by a fusing agent such as polyethylene glycol (Goding, 1996). Rodent, bovine, or human myeloma cells immortalized by transformation may be used, or rat or mouse myeloma cell lines. Because pure populations of hybridoma cells and not unfused immortalized cells are preferred, the cells after fusion are grown in a suitable medium that contains one or more substances that inhibit the growth or survival of unfused, immortalized cells. A common technique uses parental cells that lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT). In this case, hypoxanthine, aminopterin and thymidine are added to the

medium (HAT medium) to prevent the growth of HGPRT-deficient cells while permitting hybridomas to grow.

Preferred immortalized cells fuse efficiently, can be isolated from mixed populations by selecting in a medium such as HAT, and support stable and high-level expression of antibody after fusion. Preferred immortalized cell lines are murine myeloma lines, available from the American Type Culture Collection (Manassas, VA). Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human mAbs (Kozbor *et al.*, 1984; Schook, 1987).

Because hybridoma cells secrete antibody extracellularly, the culture media can be assayed for the presence of mAbs directed against VEGFmg (anti-VEGFmg mAbs). Immunoprecipitation or *in vitro* binding assays, such as radio immunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA), measure the binding specificity of mAbs (Harlow and Lane, 1988; Harlow and Lane, 1999), including Scatchard analysis (Munson and Rodbard, 1980).

Anti-VEGFmg mAb secreting hybridoma cells may be isolated as single clones by limiting dilution procedures and sub-cultured (Goding, 1996). Suitable culture media include Dulbecco's Modified Eagle's Medium, RPMI-1640, or if desired, a protein-free or -reduced or serum-free medium (*e.g.*, Ultra DOMA PF or HL-1; Biowhittaker; Walkersville, MD). The hybridoma cells may also be grown *in vivo* as ascites.

The mAbs may be isolated or purified from the culture medium or ascites fluid by conventional Ig purification procedures such as protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, ammonium sulfate precipitation or affinity chromatography (Harlow and Lane, 1988; Harlow and Lane, 1999).

The mAbs may also be made by recombinant methods (U.S. Patent No. 4166452, 1979). DNA encoding anti-VEGFmg mAbs can be readily isolated and sequenced using conventional procedures, *e.g.*, using oligonucleotide probes that specifically bind to murine heavy and light antibody chain genes, to probe preferably DNA isolated from anti-VEGFmg-secreting mAb hybridoma cell lines. Once isolated, the isolated DNA fragments are sub-cloned into expression vectors that are then transfected into host cells such as simian COS-7 cells, Chinese hamster ovary

(CHO) cells, or myeloma cells that do not otherwise produce Ig protein, to express mAbs. The isolated DNA fragments can be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4816567, 1989; Morrison *et al.*, 1987), or by fusing the Ig coding sequence to all or part of the coding sequence for a non-Ig polypeptide. Such a non-Ig polypeptide can be substituted for the constant domains of an antibody, or can be substituted for the variable domains of one antigen-combining site to create a chimeric bivalent antibody.

3. *Monovalent Abs*

The Abs may be monovalent Abs that consequently do not cross-link with each other. For example, one method involves recombinant expression of Ig light chain and modified heavy chain. Heavy chain truncations generally at any point in the F_c region will prevent heavy chain cross-linking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted, preventing crosslinking. *In vitro* methods are also suitable for preparing monovalent Abs. Abs can be digested to produce fragments, such as F_{ab} fragments (Harlow and Lane, 1988; Harlow and Lane, 1999).

4. *Humanized and human Abs*

Anti-VEGFmg Abs may further comprise humanized or human Abs. Humanized forms of non-human Abs are chimeric Igs, Ig chains or fragments (such as F_v, F_{ab}, $F_{ab'}$, $F_{(ab')2}$ or other antigen-binding subsequences of Abs) that contain minimal sequence derived from non-human Ig.

Generally, a humanized antibody has one or more amino acid residues introduced from a non-human source. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization is accomplished by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody (Jones *et al.*, 1986; Riechmann *et al.*, 1988; Verhoeyen *et al.*, 1988). Such "humanized" Abs are chimeric Abs (U.S. Patent No. 4816567, 1989), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized Abs are typically human Abs in which some CDR residues and possibly some FR residues are substituted by residues

from analogous sites in rodent Abs. Humanized Abs include human Igs (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit, having the desired specificity, affinity and capacity. In some instances, corresponding non-human residues replace F_v framework residues of the human Ig. Humanized Abs may comprise residues that are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody comprises substantially all of at least one, and typically two, variable domains, in which most if not all of the CDR regions correspond to those of a non-human Ig and most if not all of the FR regions are those of a human Ig consensus sequence. The humanized antibody optimally also comprises at least a portion of an Ig constant region (F_c), typically that of a human Ig (Jones *et al.*, 1986; Presta, 1992; Riechmann *et al.*, 1988).

Human Abs can also be produced using various techniques, including phage display libraries (Hoogenboom *et al.*, 1991; Marks *et al.*, 1991) and the preparation of human mAbs (Boerner *et al.*, 1991; Reisfeld and Sell, 1985). Similarly, introducing human Ig genes into transgenic animals in which the endogenous Ig genes have been partially or completely inactivated can be exploited to synthesize human Abs. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire (U.S. Patent No. 5545807, 1996; U.S. Patent No. 5545806, 1996; U.S. Patent No. 5569825, 1996; U.S. Patent No. 5633425, 1997; U.S. Patent No. 5661016, 1997; U.S. Patent No. 5625126, 1997; Fishwild *et al.*, 1996; Lonberg and Huszar, 1995; Lonberg *et al.*, 1994; Marks *et al.*, 1992).

5. *Bi-specific mAbs*

Bi-specific Abs are monoclonal, preferably human or humanized, that have binding specificities for at least two different antigens. For example, a binding specificity is VEGFmg; the other is for any antigen of choice, preferably a cell-surface protein or receptor or receptor subunit.

Traditionally, the recombinant production of bi-specific Abs is based on the co-expression of two Ig heavy-chain/light-chain pairs, where the two heavy chains have different specificities (Milstein and Cuello, 1983). Because of the random

assortment of Ig heavy and light chains, the resulting hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the desired bi-specific structure. The desired antibody can be purified using affinity chromatography or other techniques (WO 93/08829, 1993; Traunecker *et al.*, 1991).

To manufacture a bi-specific antibody (Suresh *et al.*, 1986), variable domains with the desired antibody-antigen combining sites are fused to Ig constant domain sequences. The fusion is preferably with an Ig heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. Preferably, the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding is in at least one of the fusions. DNAs encoding the Ig heavy-chain fusions and, if desired, the Ig light chain, are inserted into separate expression vectors and are co-transfected into a suitable host organism.

The interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers that are recovered from recombinant cell culture (WO 96/27011, 1996). The preferred interface comprises at least part of the CH3 region of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.* tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (*e.g.* alanine or threonine). This mechanism increases the yield of the heterodimer over unwanted end products such as homodimers.

Bi-specific Abs can be prepared as full length Abs or antibody fragments (*e.g.* $F_{(ab')2}$ bi-specific Abs). One technique to generate bi-specific Abs exploits chemical linkage. Intact Abs can be proteolytically cleaved to generate $F_{(ab')2}$ fragments (Brennan *et al.*, 1985). Fragments are reduced with a dithiol complexing agent, such as sodium arsenite, to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The generated $F_{ab'}$ fragments are then converted to thionitrobenzoate (TNB) derivatives. One of the $F_{ab'}$-TNB derivatives is then reconverted to the $F_{ab'}$-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount

of the other $F_{ab'}$-TNB derivative to form the bi-specific antibody. The produced bi-specific Abs can be used as agents for the selective immobilization of enzymes.

$F_{ab'}$ fragments may be directly recovered from *E. coli* and chemically coupled to form bi-specific Abs. For example, fully humanized bi-specific $F_{(ab')2}$ Abs can be produced (Shalaby *et al.*, 1992). Each $F_{ab'}$ fragment is separately secreted from *E. coli* and directly coupled chemically *in vitro*, forming the bi-specific antibody.

Various techniques for making and isolating bi-specific antibody fragments directly from recombinant cell culture have also been described. For example, leucine zipper motifs can be exploited (Kostelny *et al.*, 1992). Peptides from the *Fos* and *Jun* proteins are linked to the $F_{ab'}$ portions of two different Abs by gene fusion. The antibody homodimers are reduced at the hinge region to form monomers and then re-oxidized to form antibody heterodimers. This method can also produce antibody homodimers. The "diabody" technology (Holliger *et al.*, 1993) provides an alternative method to generate bi-specific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker that is too short to allow pairing between the two domains on the same chain. The V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, forming two antigen-binding sites. Another strategy for making bi-specific antibody fragments is the use of single-chain F_v (sF_v) dimers (Gruber *et al.*, 1994). Abs with more than two valencies are also contemplated, such as tri-specific Abs (Tutt *et al.*, 1991).

Exemplary bi-specific Abs may bind to two different epitopes on a given VEGFmg. Alternatively, cellular defense mechanisms can be restricted to a particular cell expressing the particular VEGFmg: an anti-VEGFmg arm may be combined with an arm that binds to a leukocyte triggering molecule, such as a T-cell receptor molecule (*e.g.* CD2, CD3, CD28, or B7), or to F_c receptors for IgG ($F_c\gamma R$), such as $F_c\gamma RI$ (CD64), $F_c\gamma RII$ (CD32) and $F_c\gamma RIII$ (CD16). Bi-specific Abs may also be used to target cytotoxic agents to cells that express a particular VEGFmg. These Abs possess a VEGFmg-binding arm and an arm that binds a cytotoxic agent or a radionuclide chelator.

6. *Heteroconjugate Abs*

Heteroconjugate Abs, consisting of two covalently joined Abs, have been proposed to target immune system cells to unwanted cells (4,676,980, 1987) and for treatment of human immunodeficiency virus (HIV) infection (WO 91/00360, 1991; WO 92/20373, 1992). Abs prepared *in vitro* using synthetic protein chemistry methods, including those involving cross-linking agents, are contemplated. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents include iminothiolate and methyl-4-mercaptobutyrimidate (4,676,980, 1987).

7. *Immunoconjugates*

Immunoconjugates may comprise an antibody conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (*e.g.*, an enzymatically active toxin or fragment of bacterial, fungal, plant, or animal origin), or a radioactive isotope (*i.e.*, a radioconjugate).

Useful enzymatically-active toxins and fragments include Diphtheria A chain, non-binding active fragments of Diphtheria toxin, exotoxin A chain from *Pseudomonas aeruginosa,* ricin A chain, abrin A chain, modeccin A chain, α-sarcin, *Aleurites fordii* proteins, Dianthin proteins, *Phytolaca americana* proteins, *Momordica charantia* inhibitor, curcin, crotin, *Sapaonaria officinalis* inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated Abs, such as ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y, and ^{186}Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bi-functional protein-coupling agents, such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bi-functional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), *bis*-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), *bis*-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and *bis*-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared (Vitetta *et al.*, 1987). ^{14}C-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic

acid (MX-DTPA) is an exemplary chelating agent for conjugating radionuclide to antibody (WO 94/11026, 1994).

In another embodiment, the antibody may be conjugated to a "receptor" (such as streptavidin) for utilization in tumor pre-targeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a streptavidin "ligand" (*e.g.*, biotin) that is conjugated to a cytotoxic agent (*e.g.*, a radionuclide).

8. *Effector function engineering*

The antibody can be modified to enhance its effectiveness in treating a disease, such as cancer. For example, cysteine residue(s) may be introduced into the F_c region, thereby allowing interchain disulfide bond formation in this region. Such homodimeric Abs may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC) (Caron *et al.*, 1992; Shopes, 1992). Homodimeric Abs with enhanced anti-tumor activity can be prepared using hetero-bifunctional cross-linkers (Wolff *et al.*, 1993). Alternatively, an antibody engineered with dual F_c regions may have enhanced complement lysis (Stevenson *et al.*, 1989).

9. *Immunoliposomes*

Liposomes containing the antibody may also be formulated (U.S. Patent No. 4485045, 1984; U.S. Patent No. 4544545, 1985; U.S. Patent No. 5013556, 1991; Eppstein *et al.*, 1985; Hwang *et al.*, 1980). Useful liposomes can be generated by a reverse-phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol, and PEG-derivatized phosphatidylethanolamine (PEG- PE). Such preparations are extruded through filters of defined pore size to yield liposomes with a desired diameter. $F_{ab'}$ fragments of the antibody can be conjugated to the liposomes (Martin and Papahadjopoulos, 1982) via a disulfide-interchange reaction. A chemotherapeutic agent, such as Doxorubicin, may also be contained in the liposome (Gabizon *et al.*, 1989). Other useful liposomes with different compositions are contemplated.

10. *Diagnostic applications of Abs directed against VEGFmg*

Anti-VEGFmg Abs can be used to localize and/or quantitate VEGFmg (*e.g.*, for use in measuring levels of VEGFmg within tissue samples or for use in diagnostic

methods, *etc.*). Anti-VEGFmg epitope Abs can be utilized as pharmacologically-active compounds.

Anti-VEGFmg Abs can be used to isolate a VEGFmg of choice by standard techniques, such as immunoaffinity chromatography or immunoprecipitation. These approaches facilitate purifying endogenous VEGFmg antigen-containing polypeptides from cells and tissues. These approaches, as well as others, can be used to detect a VEGFmg in a sample to evaluate the abundance and pattern of expression of the antigenic protein. Anti-VEGFmg Abs can be used to monitor protein levels in tissues as part of a clinical testing procedure; for example, to determine the efficacy of a given treatment regimen. Coupling the antibody to a detectable substance (label) allows detection of Ab-antigen complexes. Classes of labels include fluorescent, luminescent, bioluminescent, and radioactive materials, enzymes and prosthetic groups. Useful labels include horseradish peroxidase, alkaline phosphatase, β-galactosidase, acetylcholinesterase, streptavidin/biotin, avidin/biotin, umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride, phycoerythrin, luminol, luciferase, luciferin, aequorin, and ^{125}I, ^{131}I, ^{35}S or ^{3}H.

11. *Antibody therapeutics*

Abs of the invention, including polyclonal, monoclonal, humanized and fully human Abs, can be used therapeutically. Such agents will generally be employed to treat or prevent a disease or pathology in a subject. An antibody preparation, preferably one having high antigen specificity and affinity generally mediates an effect by binding the target epitope(s). Generally, administration of such Abs may mediate one of two effects: (1) the antibody may prevent ligand binding, eliminating endogenous ligand binding and subsequent signal transduction, or (2) the antibody elicits a physiological result by binding an effector site on the target molecule, initiating signal transduction.

A therapeutically effective amount of an antibody relates generally to the amount needed to achieve a therapeutic objective, epitope binding affinity, administration rate, and depletion rate of the antibody from a subject. Common ranges for therapeutically effective doses may be, as a nonlimiting example, from

about 0.1 mg/kg body weight to about 50 mg/kg body weight. Dosing frequencies may range, for example, from twice daily to once a week.

12. *Pharmaceutical compositions of Abs*

Anti-VEGFmg Abs, as well as other VEGFmg interacting molecules (such as aptamers) identified in other assays, can be administered in pharmaceutical compositions to treat various disorders. Principles and considerations involved in preparing such compositions, as well as guidance in the choice of components can be found in (de Boer, 1994; Gennaro, 2000; Lee, 1990).

