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