Because many VEGFmgs are intracellular, Abs that are internalized are preferred when whole Abs are used as inhibitors to these molecules. Otherwise, Abs that are not internalized are preferred, such as anti-osteonidogen Abs. Liposomes may also be used as a delivery vehicle for intracellular introduction. Where antibody fragments are used, the smallest inhibitory fragment that specifically binds to the epitope is preferred. For example, peptide molecules can be designed that bind a preferred epitope based on the variable-region sequences of a useful antibody. Such peptides can be synthesized chemically and/or produced by recombinant DNA technology (Marasco *et al.*, 1993). Formulations may also contain more than one active compound for a particular treatment, preferably those with activities that do not adversely affect each other. The composition may comprise an agent that enhances function, such as a cytotoxic agent, cytokine, chemotherapeutic agent, or growth-inhibitory agent.

The active ingredients can also be entrapped in microcapsules prepared by coacervation techniques or by interfacial polymerization; for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles, and nanocapsules) or in macroemulsions.

The formulations to be used for *in vivo* administration are highly preferred to be sterile. This is readily accomplished by filtration through sterile filtration membranes or any of a number of techniques.

Sustained-release preparations may also be prepared, such as semi-permeable matrices of solid hydrophobic polymers containing the antibody, which matrices are

in the form of shaped articles, *e.g.*, films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (Boswell and Scribner, U.S. Patent No. 3,773,919, 1973), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as injectable microspheres composed of lactic acid-glycolic acid copolymer, and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods and may be preferred.

Therapeutic applications of VEGFmg

1. *Pathology-related utilities*

The polynucleotides and proteins of the invention are useful in potential therapeutic applications implicated in tumors and neoplasias, hamangiomas, rheumatoid arthritis, atherosclerosis, idiopathic pulmonary fibrosis, vascular restenosis, arteriovenous malformations, meningioma, neovascular glaucoma, psoriasis, agniofibroma, hemophilic joints, hypertrophic scars, Osler-Weber syndrome, pyogenic gtranuloma retrolental fibroplasias, scleroderma, trachoma, vascular adhesion pathologies, synovitis, dermatitis, enometriosis, pterygium, diabetic retinopathy, newovascularization associated with corneal injury or grafts, wound, sore, and ulcers (skin, gastric and duodenal) healing. For example, a cDNA encoding *ARP* may be useful in gene therapy, and ARP protein may be useful when administered to a subject in need thereof. The novel nucleic acid encoding ARP, and the ARP protein of the invention, or fragments thereof, may further be useful in diagnostic applications, wherein the presence or amount of the nucleic acid or the protein are to be assessed. These materials are further useful in the generation of Abs that bind immunospecifically to the novel substances of the invention for use in therapeutic or diagnostic methods.

In addition, the instant invention may be used to determine the clinical state or pathology of a sample, such as a biopsy of cells taken from a patient. A clinical state of a growth, such as a tumor or cancer, is a classification system recognized by those of skill in the art to categorize, for example, the metastatic aggressiveness of a cancer.

2. *Agonists and antagonists*

"Antagonist" includes any molecule that partially or fully blocks, inhibits, or neutralizes a biological activity of endogenous VEGFmg. Similarly, "agonist" includes any molecule that mimics a biological activity of endogenous VEGFmg. Molecules that can act as agonists or antagonists include Abs or antibody fragments, fragments or variants of endogenous VEGFmg, peptides, antisense oligonucleotides, small organic molecules, *etc.*

3. *Identifying antagonists and agonists*

To assay for antagonists, VEGFmg is added to, or expressed in, a cell along with the compound to be screened for a particular activity. If the compound inhibits the activity of interest in the presence of the VEGFmg, that compound is an antagonist to the VEGFmg; if VEGFmg activity is enhanced, the compound is an agonist.

(a) *Specific examples of potential antagonists and agonist*

Any molecule that alters VEGFmg cellular effects, such as angiogenesis or cell survival, is a candidate antagonist or agonist. Screening techniques well known to those skilled in the art can identify these molecules. Examples of antagonists and agonists include: (1) small organic and inorganic compounds, (2) small peptides, (3) Abs and derivatives, (4) polypeptides closely related to VEGFmg, (5) antisense DNA and RNA, (6) ribozymes, (7) triple DNA helices and (8) nucleic acid aptamers.

Small molecules that bind to the VEGFmg active site or other relevant part of the polypeptide and inhibit the biological activity of the VEGFmg are antagonists. Examples of small molecule antagonists include small peptides, peptide-like molecules, preferably soluble, and synthetic non-peptidyl organic or inorganic compounds. These same molecules, if they enhance VEGFmg activity, are examples of agonists.

Almost any antibody that affects a VEGFmg's function is a candidate antagonist, and occasionally, agonist. Examples of antibody antagonists include polyclonal, monoclonal, single-chain, anti-idiotypic, chimeric Abs, or humanized versions of such Abs or fragments. Abs may be from any species in which an immune response can be raised. Humanized Abs are also contemplated.

Alternatively, a potential antagonist or agonist may be a closely related protein, for example, a mutated form of the VEGFmg that recognizes a VEGFmg-interacting protein but imparts no effect, competitively inhibiting VEGFmg action. Alternatively, a mutated VEGFmg may be constitutively activated and may act as an agonist.

Antisense RNA or DNA constructs can be effective antagonists. Antisense RNA or DNA molecules block function by inhibiting translation by hybridizing to targeted mRNA. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which depend on polynucleotide binding to DNA or RNA. For example, the 5' coding portion of the *VEGFmg* sequence is used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix) (Beal and Dervan, 1991; Cooney *et al.*, 1988; Lee *et al.*, 1979), preventing transcription and the production of the VEGFmg. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into the VEGFmg (antisense) (Cohen, 1989; Okano *et al.*, 1991). These oligonucleotides can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of the VEGFmg. When antisense DNA is used, oligodeoxyribonucleotides derived from the translation-initiation site, *e.g.*, between about -10 and +10 positions of the target gene nucleotide sequence, are preferred.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. Ribozymes act by sequence-specific hybridization to the complementary target RNA, followed by endonucleolytic cleavage. Specific ribozyme cleavage sites within a potential RNA target can be identified by known techniques (WO 97/33551, 1997; Rossi, 1994).

To inhibit transcription, triple-helix nucleic acids that are single-stranded and comprise deoxynucleotides are useful antagonists. These oligonucleotides are designed such that triple-helix formation via Hoogsteen base-pairing rules is promoted, generally requiring stretches of purines or pyrimidines (WO 97/33551, 1997).

Because a VEGFmg activity may include nucleic acid binding, molecules that compete for VEGFmg nucleic acid binding site(s) can be effective intracellular competitors. Aptamers are short oligonucleotide sequences that can be used to recognize and specifically bind almost any molecule. The systematic evolution of ligands by exponential enrichment (SELEX) process (Ausubel *et al.*, 1987; Ellington and Szostak, 1990; Tuerk and Gold, 1990) is powerful and can be used to find such aptamers. Aptamers have many diagnostic and clinical uses; almost any use in which an antibody has been used clinically or diagnostically, aptamers too may be used. In addition, are cheaper to make once they have been identified, and can be easily applied in a variety of formats, including administration in pharmaceutical compositions, in bioassays, and diagnostic tests (Jayasena, 1999).

Pharmaceutical compositions

The *VEGFmg* nucleic acid molecules, VEGFmg polypeptides, and anti-VEGFmg Abs (active compounds) of the invention, and derivatives, fragments, analogs and homologs thereof, can be incorporated into pharmaceutical compositions. Such compositions typically comprise the nucleic acid molecule, protein, or antibody and a pharmaceutically acceptable carrier. A "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration (Gennaro, 2000). Preferred examples of such carriers or diluents include, but are not limited to, water, saline, finger's solutions, dextrose solution, and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils may also be used. Except when a conventional media or agent is incompatible with an active compound, use of these compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

1. *General considerations*

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration, including intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (*i.e.*, topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include: a sterile diluent such as water for injection,

saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates, and agents for the adjustment of tonicity such as sodium chloride or dextrose. The *p*H can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

2. *Injectable formulations*

Pharmaceutical compositions suitable for injection include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, CREMOPHOR EL™ (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid so as to be administered using a syringe. Such compositions should be stable during manufacture and storage and must be preserved against contamination from microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (such as glycerol, propylene glycol, and liquid polyethylene glycol), and suitable mixtures. Proper fluidity can be maintained, for example, by using a coating such as lecithin, by maintaining the required particle size in the case of dispersion and by using surfactants. Various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, and thimerosal, can contain microorganism contamination. Isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, and sodium chloride can be included in the composition. Compositions that can delay absorption include agents such as aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*, VEGFmg or anti-VEGFmg antibody) in the required amount in an appropriate solvent with one or a combination of ingredients as required, followed by sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium, and the other

required ingredients as discussed. Sterile powders for the preparation of sterile injectable solutions, methods of preparation include vacuum drying and freeze-drying that yield a powder containing the active ingredient and any desired ingredient from a sterile solutions.

3. *Oral compositions*

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included. Tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, PRIMOGEL, or corn starch; a lubricant such as magnesium stearate or STEROTES; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

4. *Compositions for inhalation*

For administration by inhalation, the compounds are delivered as an aerosol spray from a a nebulizer or a pressurized container that contains a suitable propellant, *e.g.*, a gas such as carbon dioxide.

5. *Systemic administration*

Systemic administration can also be transmucosal or transdermal. For transmucosal or transdermal administration, penetrants that can permeate the target barrier(s) are selected. Transmucosal penetrants include, detergents, bile salts, and fusidic acid derivatives. Nasal sprays or suppositories can be used for transmucosal administration. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams.

The compounds can also be prepared in the form of suppositories (*e.g.*, with bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

6. *Carriers*

In one embodiment, the active compounds are prepared with carriers that protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Such materials can be obtained commercially from ALZA Corporation (Mountain View, CA) and NOVA Pharmaceuticals, Inc. (Lake Elsinore, CA), or prepared by one of skill in the art. Liposomal suspensions can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, such as in (Eppstein *et al.*, US Patent No. 4,522,811, 1985).

7. *Unit dosage*

Oral formulations or parenteral compositions in unit dosage form can be created to facilitate administration and dosage uniformity. Unit dosage form refers to physically discrete units suited as single dosages for the subject to be treated, containing a therapeutically effective quantity of active compound in association with the required pharmaceutical carrier. The specification for the unit dosage forms of the invention are dictated by, and directly dependent on, the unique characteristics of the active compound and the particular desired therapeutic effect, and the inherent limitations of compounding the active compound.

8. *Gene therapy compositions*

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (Nabel and Nabel, US Patent No. 5,328,470, 1994), or by stereotactic injection (Chen *et al.*, 1994). The pharmaceutical preparation of a gene therapy vector can include an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells that produce the gene delivery system.

9. *Kits for pharmaceutical compositions*

The pharmaceutical compositions can be included in a kit, container, pack, or dispenser together with instructions for administration. When the invention is supplied as a kit, the different components of the composition may be packaged in separate containers and admixed immediately before use. Such packaging of the components separately may permit long-term storage without losing the active components' functions.

Kits may also include reagents in separate containers that facilitate the execution of a specific test, such as diagnostic tests or tissue typing. For example, *VEGFmg* DNA templates and suitable primers may be supplied for internal controls.

(a) *Containers or vessels*

The reagents included in the kits can be supplied in containers of any sort such that the life of the different components are preserved, and are not adsorbed or altered by the materials of the container. For example, sealed glass ampules may contain lyophilized luciferase or buffer that have been packaged under a neutral, non-reacting gas, such as nitrogen. Ampoules may consist of any suitable material, such as glass, organic polymers, such as polycarbonate, polystyrene, *etc.*, ceramic, metal or any other material typically employed to hold reagents. Other examples of suitable containers include simple bottles that may be fabricated from similar substances as ampules, and envelopes, that may consist of foil-lined interiors, such as aluminum or an alloy. Other containers include test tubes, vials, flasks, bottles, syringes, or the like. Containers may have a sterile access port, such as a bottle having a stopper that can be pierced by a hypodermic injection needle. Other containers may have two compartments that are separated by a readily removable membrane that upon removal permits the components to mix. Removable membranes may be glass, plastic, rubber, *etc.*

(b) *Instructional materials*

Kits may also be supplied with instructional materials. Instructions may be printed on paper or other substrate, and/or may be supplied as an electronic-readable medium, such as a floppy disc, CD-ROM, DVD-ROM, Zip disc, video tape, audio tape, *etc.* Detailed instructions may not be physically associated with the kit; instead, a user may be directed to an internet web site specified by the manufacturer or distributor of the kit, or supplied as electronic mail.

Screening and detection methods

Isolated nucleic acid molecules can be used to express VEGFmg (*e.g.*, via a recombinant expression vector in a host cell in gene therapy applications), to detect *VEGFmg* mRNA (*e.g.*, in a biological sample) or a genetic lesion in a *VEGFmg*, and to modulate VEGFmg activity, as described below. In addition, VEGFmg polypeptides can be used to screen drugs or compounds that modulate VEGFmg activity or expression as well as to treat disorders characterized by insufficient or excessive production of VEGFmg or production of VEGFmg forms that have decreased or aberrant activity compared to VEGFmg wild-type protein, or modulate biological function that involve VEGFmg (*e.g.* angiogenesis). In addition, the anti-VEGFmg Abs of the invention can be used to detect and isolate VEGFmg and modulate VEGFmg activity.

To modulate cell survival means to decrease or increase probability that a cell will die in the future over a period of time as compared to cells prior to modulation.

1. *Screening assays*

The invention provides a method (screening assay) for identifying modalities, *i.e.*, candidate or test compounds or agents (*e.g.*, peptides, peptidomimetics, small molecules or other drugs), foods, dosing regimens, combinations thereof, *etc.*, that effect VEGFmg, a stimulatory or inhibitory effect, including translation, transcription, activity or copies of the gene in cells. The invention also includes compounds identified in screening assays.

Testing for compounds that increase or decrease VEGFmg activity are desirable. A compound may modulate VEGFmg activity by affecting: (1) the number of copies of the gene in the cell (amplifiers and deamplifiers); (2) increasing or decreasing transcription of the *VEGFmg* (transcription up-regulators and down-regulators); (3) by increasing or decreasing the translation of *VEGFmg* mRNA into protein (translation up-regulators and down-regulators); or (4) by increasing or decreasing the activity of VEGFmg itself (agonists and antagonists).

(a) *effects of compounds*

To identify compounds that affect VEGFmg at the DNA, RNA and protein levels, cells or organisms are contacted with a candidate compound and the corresponding change in VEGFmg DNA, RNA or protein is assessed (Ausubel *et al.*, 1987). For DNA amplifiers and deamplifiers, the amount of *VEGFmg* DNA is measured, for those compounds that are transcription up-regulators and down-regulators the amount of *VEGFmg* mRNA is determined; for translational up- and down-regulators, the amount of VEGFmg polypeptides is measured. Compounds that are agonists or antagonists may be identified by contacting cells or organisms with the compound, and then measuring, for example, angiogenesis or cell survival *in vitro*.

In one embodiment, many assays for screening candidate or test compounds that bind to or modulate the activity of *VEGFmg* or polypeptide or biologically-active portion are available. Test compounds can be obtained using any of the numerous approaches in combinatorial library methods, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptides, while the other four approaches encompass peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, 1997).

(b) *small molecules*

A "small molecule" refers to a composition that has a molecular weight of less than about 5 kD and most preferably less than about 4 kD, even more preferably less than 0.6 kD. Small molecules can be, nucleic acids, peptides, polypeptides, peptidomimetics, carbohydrates, lipids or other organic or inorganic molecules. Libraries of chemical and/or biological mixtures, such as fungal, bacterial, or algal extracts, are known in the art and can be screened with any of the assays of the invention. Examples of methods for the synthesis of molecular libraries can be found in: (Carell *et al.*, 1994a; Carell *et al.*, 1994b; Cho *et al.*, 1993; DeWitt *et al.*, 1993; Gallop *et al.*, 1994; Zuckermann *et al.*, 1994).

Libraries of compounds may be presented in solution (Houghten *et al.*, 1992) or on beads (Lam *et al.*, 1991), on chips (Fodor *et al.*, 1993), bacteria, spores (Ladner *et al.*, US Patent No. 5,223,409, 1993), plasmids (Cull *et al.*, 1992) or on phage (Cwirla *et al.*, 1990; Devlin *et al.*, 1990; Felici *et al.*, 1991; Ladner *et al.*, US Patent

No. 5,223,409, 1993; Scott and Smith, 1990). A cell-free assay comprises contacting VEGFmg or biologically-active fragment with a known compound that binds VEGFmg to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with VEGFmg, where determining the ability of the test compound to interact with VEGFmg comprises determining the ability of the VEGFmg to preferentially bind to or modulate the activity of a VEGFmg target molecule.

(c) *cell-free assays*

The cell-free assays of the invention may be used with both soluble or a membrane-bound forms of VEGFmg. In the case of cell-free assays comprising the membrane-bound form, a solubilizing agent to maintain VEGFmg in solution. Examples of such solubilizing agents include non-ionic detergents such as n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, TRITON® X-100 and others from the TRITON® series, THESIT®, Isotridecypoly(ethylene glycol ether)$_n$, N-dodecyl--N,N-dimethyl-3-ammonio-1-propane sulfonate, 3-(3-cholamidopropyl) dimethylamminiol-1-propane sulfonate (CHAPS), or 3-(3-cholamidopropyl)dimethylamminiol-2-hydroxy-1-propane sulfonate (CHAPSO).

(d) *immobilization of target molecules to facilitate screening*

In more than one embodiment of the assay methods, immobilizing either VEGFmg or a partner molecule can facilitate separation of complexed from uncomplexed forms of one or both of the proteins, as well as to accommodate high throughput assays. Binding of a test compound to VEGFmg, or interaction of VEGFmg with a target molecule in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants, such as microtiter plates, test tubes, and micro-centrifuge tubes. A fusion protein can be provided that adds a domain that allows one or both of the proteins to be bound to a matrix. For example, GST-VEGFmg fusion proteins or GST-target fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical; St. Louis, MO) or glutathione derivatized microtiter plates that are then combined with the test compound or the test compound and either the non-adsorbed target protein or VEGFmg, and the mixture is incubated under conditions conducive to complex

formation (*e.g.*, at physiological conditions for salt and pH). Following incubation, the beads or microtiter plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, complex determined either directly or indirectly, for example, as described. Alternatively, the complexes can be dissociated from the matrix, and the level of VEGFmg binding or activity determined using standard techniques.

Other techniques for immobilizing proteins on matrices can also be used in screening assays. Either VEGFmg or a target molecule can be immobilized using biotin-avidin or biotin-streptavidin systems. Biotinylation can be accomplished using many reagents, such as biotin-NHS (N-hydroxy-succinimide; PIERCE Chemicals, Rockford, IL), and immobilized in wells of streptavidin-coated 96 well plates (PIERCE Chemical). Alternatively, Abs reactive with VEGFmg or target molecules, but which do not interfere with binding of the VEGFmg to its target molecule, can be derivatized to the wells of the plate, and unbound target or VEGFmg trapped in the wells by antibody conjugation. Methods for detecting such complexes, in addition to those described for the GST-immobilized complexes, include immunodetection of complexes using Abs reactive with VEGFmg or its target, as well as enzyme-linked assays that rely on detecting an enzymatic activity associated with the VEGFmg or target molecule.

(e) *screens to identify modulators*

Modulators of VEGFmg expression can be identified in a method where a cell is contacted with a candidate compound and the expression of VEGFmg mRNA or protein in the cell is determined. The expression level of *VEGFmg* mRNA or protein in the presence of the candidate compound is compared to VEGFmg mRNA or protein levels in the absence of the candidate compound. The candidate compound can then be identified as a modulator of VEGFmg mRNA or protein expression based upon this comparison. For example, when expression of VEGFmg mRNA or protein is greater (*i.e.*, statistically significant) in the presence of the candidate compound than in its absence, the candidate compound is identified as a stimulator of VEGFmg mRNA or protein expression. Alternatively, when expression of VEGFmg mRNA or protein is less (statistically significant) in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of VEGFmg

mRNA or protein expression. The level of VEGFmg mRNA or protein expression in the cells can be determined by methods described for detecting VEGFmg mRNA or protein.

(f) *hybrid assays*

In yet another aspect of the invention, VEGFmg can be used as "bait" in two-hybrid or three hybrid assays (Bartel *et al.*, 1993; Brent *et al.*, WO94/10300, 1994; Iwabuchi *et al.*, 1993; Madura *et al.*, 1993; Saifer *et al.*, US Patent No. 5,283,317, 1994; Zervos *et al.*, 1993) to identify other proteins that bind or interact with VEGFmg (VEGFmg-binding proteins (VEGFmg-bps)) and modulate VEGFmg activity. Such VEGFmg-bps are also likely to be involved in the propagation of signals by the VEGFmg as, for example, upstream or downstream elements of a VEGFmg pathway.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. In one construct, the gene that codes for VEGFmg is fused to a gene encoding the DNA binding domain of a known transcription factor (*e.g.*, GAL4). The other construct, a DNA sequence from a library of DNA sequences that encodes an unidentified protein ("prey" or "sample") is fused to a gene that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact *in vivo*, forming a VEGFmg-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (*e.g.*, LacZ) that is operably-linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected, and cell colonies containing the functional transcription factor can be isolated and used to obtain the cloned gene that encodes the VEGFmg-interacting protein.

(g) *calcium channel regulators*

Several classes of calcium channel blocker are known and may be effective antagonists and agonists. For example, Mak *et al.* (Mak *et al.*, 1995) report the activity of the lipophilic calcium channel blockers, nicardipine, nifedipine, verapamil, and diltiazem as anti-oxidants and protectants for endothelial cells. Calcium channels

may play a significant role in the cell survival in which the genes identified herein are differentially expressed. Among the *VEGFmgs* that are significant in calcium regulation are DSCR1 and nexin. For example, those agents that stimulate the expression of DSCR1 or nexin and reduce the activity of the mitochondrial respiratory chain will promote survival and are useful to treat angiogenesis-related diseases, that is, diseases in which angiogenesis is repressed or insufficient. Agents that reduce the expression of *e.g.* DSCR1 or nexin and that increase the activity of the mitochondrial respiratory chain will induce or promote apoptosis and therefore are useful to treat diseases where the angiogenesis is stimulated.

The invention further pertains to novel agents identified by the aforementioned screening assays and uses thereof for treatments as described herein.

2. *Detection assays*

Portions or fragments of *VEGFmg* cDNA sequences identified herein (and the complete *VEGFmg* gene sequences) are useful in themselves. By way of non-limiting example, these sequences can be used to: (1) identify an individual from a minute biological sample (tissue typing); and (2) aid in forensic identification of a biological sample.

(a) *Tissue typing*

The VEGFmg sequences of the invention can be used to identify individuals from minute biological samples. In this technique, an individual's genomic DNA is digested with one or more restriction enzymes and probed on a Southern blot to yield unique bands. The sequences of the invention are useful as additional DNA markers for "restriction fragment length polymorphisms" (RFLP; (Smulson *et al.*, US Patent No. 5,272,057, 1993)).

Furthermore, the *VEGFmg* sequences can be used to determine the actual base-by-base DNA sequence of targeted portions of an individual's genome. *VEGFmg* sequences can be used to prepare two PCR primers from the 5'- and 3'-termini of the sequences that can then be used to amplify an the corresponding sequences from an individual's genome and then sequence the amplified fragment.

Panels of corresponding DNA sequences from individuals can provide unique individual identifications, as each individual will have a unique set of such DNA

sequences due to allelic differences. The sequences of the invention can be used to obtain such identification sequences from individuals and from tissue. The *VEGFmg* sequences of the invention uniquely represent portions of an individual's genome. Allelic variation occurs to some degree in the coding regions of these sequences, and to a greater degree in the noncoding regions. The allelic variation between individual humans occurs with a frequency of about once ever 500 bases. Much of the allelic variation is due to single nucleotide polymorphisms (SNPs), which include RFLPs.

Each of the sequences described herein can, to some degree, be used as a standard against which DNA from an individual can be compared for identification purposes. Because greater numbers of polymorphisms occur in noncoding regions, fewer sequences are necessary to differentiate individuals. Noncoding sequences can positively identify individuals with a panel of 10 to 1,000 primers that each yield a noncoding amplified sequence of 100 bases.

3. *Assaying VEGF-modulated genes using oligonucleotide arrays*

In addition to using the nucleotide probes, antibodies, *etc.*, described above, other methods are available to identify VEGFmg expression.

The invention provides for the use of the genes identified as differentially expressed in methods directed to screen for compounds that affect survival of endothelial cells, such as HUVECs. The simultaneous analysis of *VEGFmg* expression levels with appropriate controls can assess drugs, proteins, or other compounds and formulations. Assessing the extent of differential expression of *VEGFmgs* can be accomplished using an array or similar device containing oligonucleotides complementary to and capable of binding or hybridizing to the mRNAs corresponding to *VEGFmgs*. For example, such an array can measure mRNA levels in endothelial cells treated with, for example, a compound, and compared to mRNA levels in untreated cells. One example of this device is GeneChip™ (Affymetrix, CITY, CA), a miniaturized, high-density array of oligonucleotides complementary to and capable of binding or hybridizing to a set of mRNAs. The technical implementation of this strategy is described in detail (Lipshutz *et al.*, 1999).

Predictive medicine

The invention also pertains to the field of predictive medicine in which diagnostic assays, prognostic assays, pharmacogenomics, and monitoring clinical trials are used for prognostic (predictive) purposes to treat an individual prophylactically. Accordingly, one aspect of the invention relates to diagnostic assays for determining VEGFmg and/or nucleic acid expression as well as VEGFmg activity, in the context of a biological sample (*e.g.*, blood, serum, cells, tissue) to determine whether an individual is afflicted with a disease or disorder, or is at risk of developing a disorder, associated with aberrant VEGFmg expression or activity, including angiogenesis and cell survival. The invention also provides for prognostic (or predictive) assays for determining whether an individual is at risk of developing a disorder associated with VEGFmg, nucleic acid expression or activity. For example, mutations in *VEGFmg* can be assayed in a biological sample. Such assays can be used for prognostic or predictive purpose to prophylactically treat an individual prior to the onset of a disorder characterized by or associated with VEGFmg, nucleic acid expression, or biological activity.

Another aspect of the invention provides methods for determining VEGFmg activity, or nucleic acid expression, in an individual to select appropriate therapeutic or prophylactic agents for that individual (referred to herein as "pharmacogenomics"). Pharmacogenomics allows for the selection of modalities (*e.g.*, drugs, foods) for therapeutic or prophylactic treatment of an individual based on the individual's genotype (*e.g.*, the individual's genotype to determine the individual's ability to respond to a particular agent). Another aspect of the invention pertains to monitoring the influence of modalities (*e.g.*, drugs, foods) on the expression or activity of VEGFmg in clinical trials.

1. *Diagnostic assays*

An exemplary method for detecting the presence or absence of VEGFmg in a biological sample involves obtaining a biological sample from a subject and contacting the biological sample with a compound or an agent capable of detecting VEGFmg or *VEGFmg* nucleic acids (*e.g.*, mRNA, genomic DNA) such that the presence of a VEGFmg is confirmed in the sample. An agent for detecting *VEGFmg* mRNA or genomic DNA is a labeled nucleic acid probe that can hybridize to *VEGFmg* mRNA or genomic DNA. The nucleic acid probe can be, for example, a

full-length *VEGFmg* nucleic acid or a portion thereof, such as an oligonucleotide of at least 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to *VEGFmg* mRNA or genomic DNA.

An agent for detecting VEGFmg polypeptide is an antibody capable of binding to a VEGFmg, preferably an antibody with a detectable label. Abs can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment (*e.g.*, F_{ab} or $F(ab')_2$) can be used. A labeled probe or antibody is coupled (*i.e.*, physically linking) to a detectable substance, as well as indirect detection of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently-labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently-labeled streptavidin. The term "biological sample" includes tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. The detection method of the invention can be used to detect *VEGFmg* mRNA, protein, or genomic DNA in a biological sample *in vitro* as well as *in vivo*. For example, *in vitro* techniques for detection of *VEGFmg* mRNA include Northern hybridizations and *in situ* hybridizations. *In vitro* techniques for detection of VEGFmg polypeptide include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations, and immunofluorescence. *In vitro* techniques for detection of *VEGFmg* genomic DNA include Southern hybridizations and fluorescence in situ hybridization (FISH). Furthermore, *in vivo* techniques for detecting VEGFmg include introducing into a subject a labeled anti-VEGFmg antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

In one embodiment, the biological sample from the subject contains protein molecules, and/or mRNA molecules, and/or genomic DNA molecules. A preferred biological sample is blood.

In another embodiment, the methods further involve obtaining a biological sample from a subject to provide a control, contacting the sample with a compound or agent to detect *VEGFmg*, mRNA, or genomic DNA, and comparing the presence of *VEGFmg*, mRNA or genomic DNA in the control sample with the presence of VEGFmg, mRNA or genomic DNA in the test sample.

The invention also encompasses kits for detecting VEGFmg in a biological sample. For example, the kit can comprise: a labeled compound or agent capable of detecting VEGFmg or *VEGFmg* mRNA in a sample; reagent and/or equipment for determining the amount of VEGFmg in the sample; and reagent and/or equipment for comparing the amount of VEGFmg in the sample with a standard. The compound or agent can be packaged in a suitable container. The kit can further comprise instructions for using the kit to detect VEGFmg or nucleic acid.

2. *Prognostic assays*

The diagnostic methods described herein can furthermore be utilized to identify subjects having or at risk of developing a disease or disorder associated with aberrant VEGFmg expression or activity. For example, the assays described herein, can be used to identify a subject having or at risk of developing a disorder associated with VEGFmg, nucleic acid expression or activity. Alternatively, the prognostic assays can be used to identify a subject having or at risk for developing a disease or disorder. The invention provides a method for identifying a disease or disorder associated with aberrant VEGFmg expression or activity in which a test sample is obtained from a subject and VEGFmg or nucleic acid (*e.g.*, mRNA, genomic DNA) is detected. A test sample is a biological sample obtained from a subject. For example, a test sample can be a biological fluid (*e.g.*, serum), cell sample, or tissue.

Prognostic assays can be used to determine whether a subject can be administered a modality (*e.g.*, an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, food, *etc.*) to treat a disease or disorder associated with aberrant VEGFmg expression or activity. Such methods can be used to determine whether a subject can be effectively treated with an agent for a disorder. The invention provides methods for determining whether a subject can be effectively treated with an agent for a disorder associated with aberrant VEGFmg expression or activity in which a test sample is obtained and VEGFmg or nucleic acid is detected (*e.g.*, where the presence of VEGFmg or nucleic acid is diagnostic for a subject that can be administered the agent to treat a disorder associated with aberrant VEGFmg expression or activity).

The methods of the invention can also be used to detect genetic lesions in a VEGFmg to determine if a subject with the genetic lesion is at risk for a disorder

characterized by aberrant cell proliferation or differentiation. Methods include detecting, in a sample from the subject, the presence or absence of a genetic lesion characterized by at an alteration affecting the integrity of a gene encoding a VEGFmg polypeptide, or the mis-expression of *VEGFmg*. Such genetic lesions can be detected by ascertaining: (1) a deletion of one or more nucleotides from *VEGFmg*; (2) an addition of one or more nucleotides to a *VEGFmg*; (3) a substitution of one or more nucleotides in a *VEGFmg*, (4) a chromosomal rearrangement of a VEGFmg gene; (5) an alteration in the level of a *VEGFmg* mRNA transcripts, (6) aberrant modification of a VEGFmg, such as a change genomic DNA methylation, (7) the presence of a non-wild-type splicing pattern of a VEGFmg mRNA transcript, (8) a non-wild-type level of a *VEGFmg*, (9) allelic loss of *VEGFmg*, and/or (10) inappropriate post-translational modification of VEGFmg polypeptide. There are a large number of known assay techniques that can be used to detect lesions in a *VEGFmg*. Any biological sample containing nucleated cells may be used.

In certain embodiments, lesion detection may use a probe/primer in a polymerase chain reaction (PCR) (*e.g.*, (Mullis, US Patent No. 4,683,202, 1987; Mullis *et al.*, US Patent No. 4,683,195, 1987), such as anchor PCR or rapid amplification of cDNA ends (RACE) PCR, or, alternatively, in a ligation chain reaction (LCR) (*e.g.*, (Landegren *et al.*, 1988; Nakazawa *et al.*, 1994), the latter is particularly useful for detecting point mutations in *VEGFmg*-genes (Abravaya *et al.*, 1995). This method may include collecting a sample from a patient, isolating nucleic acids from the sample, contacting the nucleic acids with one or more primers that specifically hybridize to a *VEGFmg* under conditions such that hybridization and amplification of the *VEGFmg* (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample. It is anticipated that PCR and/or LCR may be desirable to use as a preliminary amplification step in conjunction with any of the techniques used for detecting mutations described herein.

Alternative amplification methods include: self-sustained sequence replication (Guatelli *et al.*, 1990), transcriptional amplification system (Kwoh *et al.*, 1989); Qβ Replicase (Lizardi *et al.*, 1988), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to

those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules present in low abundance.

Mutations in a *VEGFmg* from a sample can be identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis and compared. Differences in fragment length sizes between sample and control DNA indicates mutations in the sample DNA. Moreover, the use of sequence specific ribozymes can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site.

Hybridizing a sample and control nucleic acids, *e.g.*, DNA or RNA, to high-density arrays containing hundreds or thousands of oligonucleotides probes, can identify genetic mutations in *VEGFmg* (Cronin *et al.*, 1996; Kozal *et al.*, 1996). For example, genetic mutations in *VEGFmg* can be identified in two-dimensional arrays containing light-generated DNA probes (Cronin, *et al.*, 1996). Briefly, a first hybridization array of probes can be used to scan through long stretches of DNA in a sample and control to identify base changes between the sequences by making linear arrays of sequential overlapping probes. This step allows the identification of point mutations. This is followed by a second hybridization array that allows the characterization of specific mutations by using smaller, specialized probe arrays complementary to all variants or mutations detected. Each mutation array is composed of parallel probe sets, one complementary to the wild-type gene and the other complementary to the mutant gene.

In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence the *VEGFmg* of interest and detect mutations by comparing the sequence of the sample VEGFmg-with the corresponding wild-type (control) sequence. Examples of sequencing reactions include those based on classic techniques (Maxam and Gilbert, 1977; Sanger *et al.*, 1977). Any of a variety of automated sequencing procedures can be used when performing diagnostic assays (Naeve *et al.*, 1995) including sequencing by mass spectrometry (Cohen *et al.*, 1996; Griffin and Griffin, 1993; Koster, WO94/16101, 1994).

Other methods for detecting mutations in a *VEGFmg* include those in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers *et al.*, 1985). In general, the technique of "mismatch cleavage" starts by providing heteroduplexes formed by hybridizing (labeled) RNA or DNA containing the wild-type *VEGFmg* sequence with potentially mutant RNA or DNA obtained from a sample. The double-stranded duplexes are treated with an agent that cleaves single-stranded regions of the duplex such as those that arise from base pair mismatches between the control and sample strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with S_1 nuclease to enzymatically digest the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. The digested material is then separated by size on denaturing polyacrylamide gels to determine the mutation site (Grompe *et al.*, 1989; Saleeba and Cotton, 1993). The control DNA or RNA can be labeled for detection.

Mismatch cleavage reactions may employ one or more proteins that recognize mismatched base pairs in double-stranded DNA (DNA mismatch repair) in defined systems for detecting and mapping point mutations in *VEGFmg* cDNAs obtained from samples of cells. For example, the mutY enzyme of *E. coli* cleaves A at G/A mismatches and the thymidine DNA glycosylase from HeLa cells cleaves T at G/T mismatches (Hsu *et al.*, 1994). According to an exemplary embodiment, a probe based on a wild-type *VEGFmg* sequence is hybridized to a cDNA or other DNA product from a test cell(s). The duplex is treated with a DNA mismatch repair enzyme, and the cleavage products, if any, can be detected from electrophoresis protocols or the like (Modrich *et al.*, US Patent No. 5,459,039, 1995).

Electrophoretic mobility alterations can be used to identify mutations in *VEGFmg*. For example, single strand conformation polymorphism (SSCP) may be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Cotton, 1993; Hayashi, 1992; Orita *et al.*, 1989). Single-stranded DNA fragments of sample and control *VEGFmg* nucleic acids are denatured and then renatured. The secondary structure of single-stranded nucleic acids varies according to sequence; the resulting alteration in electrophoretic mobility allows detection of

even a single base change. The DNA fragments may be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a sequence changes. The subject method may use heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen *et al.*, 1991).

The migration of mutant or wild-type fragments can be assayed using denaturing gradient gel electrophoresis (DGGE; (Myers *et al.*, 1985). In DGGE, DNA is modified to prevent complete denaturation, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. A temperature gradient may also be used in place of a denaturing gradient to identify differences in the mobility of control and sample DNA (Rossiter and Caskey, 1990).

Examples of other techniques for detecting point mutations include, but are not limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension. For example, oligonucleotide primers may be prepared in which the known mutation is placed centrally and then hybridized to target DNA under conditions that permit hybridization only if a perfect match is found (Saiki *et al.*, 1986; Saiki *et al.*, 1989). Such allele-specific oligonucleotides are hybridized to PCR-amplified target DNA or a number of different mutations when the oligonucleotides are attached to the hybridizing membrane and hybridized with labeled target DNA.

Alternatively, allele specific amplification technology that depends on selective PCR amplification may be used. Oligonucleotide primers for specific amplifications may carry the mutation of interest in the center of the molecule (so that amplification depends on differential hybridization (Gibbs *et al.*, 1989)) or at the extreme 3'-terminus of one primer where, under appropriate conditions, mismatch can prevent, or reduce polymerase extension (Prosser, 1993). Novel restriction site in the region of the mutation may be introduced to create cleavage-based detection (Gasparini *et al.*, 1992). Certain amplification may also be performed using *Taq* ligase for amplification (Barany, 1991). In such cases, ligation occurs only if there is a perfect match at the 3'-terminus of the 5' sequence, allowing detection of a known mutation by scoring for amplification.

The described methods may be performed, for example, by using pre-packaged kits comprising at least one probe (nucleic acid or antibody) that may be conveniently used, for example, in clinical settings to diagnose patients exhibiting symptoms or family history of a disease or illness involving VEGFmg.

Furthermore, any cell type or tissue in which VEGFmg is expressed may be utilized in the prognostic assays described herein.

3. *Pharmacogenomics*

Agents, or modulators that have a stimulatory or inhibitory effect on VEGFmg activity or expression, as identified by a screening assay can be administered to individuals to treat, prophylactically or therapeutically, disorders, including insufficient blood supply or improper cell survival. In conjunction with such treatment, the pharmacogenomics (*i.e.*, the study of the relationship between a subject's genotype and the subject's response to a foreign modality, such as a food, compound or drug) may be considered. Metabolic differences of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. Thus, the pharmacogenomics of the individual permits the selection of effective agents *(e.g.*, drugs) for prophylactic or therapeutic treatments based on a consideration of the individual's genotype. Pharmacogenomics can further be used to determine appropriate dosages and therapeutic regimens. Accordingly, the activity of VEGFmg, expression of *VEGFmg* nucleic acid, or *VEGFmg* mutation(s) in an individual can be determined to guide the selection of appropriate agent(s) for therapeutic or prophylactic treatment.

Pharmacogenomics deals with clinically significant hereditary variations in the response to modalities due to altered modality disposition and abnormal action in affected persons (Eichelbaum and Evert, 1996; Linder *et al.*, 1997). In general, two pharmacogenetic conditions can be differentiated: (1) genetic conditions transmitted as a single factor altering the interaction of a modality with the body (altered drug action) or (2) genetic conditions transmitted as single factors altering the way the body acts on a modality (altered drug metabolism). These pharmacogenetic conditions can occur either as rare defects or as nucleic acid polymorphisms. For example, glucose-6-phosphate dehydrogenase (G6PD) deficiency is a common

inherited enzymopathy in which the main clinical complication is hemolysis after ingestion of oxidant drugs (anti-malarials, sulfonamides, analgesics, nitrofurans) and consumption of fava beans.

As an illustrative embodiment, the activity of drug metabolizing enzymes is a major determinant of both the intensity and duration of drug action. The discovery of genetic polymorphisms of drug metabolizing enzymes (*e.g.*, N-acetyltransferase 2 (NAT 2) and cytochrome P450 enzymes CYP2D6 and CYP2C19) explains the phenomena of some patients who show exaggerated drug response and/or serious toxicity after taking the standard and safe dose of a drug. These polymorphisms are expressed in two phenotypes in the population, the extensive metabolizer (EM) and poor metabolizer (PM). The prevalence of PM is different among different populations. For example, the *CYP2D6* gene is highly polymorphic and several mutations have been identified in PM, which all lead to the absence of functional CYP2D6. Poor metabolizers due to mutant *CYP2D6* and *CYP2C19* frequently experience exaggerated drug responses and side effects when they receive standard doses. If a metabolite is the active therapeutic moiety, PM shows no therapeutic response, as demonstrated for the analgesic effect of codeine mediated by its CYP2D6-formed metabolite morphine. At the other extreme are the so-called ultra-rapid metabolizers who are unresponsive to standard doses. Recently, the molecular basis of ultra-rapid metabolism has been identified to be due to CYP2D6 gene amplification.

The activity of VEGFmg, expression of *VEGFmg* nucleic acid, or mutation content of *VEGFmg* in an individual can be determined to select appropriate agent(s) for therapeutic or prophylactic treatment of the individual. In addition, pharmacogenetic studies can be used to apply genotyping of polymorphic alleles encoding drug-metabolizing enzymes to the identification of an individual's drug responsiveness phenotype. This knowledge, when applied to dosing or drug selection, can avoid adverse reactions or therapeutic failure and thus enhance therapeutic or prophylactic efficiency when treating a subject with a VEGFmg modulator, such as a modulator identified by one of the described exemplary screening assays.

4. *Monitoring effects during clinical trials*

Monitoring the influence of agents (*e.g.*, drugs, compounds) on the expression or activity of VEGFmg (*e.g.*, the ability to modulate aberrant cell proliferation and/or differentiation) can be applied not only in basic drug screening, but also in clinical trials. For example, the effectiveness of an agent determined by a screening assay to increase *VEGFmg* expression, protein levels, or up-regulate VEGFmg activity can be monitored in clinical trails of subjects exhibiting decreased *VEGFmg* expression, protein levels, or down-regulated VEGFmg activity. Alternatively, the effectiveness of an agent determined to decrease *VEGFmg* expression, protein levels, or down-regulate VEGFmg activity, can be monitored in clinical trails of subjects exhibiting increased *VEGFmg* expression, protein levels, or up-regulated VEGFmg activity. In such clinical trials, the expression or activity of VEGFmg and, preferably, other genes that have been implicated in, for example, angiogenesis or apoptosis, can be used as a "read out" or markers for a particular cell's responsiveness.

For example, genes, including *VEGFmg*, that are modulated in cells by treatment with a modality (*e.g.*, food, compound, drug or small molecule) can be identified. To study the effect of agents on cellular proliferation disorders, for example, in a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of *VEGFmg* and other genes implicated in the disorder. The gene expression pattern can be quantified by Northern blot analysis, nuclear run-on or RT-PCR experiments, or by measuring the amount of protein, or by measuring the activity level of VEGFmg or other gene products. In this manner, the gene expression pattern itself can serve as a marker, indicative of the cellular physiological response to the agent. Accordingly, this response state may be determined before, and at various points during, treatment of the individual with the agent.

The invention provides a method for monitoring the effectiveness of treatment of a subject with an agent (*e.g.*, an agonist, antagonist, protein, peptide, peptidomimetic, nucleic acid, small molecule, food or other drug candidate identified by the screening assays described herein) comprising the steps of (1) obtaining a pre-administration sample from a subject; (2) detecting the level of expression of a VEGFmg, mRNA, or genomic DNA in the preadministration sample; (3) obtaining one or more post-administration samples from the subject; (4) detecting the level of expression or activity of the VEGFmg, mRNA, or genomic DNA in the

post-administration samples; (5) comparing the level of expression or activity of the VEGFmg, mRNA, or genomic DNA in the pre-administration sample with the VEGFmg, mRNA, or genomic DNA in the post administration sample or samples; and (6) altering the administration of the agent to the subject accordingly. For example, increased administration of the agent may be desirable to increase the expression or activity of VEGFmg to higher levels than detected, *i.e.*, to increase the effectiveness of the agent. Alternatively, decreased administration of the agent may be desirable to decrease expression or activity of VEGFmg to lower levels than detected, *i.e.*, to decrease the effectiveness of the agent.

5. *Methods of treatment*

The invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a disorder or having a disorder associated with aberrant VEGFmg expression or activity.

6. *Diseases and disorders*

Diseases and disorders that are characterized by increased VEGFmg levels or biological activity may be treated with therapeutics that antagonize (*i.e.*, reduce or inhibit) activity. Antognists may be administered in a therapeutic or prophylactic manner. Therapeutics that may be used include: (1) VEGFmg peptides, or analogs, derivatives, fragments or homologs thereof; (2) Abs to a VEGFmg peptide; (3) *VEGFmg* nucleic acids; (4) administration of antisense nucleic acid and nucleic acids that are "dysfunctional" (*i.e.*, due to a heterologous insertion within the coding sequences) that are used to eliminate endogenous function of by homologous recombination (Capecchi, 1989); or (5) modulators (*i.e.*, inhibitors, agonists and antagonists, including additional peptide mimetic of the invention or Abs specific to VEGFmg) that alter the interaction between VEGFmg and its binding partner.

Diseases and disorders that are characterized by decreased VEGFmg levels or biological activity may be treated with therapeutics that increase (*i.e.*, are agonists to) activity. Therapeutics that upregulate activity may be administered therapeutically or prophylactically. Therapeutics that may be used include peptides, or analogs, derivatives, fragments or homologs thereof; or an agonist that increases bioavailability.

Increased or decreased levels can be readily detected by quantifying peptide and/or RNA, by obtaining a patient tissue sample (*e.g.*, from biopsy tissue) and assaying *in vitro* for RNA or peptide levels, structure and/or activity of the expressed peptides (or *VEGFmg* mRNAs). Methods include, but are not limited to, immunoassays (*e.g.*, by Western blot analysis, immunoprecipitation followed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, immunocytochemistry, *etc.*) and/or hybridization assays to detect expression of mRNAs (*e.g.*, Northern assays, dot blots, *in situ* hybridization, and the like).

7. *Prophylactic methods*

The invention provides a method for preventing, in a subject, a disease or condition associated with an aberrant VEGFmg expression or activity, by administering an agent that modulates VEGFmg expression or at least one VEGFmg activity. Subjects at risk for a disease that is caused or contributed to by aberrant VEGFmg expression or activity, such as tumorigenesis or metastasis, can be identified by, for example, any or a combination of diagnostic or prognostic assays. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the VEGFmg aberrancy, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of VEGFmg aberrancy, for example, a VEGFmg agonist or VEGFmg antagonist can be used to treat the subject. The appropriate agent can be determined based on screening assays.

VEGFmg nucleic acids, or fragments, may also be useful in diagnostic applications, wherein the presence or amount of the nucleic acid or the protein is to be assessed. A further use could be as an anti-bacterial molecule (*i.e.*, some peptides have been found to possess anti-bacterial properties). These materials are further useful in the generation of Abs that immunospecifically bind to the novel substances of the invention for use in therapeutic or diagnostic methods.

8. *Therapeutic methods*

Another aspect of the invention pertains to methods of modulating VEGFmg expression or activity for therapeutic purposes. The modulatory method of the invention involves contacting a cell with an agent that modulates one or more of the activities of VEGFmg activity associated with the cell. An agent that modulates

VEGFmg activity can be a nucleic acid or a protein, a naturally occurring cognate ligand of VEGFmg, a peptide, a VEGFmg peptidomimetic, or other small molecule. The agent may stimulate VEGFmg activity. Examples of such stimulatory agents include active VEGFmg and a *VEGFmg* nucleic acid molecule that has been introduced into the cell. In another embodiment, the agent inhibits VEGFmg activity. Examples of inhibitory agents include antisense *VEGFmg* nucleic acids and anti-VEGFmg Abs. Modulatory methods can be performed *in vitro* (*e.g.*, by culturing the cell with the agent) or, alternatively, *in vivo* (*e.g.*, by administering the agent to a subject). As such, the invention provides methods of treating an individual afflicted with a disease or disorder characterized by aberrant expression or activity of a VEGFmg or nucleic acid molecule. In one embodiment, the method involves administering an agent (*e.g.*, an agent identified by a screening assay), or combination of agents that modulates (*e.g.*, up-regulates or down-regulates) VEGFmg expression or activity. In another embodiment, the method involves administering a VEGFmg or nucleic acid molecule as therapy to compensate for reduced or aberrant VEGFmg expression or activity.

Stimulation of VEGFmg activity is desirable in situations in which VEGFmg is abnormally down-regulated and/or in which increased VEGFmg activity is likely to have a beneficial effect. One example of such a situation is where a subject has a disorder characterized by aberrant cell proliferation and/or differentiation (*e.g.*, cancer or immune associated disorders).

9. *Determination of the biological effect of the therapeutic*

Suitable *in vitro* or *in vivo* assays can be performed to determine the effect of a specific therapeutic and whether its administration is indicated for treatment of the affected tissue.

In various specific embodiments, *in vitro* assays may be performed with representative cells of the type(s) involved in the patient's disorder, to determine if a given therapeutic exerts the desired effect upon the cell type(s). Modalities for use in therapy may be tested in suitable animal model systems including, but not limited to rats, mice, chicken, cows, monkeys, rabbits, and the like, prior to testing in human subjects. Similarly, for *in vivo* testing, any of the animal model system known in the

art may be used prior to administration to human subjects. Various assays directed at measuring angiogenesis and cell survival may be used.

10. *Anti-sense nucleic acids*

Using antisense and sense VEGFmg oligonucleotides can prevent VEGFmg polypeptide expression. These oligonucleotides bind to target nucleic acid sequences, forming duplexes that block transcription or translation of the target sequence by enhancing degradation of the duplexes, terminating prematurely transcription or translation, or by other means.

Antisense or sense oligonucleotides are singe-stranded nucleic acids, either RNA or DNA, which can bind target *VEGFmg* mRNA (sense) or *VEGFmg* DNA (antisense) sequences. Anti-sense nucleic acids can be designed according to Watson and Crick or Hoogsteen base pairing rules. The anti-sense nucleic acid molecule can be complementary to the entire coding region of *VEGFmg* mRNA, but more preferably, to only a portion of the coding or noncoding region of *VEGFmg* mRNA. For example, the anti-sense oligonucleotide can be complementary to the region surrounding the translation start site of *VEGFmg* mRNA. Antisense or sense oligonucleotides may comprise a fragment of the VEGFmg DNA coding region of at least about 14 nucleotides, preferably from about 14 to 30 nucleotides. In general, antisense RNA or DNA molecules can comprise at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 bases in length or more. Among others, (Stein and Cohen, 1988; van der Krol *et al.*, 1988a) describe methods to derive antisense or a sense oligonucleotides from a given cDNA sequence.

Examples of modified nucleotides that can be used to generate the anti-sense nucleic acid include: 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, β-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, β-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 anti-sense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been sub-cloned in an anti-sense orientation such that the transcribed RNA will be complementary to a target nucleic acid of interest.

To introduce antisense or sense oligonucleotides into target cells (cells containing the target nucleic acid sequence), any gene transfer method may be used. Examples of gene transfer methods include (1) biological, such as gene transfer vectors like Epstein-Barr virus or conjugating the exogenous DNA to a ligand-binding molecule, (2) physical, such as electroporation and injection, and (3) chemical, such as $CaPO_4$ precipitation and oligonucleotide-lipid complexes.

An antisense or sense oligonucleotide is inserted into a suitable gene transfer retroviral vector. A cell containing the target nucleic acid sequence is contacted with the recombinant retroviral vector, either *in vivo* or *ex vivo*. Examples of suitable retroviral vectors include those derived from the murine retrovirus M-MuLV, N2 (a retrovirus derived from M-MuLV), or the double copy vectors designated DCT5A, DCT5B and DCT5C (WO 90/13641, 1990). To achieve sufficient nucleic acid molecule transcription, vector constructs in which the transcription of the anti-sense nucleic acid molecule is controlled by a strong pol II or pol III promoter are preferred.

To specify target cells in a mixed population of cells cell surface receptors that are specific to the target cells can be exploited. Antisense and sense oligonucleotides are conjugated to a ligand-binding molecule, as described in (WO 91/04753, 1991). Ligands are chosen for receptors that are specific to the target cells. Examples of suitable ligand-binding molecules include cell surface receptors, growth factors, cytokines, or other ligands that bind to cell surface receptors or molecules. Preferably, conjugation of the ligand-binding molecule does not substantially interfere with the ability of the receptors or molecule to bind the ligand-binding molecule conjugate, or block entry of the sense or antisense oligonucleotide or its conjugated version into the cell.

Liposomes efficiently transfer sense or an antisense oligonucleotide to cells (WO 90/10448, 1990). The sense or antisense oligonucleotide-lipid complex is preferably dissociated within the cell by an endogenous lipase.

The anti-sense nucleic acid molecule of the invention may be 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 α-units, the strands run parallel to each other (Gautier *et al.*, 1987). The anti-sense nucleic acid molecule can also comprise a 2'-o-methylribonucleotide (Inoue *et al.*, 1987a) or a chimeric RNA-DNA analogue (Inoue *et al.*, 1987b).

In one embodiment, an anti-sense nucleic acid of the invention is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity that are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes, such as hammerhead ribozymes (Haseloff and Gerlach, 1988) can be used to catalytically cleave *VEGFmg* mRNA transcripts and thus inhibit translation. A ribozyme specific for a VEGFmg-encoding nucleic acid can be designed based on the nucleotide sequence of a VEGFmg cDNA. 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 a VEGFmg-encoding mRNA (Cech *et al.*, U.S. Patent No. 5,116,742, 1992; Cech *et al.*, U.S. Patent No. 4,987,071, 1991). *VEGFmg* mRNA can also be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules (Bartel and Szostak, 1993).

Alternatively, *VEGFmg* expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the *VEGFmg* (*e.g.*, the *VEGFmg* promoter and/or enhancers) to form triple helical structures that prevent transcription of the *VEGFmg* in target cells (Helene, 1991; Helene *et al.*, 1992; Maher, 1992).

Modifications of antisense and sense oligonucleotides can augment their effectiveness. Modified sugar-phosphodiester bonds or other sugar linkages (WO 91/06629, 1991), increase *in vivo* stability by conferring resistance to endogenous nucleases without disrupting binding specificity to target sequences. Other modifications can increase the affinities of the oligonucleotides for their targets, such as covalently linked organic moieties (WO 90/10448, 1990) or poly-(L)-lysine. Other

attachments modify binding specificities of the oligonucleotides for their targets, including metal complexes or intercalating (*e.g.* ellipticine) and alkylating agents. For example, the deoxyribose phosphate backbone of the nucleic acids can be modified to generate peptide nucleic acids (Hyrup and Nielsen, 1996). "Peptide nucleic acids" or "PNAs" refer to nucleic acid mimics (*e.g.*, DNA mimics) in that the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs allows for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols (Hyrup and Nielsen, 1996; Perry-O'Keefe *et al.*, 1996). PNAs of VEGFmg can be used in therapeutic and diagnostic applications. For example, PNAs can be used as anti-sense or antigene agents for sequence-specific modulation of gene expression by inducing transcription or translation arrest or inhibiting replication. VEGFmg PNAs may also be used in the analysis of single base pair mutations (*e.g.*, PNA directed PCR clamping; as artificial restriction enzymes when used in combination with other enzymes, *e.g.*, S_1 nucleases (Hyrup and Nielsen, 1996); or as probes or primers for DNA sequence and hybridization (Hyrup and Nielsen, 1996; Perry-O'Keefe *et al.*, 1996).

PNAs of VEGFmg can be modified to enhance their stability or cellular uptake. Lipophilic or other helper groups may be attached to PNAs, PNA-DNA dimmers formed, or the use of liposomes or other drug delivery techniques. For example, PNA-DNA chimeras can be generated that may combine the advantageous properties of PNA and DNA. Such chimeras allow DNA recognition enzymes (*e.g.*, RNase H and DNA polymerases) to interact with the DNA portion while the PNA portion provides high binding affinity and specificity. PNA-DNA chimeras can be linked using linkers of appropriate lengths selected in terms of base stacking, number of bonds between the nucleobases, and orientation (Hyrup and Nielsen, 1996). The synthesis of PNA-DNA chimeras can be performed (Finn *et al.*, 1996; Hyrup and Nielsen, 1996). For example, a DNA chain can be synthesized on a solid support using standard phosphoramidite coupling chemistry, and modified nucleoside analogs, *e.g.*, 5'-(4-methoxytrityl)amino-5'-deoxy-thymidine phosphoramidite, can be used between the PNA and the 5' end of DNA (Finn *et al.*, 1996; Hyrup and Nielsen,

1996). PNA monomers are then coupled in a stepwise manner to produce a chimeric molecule with a 5' PNA segment and a 3' DNA segment (Finn *et al.*, 1996). Alternatively, chimeric molecules can be synthesized with a 5' DNA segment and a 3' PNA segment (Petersen *et al.*, 1976).

The oligonucleotide may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (Lemaitre *et al.*, 1987; Letsinger *et al.*, 1989) or PCT Publication No. WO88/09810) or the blood-brain barrier (*e.g.*, PCT Publication No. WO 89/10134). In addition, oligonucleotides can be modified with hybridization-triggered cleavage agents (van der Krol *et al.*, 1988b) or intercalating agents (Zon, 1988). The oligonucleotide may be conjugated to another molecule, *e.g.*, a peptide, a hybridization triggered cross-linking agent, a transport agent, a hybridization-triggered cleavage agent, and the like.

The following examples illustrate by way of non-limiting example various aspects of the invention.

EXAMPLES

Example 1 *Differential gene expression in human umbilical cord endothelial cells (HUVECs).*

1. Background

To obtain a comprehensive profile of those genes whose expression is modulated during VEGF-dependent, or mutant VEGFR1-dependent, survival pathway, GeneCalling™ s technology (Rothberg *et al.*, US Patent No. 5,871,697, 1999; Shimkets *et al.*, 1999), was applied to serum-starved human umbilical cord endothelial cells treated with a set of growth factors and to reference HUVEC cells grown in the presence of 10% serum. Cells grown in the absence of both any growth factor and serum served as the negative control. GeneCalling™ technology relies on Quantitative Expression Analysis to generate the gene expression profile of a given sample and then generates differential expression analysis of pairwise comparison of these profiles to controls containing no addition. Polynucleotides exhibiting differential expression are confirmed by conducting a PCR reaction according to the

GeneCalling™ protocol with the addition of a competing unlabelled primer that prevents the amplification from being detected.

2. Growth factors used

(a) VEGF

A principal growth factor employed in this example is VEGF, which binds to both VEGFR1 and VEGFR2. In addition, a mutant of VEGF that binds only VEGFR1 (VEGFR1s) was used. The other growth factors used in this study bind to receptors other than VEFGR1 and have different angiogenic potential. They are included as positive (VEGF, VEGFR1s) and negative (PlGF, bFGF, HGS/SF) controls to focus the analysis on the VEGFR1 pathway.

(b) bFGF

Basic fibroblast growth factor (bFGF) is expressed in vascular endothelium during tumor neovascularization and angioproliferative diseases. VEGF and bFGF are potently synergistic in their combined mitogenic activity. A possible explanation for this synergism is the evidence that bFGF induces the expression of VEGF receptor VEGFR1 and of VEGF itself (Hata *et al.*, 1999). Treatment with bFGF will modulate a set of genes overlapping with those modulated by VEGF and VEGFR1s.

(c) HGF/SF

Hepatocyte growth factor/scatter factor (HGF/SF) is a pleiotropic growth factor that stimulates proliferation and migration of endothelial cells. Similarly to bFGF, HGF and VEGF are synergistic in their combined angiogenic activity (Van Belle *et al.*, 1997). HGF induces VEGF expression (Gille *et al.*, 1998). Therefore it could be expected that treatment with bFGF will modulate a set of genes overlapping with those modulated by VEGF.

(d) PlGF

Placenta growth factor (PlGF) belongs to the family of VEGFs (VEGFs). Three PlGF isoforms are produced by alternative splicing and all induce migration of endothelial cells while having no effect on cell proliferation (Migdal *et al.*, 1998). They ligate VEGFR2 receptor but not to VEGFR1 that is thought to mediate most of the angiogenic and proliferative effects of VEGF. Treatment with PlGF will modulate a set of genes overlapping with those modulated by VEGF but not with

those modulated by VEGFR1s. This observation allows for the identification of the set of genes specifically modulated by VEGF via the VEGFR1 receptor.

3. Genes analysed and corresponding GenBank accession

Table E1 provides the GenBank Accession numbers for the genes whose expression was analyzed in this example.

Table E1 GenBank Accessions for analysed genes

Gene Name	GenBank Accession
Nexin/Glia derived neurite promoting factor (GDNPF)	A03911
placental protein 5 (PP5)/tissue factor pathway inhibitor 2	5730090 D29992
heparin-binding EGF-like growth factor (HB-EGF)	4503412
Regulator of G-protein signaling 3 (RGS3)	U27655
Gravin/myasthenia gravis autoantigen	U81607
MKP-1 like protein tyrosine phosphatase (MKP1LPTP)	AF038844
amyloid precursor-like protein 2 (APLP2)	L27631
Osteonidogen, nidogen-2 precursor	D86425
amyloid precursor protein (APP)	D87675
hVPS41p	U87309
arginine-rich protein (ARP)	5174392/M83751
Down's syndrome critical region protein 1 (DSCR1)	4758195/ U28833
insulin induced protein 1 (INSIG1)	5031800/ U96876
cytochrome oxidase subunit I (MTCO1)	AF035429
NADH-ubiquinone oxidoreductase chain 1 (NH1)	DNHUN1
NADH-ubiquinone oxidoreductase chain 4 (NH4)	DNHUN4
decidual protein induced by progesterone (DEPP)	AB022718
connective tissue growth factor (CTGF)	X78947

4. Results

HUVECs were treated with various growth factors, or none, and harvested after 6 or 24 hours. This permits distinguishing between those genes that are more directly regulated by growth factor treatment (after 6 hours) vs. those that may be indirectly regulated, and so appear to be modulated only after 24 hours.

The results of this analysis are summarized in Table E2.

The serum-starved HUVECs represent a valid *in vitro* model because 30% of the cells undergo apoptosis after serum deprivation, representing a 6 fold increase over non-serum starved controls. VEGF or VEGFR1s addition strongly decreases the number of apoptotic cells, while PlGF addition does not stimulate survival (Gerber *et al.*, 1998). These results show that signaling via VEGFR1 and not via the PlGF receptor is important for VEGF activity.

Table E2 GeneCalling™ results

		Treatment															
		Serum		VEGF		Serum		VEGF		VEGFR1s		BFGF		HGF		P1GF	
	Time (hours):	6	24	6	24	6	24	6	24	6	24	6	24	6	24	6	24
Gene	**GeneCalling bands**																
Nexin	f0n0-178.8	-	-	+1.2	+2.7	-	-	-	-	-	-	-	-	-	-	-	-
PP5	b1i0-190.7 d0l0-227.9 i0u0-108.1	-1.6	-1.4	+1.9	+2.7	-	-	+2.4	+2.4	+2.1	+2	+2.3	-	-	-	-	+1.4
HB-EGF	u0f0-157.6	-	+3.9	+3.5	+4.1	+3	-2.6	+2.3	-	-	-	-	+2.1	-	-	-	-
RGS33	b1i0-75.5	-	-2.3	+2.2	+2.6	-	-2.9	-	-	-	+2.2	-	-	-	-	-	-
gravin	d0y0-108.1 y0h0-123.3	+1.4	+5.7	+1.6	+5.7	+4.1	-	-	+2.7	-	-	-	-	-	-	-	-
MKP1LPTP	l1c0-184.5	+3	-	+2.1	+1.6	-	-	-	-	-	-	-	-	-	-	-	-
APLP2	d0v0-324.8	-	-	-1.2	-	-1.4	+1.9	+1.2	+3.2	+1.3	-	-	-	-1.5	+2.3	-	-
Osteonidogen	h0a0-166.1	-	-2.2	+2.1	+2.4	-	-	-	-	-	-	-	-	-	-	-	-
APP	n0s0-112.8	-2	+2.8	-	-1.9	-2	+6.5	-	-	-	+5.6	-	-	-	-	-	-
hVPS41p	i0r0-152.3 w0c0-259	+1.6	+2.8	-2	-2.1	-	+3.7	-	-	-	-	-	-	-	-	-	-
ARP	i0c0-224.3	-	+2.1	-	-	+2.2	-	+2.3	-	+2.8	+2.4	+1.6	-	+1.3	-	-	-
DSCR1	h0a0-78.1	+1.2	-1.1	+6.3	+4.8	-	-	+3	+2.5	+3.1	-	-	+1.8	-	-	-	-

	i0n0-136.2 i0n0-136.3																						
INSIG1	g1n0-43.2	-	-	-	+2.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MTCO1	l0r0-215.7 r0y0-360.5	-	-1.4	-	-2.3	-1.5	-1.6	-	-	-6.2	-1.8	-	-	-1.4	-1.3	-1.5	+6.3						
NH1		+2.5	+11.5	+1.9	+4.2	+8.2	+2.8	-	-3.8	+2	-7	+2.2	-6.8	-	-2.3	-	-7.1						
NH4	u0w0-153.5	-	-	-	-1.7	-	-	-2.3	-2.6	-	-2	-	-1.9	-	-1.4	-	-						
DEPP	s0h0-217.2	-	-	-	-	-	-	+4.7	-	-	-	-	-	-	+3.2	-	-2.8						
CTGF	m0a0-399.6	+3.4	-	+1.4	+1.6	-	+2.5	-	+3.1	+1.8	+5.1	-	-	-2.8	-	-4.4	+2.6						

For each gene, this Table lists the GeneCalling generated cDNA fragments (bands) that were positively associated with that gene by confirmation and the modulation levels observed in each GeneCalling job for those bands.

Example 2 TaqMan™ analysis of differential gene expression in HUVECs

Genes that were shown to be modulated in the GeneCalling analysis were then subjected to Taqman™ analysis (TaqMan™ polymerase chain reaction detection; Perkin Elmer, Applied Biosystems Division, Foster City, CA).

100 ng of total RNA was added to a 50 µl RT-PCR reaction (PCR-Access, Promega). Primers and probes for real time PCR analysis were designed using the Oligo Version 4.0 program (National Bioscience, Plymouth, MN) (Heid *et al.*, 1996). RT-PCR reactions and the resulting relative increase in reporter fluorescent dye emission were monitored in real time with the 7700 Sequence Detector (Perkin Elmer, Foster City, CA). Signals were analyzed using the sequence detector 1.0 program (PE). Conditions were as follows: 1 cycle 48° C for 45 min., 1 cycle 94° C for 2 min., 40 cycles 94° C, 30 sec., 60° C, 1 min., 68° C, 2 min.

The results are shown in Table E3.

Table E3 TaqMan™ analysis results.

		SERUM			VEGF			
Gene	**Bands**	6 h	18 h	24 h	6 h	18 h	24 h	32 h
PP5	b1i0-190.7 d0l0-227.9 i0u0-108.1	nd	nd	nd	nd	5	nd	6
HB-EGF	U0f0-157.6	nd	nd	4	4	nd	4.9	nd
RGS3	B1i0-75.5	nd	nd	nd	4	4	nd	Nd
Gravin	D0y0-108.1 y0h0-123.3	nd	nd	nd	5	4	nd	4.5
MKP1LPTP	L1c0-184.5	1	2	nd	3	2	nd	Nd
APLP2	D0v0-324.8	1	nd	nd	1.6	nd	5	Nd
Osteonidogen	H0a0-166.1	nd	nd	4	3	3	5.3	Nd
hVPS41p	I0r0-152.3 w0c0-259	nd	nd	nd	nd	2	nd	3
ARP	I0c0-224.3	nd	nd	nd	3	2	nd	3
DSCR1	H0a0-78.1 i0n0-136.2 i0n0-136.3	nd	nd	nd	4	6	nd	5
Nexin	F0n0-178.8	1	nd	1	1.5	nd	nd	2.4
INSIG1	G1n0-43.2	1	nd	1	1.5	nd	nd	3.2
CTGF	M0a0-399.6	nd	nd	nd	nd	1.7	nd	6
For each gene, the GeneCalling generated cDNA fragments (bands) that were positively associated with that gene by confirmation and the modulation levels observed by TaqMan analysis.								

Table E7 Probe Primer sets used for Real-time RT-PCR analysis.

	Probe sequence	#	Forward primer	#	Reverse primer	#
HSPP5.P/#43	aaagttcccaaagtttgccggctgc	45	cgatgcttgctggaggataga	46	acactggtcgtccacactcact	47
HVPS41 1667.FP/#50	ttcgcccagacatgtatccctgcag	48	atgtgccccgggatgatata	49	gtcccccagccaataatcagt	50
HSARP 560.FP/#51	aggtatcaaagcctctggcccacca	51	gcagccaccaaaatcatcaat	52	tcacagatcttctccacagggat	53
HSDSCR1 1113.FP/#52	aggttgtgaaaacagcagcaatgcaatgt	54	ccacaggaagccgcctagt	55	tgagggaagaaaggaaacgct	56
HSGRAVIN 4118.FP/#53	ctgaggcatcattcactctaacagcggc	57	gaggaggcagtatgcaccaaa	58	tgcaggctccaacgtttca	59
HSDOCK 180hlg.259.FP#	agaatgccgcgtgctttctcctgac	60	atgtaggacagaacgggcctt	61	gttttgaattgcattgcccc	62
HSRGS3 1696.FP/#55	aggacaacctgcagagcgtcacgc	63	aagatgcgcttctgtgcca	64	aacctggactcctacacgcg	65
HSPDK-1 1059.FP	tgtgaggaaatggaaggatacggacctcttaaa	66	gatgccacaaagcggttagg	67	gtgacggactcgaagaacgg	68
HSPTPLC100hlg 183.FP/#57	tacaactgggtgaaagcccggcg	69	acaacgtgtgcctgctgga	70	cctacgttgggcctgatgac	71
HSVEGF.294.FP/#92	tgtgcccactgaggagtccaacatca	72	aatgacgagggcctggagt	73	ttgatccgcataatctgcatg	74
HSHB-EGF.300.FP/	ctggctgcagttctctcggcactg	75	tgaacagtgaggtatgctgaact	76	ctccaggctctcgccagtc	77
HSFlt-1-2449T/#175	accaaccagaagggctctgtggaa	78	aaggtgtctatcactgcaaagc	79	tgaacagtgaggtatgctgaact	80
HSKDR.1180.RP/#93	agacaggtcgggtgagggcg	81	cgcctctgtgggtaagga	82	ccgagttagatctggctttca	83

#, SEQ ID NO:.

Example 3 ^{33}P-hybridization analysis of differential gene expression

Formalin fixed, paraffin-embedded human tissues were investigated for *in situ* mRNA expression. Tissues included first trimester (14-15 week) placenta, adult adrenal cortex, aorta, muscular artery with atherosclerosis, brain, gall bladder, heart, pancreas, prostate, stomach, eye with age related macular degeneration (AMD), and inflamed appendix, pulmonary adenocarcinoma, ductal mammary adenocarcinoma, kidney with renal cell carcinoma, hepatocellular carcinoma, squamous cell carcinoma, osteosarcoma, and chondrosarcoma. *In vitro* transcription and [^{33}P] labeling of sense and anti-sense riboprobes was performed as follows: Sequences for the genes to be analyzed were PCR-amplified from plasmid DNA using gene-specific primers that encoded T3 or T7 RNA polymerase initiation sites. Sense and antisense riboprobes were prepared by *in vitro* transcription from the PCR -amplified templates and diluted in hybridization buffer to a specific activity of 1 x 10^6 cpm/ml .Tissue sections 5 micrometers thick were deparaffinized, deproteinated in 4 µg/ml of proteinase K for 30 minutes at 37°C, hybridized at 55°C overnight, then washed at high stringency (55°C in 0.1 X SSC for 2 hours). Glass slides were dipped in NBT2 nuclear track emulsion (Eastman Kodak), exposed in sealed plastic slide boxes containing dessicant for 4 weeks at 4°C, developed and counterstained with hematoxylin and eosin.

The results of the in-situ hybridization experiments are shown in Table E4.

Table E4 ***In situ*** **hybridization analysis**

	DSCR1	PP5	RGS3	ARP	hVPS41p	HB-EGF	Gravin	MKP1-LPTP	CTGF	nexin	Osteo-nidogen
HUVEC: ct values	23.1	18.7	21.7	20.5	22.4	25.9	19.3	23	21	-	22
tumor: vascular	-	-	++	-	-	-	(+)	-	++	-	++
tumor : non vascular	+	-	+++	++	++	++	++	+/++	stromal	+/++	++
fetal: vascular	++	+++	-	-	-	-	++	-	+++	-	++
fetal: non vascular	++	+	+	++	-	+/++	++		+++	+/++	++
adult vascular	-	-	-	-	-	-	(+)	-	++	-	+
adult non vascular	+	-	+	-	-	+	-	-	++	(+)	+
Inflammation	-	-	+	+++	-	+++	?	-	++		++

+ weak expression, ++ moderate expression, +++ strong expression

The results in Table E4 show that in fetal vascular tissue certain of the differentially expressed genes identified by GeneCalling are also differentially identified by in-situ hybridization. In adult vascular tissue, however, only pathological states, such as presence of a tumor or of inflammation, lead to significant modulation of genes among the set of differentially expressed genes.

Example 4 Clinical stage correlation of ovarian tumors with differential expression of VEGF-modulated genes

In order to test, whether the correlation between VEGF stimulation and DSCR1 expression observed in tissue culture conditions *in vitro* did also translate *in vivo* in tumors associated with high VEGF expression, we have analyzed 3 matched sets of RNA derived from ovarian tumors and control tissues from the same patients (Clonetech) by real-time RT-PCR. VEGF overespression is thought to play a major role in the progression of ovarian cancer by promoting the neovascularization and subsequent growth of solid intraperitoneal tumors and by inducing ascites formation by increasing the permeability of the tumor vasculature (Mesiano et al, Am. J. Pathol, 153, p1249, 1998). VEGF mRNA levels in ovarian carcinomas are significantly higher than in normal ovaries. The average levels of VEGF expression in normal versus tumor tissues was increased 3.2 fold and correlated with the 2.7 fold increase in DSCR1 expression in the tumor RNA.

Two thirds of patients with epithelial ovarian carcinomas have advanced disease at diagnosis and have poor prognosis because of the presence of highly invasive carcinoma cells (CA) and rapidly accumulating ascites fluid. One third of patients with low metastatic epithelial adenocarcinomas (low malignant potential=LMPs), have extremely favorable long term outcomes. Previous studies indicated not only a correlation between disease and VEGF expression, but identified VEGF as key regulator of angiogenesis and ascites formation in ovarian cancer. (Fujimoto et al, Cancer, 83, p.2532, 1998.)

A series of total RNAs isolated from 12 patients with LMPs and 9 patients with CAs for expression of VEGF were tested, VEGF receptors and DSCR1 by real-time RT-PCR. Expression levels were normalized to the levels of GAPDH or β-actin (data not shown). Based on these expression levels, statistical analysis using StatView

statistical analysis sofware program, lead to the identification of a correlation between VEGF, VEGF receptors and the expression levels of DSCR1 (Table E5). In addition, a correlation between clinical stage (R=), KDR (R=0.834) and VEGF (R=) expression. These findings indicate that gene profiling experiments in endothelial cells grown in tumor like conditions mimicked by the presence of VEGF, might be instrumental in the search of novel VEGF target genes that are specifically upregulated in tumors or the tumor vasculature. Moreover, the correlation between with clinical stages of tumor development and DSCR1 levels opens the question whether DSCR can serve as a predictive marker for tumor progression in ovarian tumor patients and in other indications.

Total RNA was isolated from tumor biopsies of 12 patients with Low Malignant Potential (LMP) ovarian tumor and from 9 patients with the more malignant Cystoadeno Carcinoma (CA) ovarian tumor. The RNA was analyzed for the expression of VEGF, VEGF receptors and VEGF target genes by TaqMan™ as described above. RNA was run in triplicate, a standard curve with HUVE cell RNA was generated for each probe and relative expression levels were calculated using as a standard the housekeeping gene β-glucuronidase (GUS) and the endothelial marker CD31 to correct for the amount of endothelial cells present. The results are summarized in Table E5. The first row reports the results of ANOVA analysis between the expression of a given gene and grouping the tumor samples based on the clinical stage, LMP vs CA. The second and third rows report the correlation between expression of a given gene and the expression of VEGF or VEGFR1 receptor by the tumor samples. They indicate that there is a positive correlation between high metastatic potential and increased expression level for DSCR1 and ARG rich genes.

Table E5 Ovarian tumor clinical stage correlation analysis

	Ovarian tumor RNA										
Correlation with:	DSCR1	PP5	RGS3	ARP	HVPS41	HB-EGF	Gravin	MKP1LP TP	CTGF	Nexin	Osteonid ogen
clinical stage (LMP/CA)	p=0.0157	-	-	p=0.0157	-	nd	-	-	nd	nd	nd
VEGF expression	-	R=0.949 p=0.0001	R=0.590 p=0.0049	-	R=0.665 p=0.001	nd	R=0.956 p=0.0001	-	nd	nd	nd
VEGFR1 expression:	R=0.834 p<0.0001	-	R=0.667 p=0.0009	R=0.799 p=0.0001	R=0.662 p=0.0011	nd	-	R=0.662 p=0.0034	nd	nd	nd

Example 5 Survival of endothelial cells transfected with VEGFmgs

In order to study whether DSCR1 directly regulates endothelial cell survival, we transiently cotransfected epitope tagged version of DSCR1 with an expression vectors for EGFP and quantifed the ratio between EGFP positive and healthy and apoptotic endothelial cells by fluorescenz micropscopy. As shown in Figure 1, transient overexpression of epitope tagged version of DSCR1 (DSCR1-FLAG) led to a modest decrease in cell viabilty. Overexpression of the antisense construct, in contrast, increased survival to similar extends as observed for a constitutive active form of Akt (Akt 179). These findings excluded a direct survival effect of DSCR1 when overexpressed in endothelial cells and suggested a decrease in viablity under serum starvation conditions. However, no such decrease in viablity was observed in cells grown in 5 % serum conditions (Fig 1).

It is seen that in the control, DSCR1 removal induces apoptosis; at 66 hours only about 25% of the cells are alive. On the other hand, about 80% of the cells transfected with Akt2D survive. Cells transfected with DSCR1 have a survival rate similar to Akt2d while transfection with the sense strand of DSCR1, presumably leading to higher expression, induces faster cell death.

Experimental details:

Expression in HUVECs of sense and antisense polynucleotides corresponding to genes in this invention was carried out as follows:

a) Cells:

HUVEC, p6 (Cell system) in 6 cm tissue culture dish (Falcon 3802, primaria, surface modified polystyrene). grown on gelatin coated plastic.

6 cm dishes were coated for >20 min with 0.2% gelatin in PBS, before applying the cells.

Cells were coated at a density of 140,000 cells per 6 cm dish, i.e., ca. 5000 cells/cm2

Cells should attain at least 60% confluency, since otherwise increased toxicity was observed. At high density, low transfection efficiency was observed.

For microvascular cells, other DNA/lipofectin ratios have to be determined, otherwise increased toxicity is found.

control samples:

#	VEGF (50ng/ml)	GFP	Annexin-PE
1	+	-	-
2	+	+	-
3	+	-	+
4	-	-	-
5	-	+	-
6	-	-	-

DNA: 3.0 µg total DNA/ 6 cm dish: 2. 0 µg test, 1.0 µg Green Fluorescent Protein (GFP)

F1: 4 µl/ 6 cm dish

OPTIMEM: 1.3 ml per 6 cm dish
Use Falcon clear tubes (polystyrene)

For HMVE cells: 2µg DNA+4 µl F1

b) Procedure:
Day1: Split cells 24 hours before Lipofectin,

Day 2: 4pm to 6pm: Vortex Lipofectin (Life Technologies, Inc. , Rockville, MD) thoroughly in clear Falcon tubes for 20 sec before using.

First add 1.35 ml/sample of OPTIMEM (Gibco BRL Cat No. 31985). Next add 3 µg total DNA per sample and mix well by vortexing. Then add 4 µl of F1 per sample and mix well by vortexing. Mix DNA+Lipofectin+OPTIMEM and incubate in a water bath at 37 C for 20 to 30 min; then wash the cells twice with OPTIMEM.

Add 1.35 ml of the transfection mix and incubate for 2 h at 37 C. After 2h, add 3 ml of complete medium and incubate 16 to19 hours.

Day 3: 10 am: replace media next morning to 10% serum-containing medium, but do not wash the cells. Alternatively, leave the transfection mix for another 24 hours; this will lead to a higher transfection efficiency but also lead to increased cell death.

If apoptosis is being determined:

Day 3: evening: The cells are washed with 2x PBS and the medium is changed to serum starvation, then GF+WM are added.

Day 4: late afternoon: The cells are analyzed by using FACS set to detect annexin-PE and FITC channels for % apoptotic cells (30 h time point). Up to 32% transfection efficiency after 72 h was observed when Green Lantern was transfected .

If survival is being studied:

Day 4, morning: The cells are washed with 2x PBS and the medium is changed to serum starvation, then GF+WM are added.

Day 4, evening: count GFP positive cells and compare apoptotic/ healthy

Day 5 (24 h later): The cells are harvested for FACS analysis.

c) FACS analysis:

1. The supernatant (3ml) is pulled off and added to prelabelled 5 ml Falcon tubes with a filter on top at 0 C, and the tubes were spun down at 2000 rpm for 3 min. In the meantime:
2. The cells were washed carefully with 3 ml PBS.
3. 0.5 ml 2x Trypsin was added, and the mixture was incubated for 3 min. in the 37 C incubator
4. After 3 min, 3 ml of medium was added, containing 10% serum, to stop the digestion.

5. The supernatant from step 1 was drawn off by aspiration and 3.5ml from step 4 were added to the tubes containing the cell pellets.

6. The cells were pelleted at 2000 rpm for 3 min.

7. The pellets were washed 1x with 2 ml of 1x Ca binding buffer.

8. The cells were pelleted at 2000 rpm for 3 min, and the supernatant was aspirated off.

9. The pellet was taken up in 0.5 ml Ca-binding buffer (generate pool containing Annexin-PE, or simple 1xCa-binding buffer for control samples), and the cells were disaggregated by pipetting up and down 6 times.

10. Add 10 µl of Annexin-PE to the control samples , or 1 µl of the BioVison annexin-Cy3 stock solution.

11. The tubes were kept on ice and submitted to the FACS lab for analysis.

d) Materials

F1: targeting systems, Targfect F-1 (2mg/ml), Cat No #001 (1ml)or #002 (4x1ml).

Growth Factors: for 5 ml medium in 6 cm dishes

VEGF: 10µl of 0.1mg/ml stock+650µl serum-free medium. 100 µl of this stock was added to 5 ml medium present in 6 cm dish to give a 30 ng/ml final concentration.

Wortmannin (a potent, irreversible inhibitor of phosphatidylinositol 3-kinase; BIOMOL, #ST-415; Catalogue Number 1232,Tocris Cookson, United Kingdom)

The contents of the vial (5 mg) were taken up in 500 µl dimethylsulfoxide (stock: 10mg/ml stock; 23.3 mM). 4.3 µl of the 10mg/ml stock solution was diluted in 1 ml medium to give a 100 µM solution. 10 µl of this stock was diluted in 650 µl serum-free medium, and 100 µl was added to the 5 ml medium present in the 6 cm dishes.

e) DNA

empty vector: pRLCMV,	1.3 µg/µl	2.7 µl/dish
GreenLantern™:	0.7 µg/µl	1.5 µl/dish

f) FACS:

Use Annexin-Cy3, GFP and Pi (works well)

Annexin-PE (R&D), add 10 µl of stock, undiluted, to the cells. Rest as before

Annexin -Cy3, BioVision, 1002-1000

Opti-MEM-1 Gibco, BRL Cat No. 31985, 0.5 l

CSC medium, Cat. No. 4Z0-500,

noGF. no serum Cat. no 4Z3-500-S,

Endothelial cells were transfected with pRLCMV (empty vector, negative control) or with pRLCMV further containing nucleotide sequences expressing either DSCR1 in the sense direction (DSCR1), or DSCR1 in the anti-sense direction (DSCR1 AS), or the activated mutant of AKT (Akt2D, a positive control that induces cell survival) as outlined above. The cells were co-transfected with Green Lantern expressing Green Fluorescent Protein that gives an indication of the efficiency of transfection and provides a visible marker for surviving cells. 18 hours after transfection, serum was removed from the media.

Example 6 Further analysis of DSCR1

1. Introduction

Down's Syndrome induces mental retardation and congenital heart malformations. The open reading frame encoding DSCR1 was one of several located within the minimal region on chromosome 21 capable to induces the down syndrome phenotype (Fuentes et al, Hum Mol Genet 1995 Oct;4(10):1935-44). More recently, DSCR1 was found to interact physically and functionally with calcineurin A, the catalytic subunit of the Ca(2+)/calmodulin-dependent protein phosphatase PP2B. Transient overexpression of DSCR1 blocked calcineurin-dependent gene transcription

through the inhibition of the nuclear translocation of nuclear factor of activated T cells (NFAT). (Fuentes JJ, Hum Mol Genet 2000 Jul 1;9(11):1681-90).

NFAT was originally described as transcription factor that supported the activation of cytokine gene expression in T-cells and as the primary target of the immunoregulatory effects of cyclosporin A (CsA) and FK506. Elevated levels of NFAT in activated endothelial cells were first observed by Cockerill et al (Blood 1995 Oct 1;86(7):2689-98) and interference with NFAT activity by CsA resulted in a 40 % reduction of E-selection expression on endothelial cells stimulated with TNF-α as well as a 29 % decrease in neutrophil adhesion. These findings suggested a biological role of DSCR1 to regulate NFAT activity and the expression of cell adhesion molecules on activated endothelial cells.

2. Materials and methods

(a) cells

Human umbilical vein endothelial cells (HUVECs) were purchased from Cell Systems and were grown in endothelial growth medium (CS-C medium, Cell Systems)) complemented to a final concentration of 5% serum. Cells were split at a cell density of 19,000 cell/cm^2, and experiments were run in triplicates. 24 hours after seeding, the cells were washed three times with phosphate buffered saline (PBS) and media, 0.1 % BSA or 0.1 % BSA and VEGF (10ng/ml) or 5 % serum.

(b) RNA harvest and Real Time RT-PCR analysis

Medium was aspirated from the cultures, and 10 ml of Trizol (Gibco) was added to 1x 10^6 cells. The tissue cultur flasks were incubated on vertical shaker for 10 min. RNA isolation and cDNA synthesis and data analysis were as described elsewhere (Kahn *et al.*, 2000). For tissues, RNA was isolated from frozen tumor tissue harvested at necropsy from five specimens of each treatment group using the STAT 60 method (TEL-TEST "B"; Friendswood, TX), and purified on RNeasy Quick spin columns (Qiagen; Valencia, CA). One hundred ng of total RNA/reaction was analyzed using the RT-PCR kit from Perkin Elmer, following the manufacturer's instructions (PE Applied Biosystems, Foster City, CA). Reactions were run in 96 well plates in a Model 7700 Sequence Detector (PE Applied Biosystems, Foster City, CA) and results were analyzed using Sequence Detection Software (PE Applied

Biosystems, Foster City, CA). RT-PCR conditions were 30 min at 48°C, 10 min at 95°C, and 40 cycles of 30 seconds at 95° C, 90 seconds at 60°C. Relative RNA equivalents for each sample were obtained by standardizing to GAPDH levels. Each of the five samples per group was run in duplicates to determine sample reproducibility, and the average relative RNA equivalents per sample pair was used for further analysis. Statistical analysis was performed using ANOVA software (Abacus Concepts, Inc., Berkeley, CA). Species specificity of the probe primer sets was verified by testing total RNA derived from human epithelial cells or mouse kidney RNA (data not shown). Expression levels were standardized to the probe/primer sets specific for human or murine GAPDH, respectively.

(c) Transient transfection of primary human endothelial cells

Used HUVE cells before they reach passage 6 and HMVEC before reaching passage 4.

Use Falcon primaria 6 well dishes uncoated. (coating with gelatin is not recommended).

Harvest cells by incubation with 2x trypsin at rt for 3 to 5 min, dilute trypsinized cells in 3 volumes complete medium (do not trypsinize too long).

Count 10 µl of mix on the hemocytometer

Spin cells 5 min at 2 krp in the meantime.

Remove sn and dilute cells with complete medium to 0.5 x10e5 cells in 3ml of compelete medium, make a pool

Add 3 ml of cells from the pool to each well (50000 cells/well, (5000 cells/cm2)

Cells should not be < 60% confluent, otherwise increased toxicity might be observed. At cell densities > 80%, lower transfection efficiency was observed.

Lipofection:

For HUVE and HMVE cells:

The following amounts were calculated for transfection of 3 wells. It is advisable to generate a pool of 3 transfections in order to have duplicate or triplicates for each gene tested.

Pipette DNA into 15 ml Falcon clear tubes (polystyrene), best results when DNA conentration measured immediately prior to experiment:

11.25 µg of expression vector (pRKN driven)

3.75 µg of luciferase reporter

1.0 µg of SV-renilla refference reporters

Add 4.5 ml of Optimem (serum free)

9.) Vortex F1 targefectin solution for 30 sec and add 14 µl of F1 to the mix.

10.) Mix the lipofection mix by inversion and incubate samples in 37 C water-bath for 20 to 30 minutes

10.) Wash cells once with PBS, remove PBS and add 1.5 ml of lipofection mix using 5ml plastic pipette per dish.

11.) Incubate cells for 2.5 hours in CO2 incubator,

12.) Add 3 ml of complete medium and incubate overnight (12 to 16 hours). The effects of prolonged incubation are not determined yet.

13.) Wash cells 1x PBS

14.) Add 3 ml of complete medium, wait for 24 hours before dosing.

15.) harvest cells after 36 hours after lipfection or 6 to 9 hours after dosing.

Serum Starvation (0.5 % FCS):

1.) Next morning: wash cells 1x with 3 ml PBS

2.) Add 3 ml of 0.5 % FCS medium, 0.2 % BSA, Pen/Step, fungizone

Up to 32% transfection efficiency after 72 h was observed when EGFP was transfected .\

Cell Harvest and luciferase measurement:

Remove medium by aspiration, wash carefully 1x with PBS and add 300 µl 1 x passive lysis buffer, sample can be stored at –20 C at this point, however activity might decrease up to 50 %.

Luminometer:

Prefill tube with 100 µl luciferast substrate solution

Add 30 µl extract

Add 100 µl STOP and GLOW

Additional materials

Materials: F1: targeting systems, Targfect F-1 (2mg/ml), Cat No #001 (1ml)or #002 (4x1ml), (Targeting systems, Tel 619 562 15 18, Rhumpia)

Culture dishes: 60 mm cell culture dishes, Falcon 3802, primaria, surface modified polystyrene.

Cells: HUVEC: Cell systems, 2VO-C75
HDMEC, Cell Systems, 2M1-C75

Medium:
5% serum containing:
Opti-MEM-1 Gibco, BRL Cat No. 31985, 0.5 l
CSC medium, Cat. no. 4Z0-500, 110$
noGF. no serum Cat. no 4Z3-500-S, 90$

3. DSCR1 is expressed in tumor vasculature and in neoplastic cells

In order to study the cellular localization of DSCR1 expression within various human tumors and other malignancies, in situ hybridization experiments including a series of different human tumors as well on sections prepared from a variety of healthy human organs were performed. During fetal development in humans, DSCR1 was found to be expressed in the fetal liver and in dorsal root ganglia, in cells of the atrio-ventricular junction near the A-V valve insertions and focally in the cells within the subendocardial layer of the left ventricular septum and right ventricular apex. There was weak expression in embryonic large hepatic vein endothelium and small vessel endothelium. In the embryonic spinal cord, there was expression in neurons. When studied in adult chimpansees, DSCR1 expression was further detected in myoepithelial cells surrounding normal mammary ducts and in normal chimp parathyroid. In adult liver, expression was localized to hepatocytes and bile duct

epithelium of cirrhotic, but not normal liver. There was focal expression within human adenocarcinomas of the mammary gland as well as in renal cell carcinoma and. Sense control were run on all samples and revealed no background signals (data not shown). These findings might reflect some degree of redundancy in the signal transduction pathways regulating DSCR1 expression on endothelial cells and transformed tumor cells. Alternatively, upregulation of VEGF receptors on tumors cells and stimulation of the VEGF specific signal transduction pathways could help to explain our findings. In summary, we found DSCR1 gene expression in fetal vasculature during normal ontogeny as well as in neoplastic tumor cells in adults and therefore identified DSCR1 as a member of the oncofetal family of genes.

4. Functional analysis of DSCR1 by transient transfection of primary human endothelial cells

Recently it was shown in yeast two hybrid experiments, that DSCR1 interacts physically and functionally with calcineurin A, the catalytic subunit of the Ca2+/calmodulin-dependent protein phosphatase PP2B. In studies in T-cells, transient overexpression of DSCR1 inhibited the transcriptional activation of the interleukin 2 promoter in response to PMA/calcium stimulation. In DSCR1 transfected cells, NFAT was unable to accumulate in the nucleus after stimulation with calcium ionophores such as ionomycine.

Overexpression of DSCR in primary human endothelial cells had any effect on the NFAT activation after stimulating the cells with PMA and the calcium Ionophore A23187 was tested. Transient cotransfection experiment of expression vector encoding DSCR1-FLAG and a luciferase reporter construct containing three NFAT binding sites (NFAT-Luc) revealed complete ablation of NFAT activity in response to PMA and ionophore after 6 hours of stimulation. Enforced expression of DSCR in endothelial cells leads to a significant downregulatin of calcineurin regulated signal transduction pathways, presumably via interference with calcineurin regulated signal transduction pathways.

EQUIVALENTS

Although particular embodiments have been disclosed herein in detail, this has been done by way of example for purposes of illustration only, and is not intended to be limiting with respect to the scope of the appended claims that follow. In particular, it is contemplated by the inventors that various substitutions, alterations, and modifications may be made to the invention without departing from the spirit and scope of the invention as defined by the claims. The choice of nucleic acid starting material, clone of interest, or library type is believed to be a matter of routine for a person of ordinary skill in the art with knowledge of the embodiments described herein. Other aspects, advantages, and modifications considered to be within the scope of the following claims.

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CLAIMS

1. An isolated polypeptide comprising an amino acid sequence having at least 80% sequence identity to the sequence SEQ ID NO:3 or SEQ ID NO:22.

2. The polypeptide of claim 1, wherein said polypeptide is an active ARP polypeptide.

3. The polypeptide of claim 2, having at least 90% sequence identity to the sequence SEQ ID NO:3 or SEQ ID NO:22.

4. The polypeptide of claim 2, having at least 98% sequence identity to the sequence SEQ ID NO:3 or SEQ ID NO:22.

5. An isolated polynucleotide encoding the polypeptide of any one of claims 1-4, or a complement of said polynucleotide.

6. An isolated polynucleotide comprising a nucleotide sequence having at least 80% sequence identity to the sequence SEQ ID NO:2 or SEQ ID NO:21, or a complement of said polynucleotide.

7. The polynucleotide of claim 6, having at least 90% sequence identity to the sequence SEQ ID NO:2 or SEQ ID NO:21, or a complement of said polynucleotide.

8. The polynucleotide of claim 6, having at least 98% sequence identity to the sequence SEQ ID NO:2 or SEQ ID NO:21, or a complement of said polynucleotide.

9. An antibody that specifically binds to the polypeptide of any one of claims 1-4.

10. A method of modulating angiogenesis comprising modulating the activity of at least one VEGF-modulated gene polypeptide.

11. The method of claim 10 wherein said modulating angiogenesis is increasing angiogenesis, and said modulating the activity comprises increasing the activity of at least one polypeptide selected from the group consisting of nexin, placental protein 5 (PP5), amyloid precursor-like protein 2 (APLP2), regulator of G-protein signaling-3 (RGS3), gravin, arginine-rich protein (ARP), Down's syndrome critical region protein-1 (DSCR1), insulin induced gene-1 (INSIG1), decidual protein induced by progesterone (DEPP), NADH-ubiquinone oxidoreductase chain 1 (ND1), heparin-binding EGF-like growth factor (HB-EGF), MKP-1 like protein tyrosine phosphatase, osteonidogen and connective tissue growth factor (CTGF).

12. The method of claim 10 wherein said modulating angiogenesis is decreasing angiogenesis, and said modulating the activity comprises increasing the activity of at least one polypeptide selected from the group consisting of amyloid precursor protein (APP), Human gene similar to yeast VPS41 (hVPS41p), cytochrome oxidase subunit I (MTCO1), NADH-ubiquinone oxidoreductase chain 4 (ND4).

13. The method of claim 10 wherein said modulating angiogenesis is decreasing angiogenesis, and said modulating the activity comprises decreasing the activity of at least one polypeptide selected from the group consisting of nexin, PP5, APLP2, RGS3, gravin, ARP, DSCR1, INSIG1, DEPP, ND1, HB-EGF, MKP-1 like protein tyrosine phosphatase, osteonidogen and CTGF.

14. The method of claim 10 wherein said modulating angiogenesis is increasing angiogenesis, and said modulating the activity comprises decreasing the activity of at least one polypeptide selected from the group consisting of APP, hVPS41p, MTCO1 and ND4.

15. The method of claim 11 or 12 wherein said increasing activity comprises increasing the expression of said at least one polypeptide.

16. The method of claim 13 or 14 wherein said decreasing activity comprises decreasing the expression of said at least one polypeptide.

17. The method of claim 15 wherein said increasing expression comprises transforming a cell to increase expression of a polynucleotide encoding said at least one polypeptide.

18. The method of claim 16 wherein said decreasing expression comprises transforming a cell to express a polynucleotide anti-sense to at least a portion of an endogenous polynucleotide encoding said at least one polypeptide.

19. The method of claim 13 or 14 wherein said decreasing activity comprises transforming a cell to express an aptamer to said at least one polypeptide.

20. The method of claim 13 or 14 wherein said decreasing activity comprises introducing into a cell an aptamer to said at least one polypeptide.

21. The method claim 13 or 14 wherein said decreasing activity comprises administering to a cell an antibody that selectively binds to said at least one polypeptide.

22. A method of treating tumors comprising decreasing angiogenesis by the method of claim 12 or 13.

23. A method of treating cancer comprising treating a cancerous tumor by the method of claim 22.

24. A method of treating myocardial infarction comprising increasing angiogenesis by the method of claim 11 or 14.

25. A method of promoting healing comprising increasing angiogenesis by the method of claim 11 or 14.

26. A method of measuring a VEGF-modulated gene transcriptional up-regulation or down-regulation activity of a compound, comprising:

contacting said compound with a composition comprising a RNA polymerase and said gene and measuring the amount of VEGF-modulated gene transcription.

27. The method of claim 26, wherein said composition is in a cell.

28. A method of measuring VEGF-modulated gene translational up-regulation or down-regulation activity of a compound, comprising:

contacting said compound with a composition comprising a ribosome and a polynucleotide corresponding to a mRNA of said gene and measuring the amount of VEGF-modulated gene translation.

29. The method of claim 28, wherein said composition is in a cell.

30. A vector, comprising the polynucleotide of any one of claims 5-8.

31. A cell, comprising the vector of claim 30.

32. A method of screening a tissue sample for tumorigenic potential, comprising:

measuring expression of at least one VEGF-modulated gene in said tissue sample.

33. The method of claim 32, wherein said measuring is measuring an amount of a polypeptide encoded by said at least one VEGF-modulated gene.

34. The method of claim 32, wherein said measuring expression is measuring an amount of mRNA corresponding to said at least one VEGF-modulated gene.

35. A transgenic non-human animal, having a disrupted *ARP*.

36. The transgenic non-human animal of claim 35, wherein the non-human animal is a mouse.

37. A transgenic non-human animal, comprising an exogenous polynucleotide having at least 80% sequence identity to the sequence SEQ ID NO:2 or SEQ ID NO:21, or a complement of said polynucleotide.

38. The transgenic non-human animal of claim 37, wherein said exogenous polynucleotide has at least 90% sequence identity to the sequence SEQ ID NO:2 or SEQ ID NO:21, or a complement of said polynucleotide.

39. The transgenic non-human animal of claim 37, wherein said exogenous polynucleotide has at least 98% sequence identity to the sequence SEQ ID NO:2 or SEQ ID NO:21, or a complement of said polynucleotide.

40. A method of screening a sample for an ARP mutation, comprising:

comparing an ARP nucleotide sequence in the sample with SEQ ID NO:2 or SEQ ID NO:21.

41. A method of modulating cell survival by modulating the activity of at least one VEGF-modulated gene polypeptide selected from the group consisting of nexin, PP5, APLP2, APP, gravin, ARP, DSCR1, MTCO1, ND1, ND4, HB-EGF, MKP-1 like protein tyrosine phosphatase, osteonidogen and CTGF.

42. The method of claim 41 wherein said modulating cell survival is increasing cell survival, and said modulating the activity comprises increasing the activity of at least one polypeptide selected from the group consisting of nexin, PP5, APLP2, APP, gravin, ARP, DSCR1, MTCO1, ND1, ND4, HB-EGF, osteonidogen and CTGF.

43. The method of claim 41 wherein said modulating cell survival is decreasing cell survival, and said modulating the activity comprises increasing the activity of at least one VEGF-modulated gene polypeptide, wherein said VEGF-modulated gene polypeptide is MKP-1 like protein tyrosine phosphatase.

44. The method of claim 41 wherein said modulating cell survival is decreasing cell survival, and said modulating the activity comprises decreasing the activity of at least one polypeptide selected from the group consisting of nexin, PP5, APLP2, APP, gravin, ARP, DSCR1, MTCO1, ND1, ND4, HB-EGF, osteonidogen and CTGF.

45. The method of claim 41 wherein said modulating cell survival is increasing cell survival, and said modulating activity comprises decreasing the activity of at least one VEGF-modulated gene polypeptide, wherein said VEGF-modulated gene polypeptide is MKP-1 like protein tyrosine phosphatase.

46. The method of claim 42 or 43 wherein said increasing activity comprises increasing the expression of said at least one polypeptide.

47. The method of claim 44 or 45 wherein said decreasing activity comprises decreasing the expression of said at least one polypeptide.

48. The method of claim 46 wherein said increasing expression comprises transforming a cell to increase expression of a polynucleotide encoding said at least one polypeptide.

49. The method of claim 47 wherein said decreasing expression comprises transforming a cell to decrease expression of a polynucleotide anti-sense to at least a portion of an endogenous polynucleotide encoding said at least one polypeptide.

50. The method of claim 44 or 45 wherein said decreasing activity comprises transforming a cell to express an aptamer to said at least one polypeptide.

51. The method of claim 44 or 45 wherein said decreasing activity comprises introducing into a cell an aptamer to said at least one polypeptide.

52. The method claim 44 or 45 wherein said decreasing activity comprises administering to a cell an antibody that selectively binds to said at least one polypeptide.

53. A method of treating tumors comprising decreasing cell survival by the method of claim 43 or 44.

54. A method of treating cancer comprising treating a cancerous tumor by the method of claim 53.

55. The method of any one of claims 41, 42, 44 and 46-54, wherein said at least one VEGF-modulated gene is DSCR1.

56. A method of determining the clinical stage of tumor comprising comparing expression of at least one VEGF-modulated gene in a sample with expression of said at least one gene in control samples.

57. The method of claim 56, wherein said at least one VEGF-modulated gene comprises at least one member selected from the group consisting of DSCR1 and ARP.

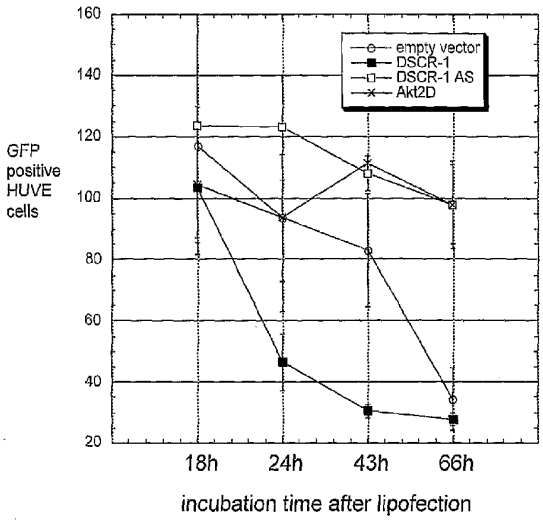
58. The method of claim 56 or 57, wherein said sample is a sample from an ovarian tumor.

59. A method of determining if a tumor has a potential for metastasis comprising determining the clinical stage of said tumor by the method of any one of claims 56-58.

60. The method of any one of claims 26-29, wherein said compound is a calcium channel regulator.

61. The method of claim 60, wherein said calcium channel regulator is selected from the group consisting of nicardiphine, nifedipine, verapamil, and diltiazem.

Fig. 1


160
140
120
100
80
60
40
20
GFP positive HUVE cells
empty vector
DSCR-1
DSCR-1 AS
Akt2D
18h
24h
43h
66h
incubation time after lipofection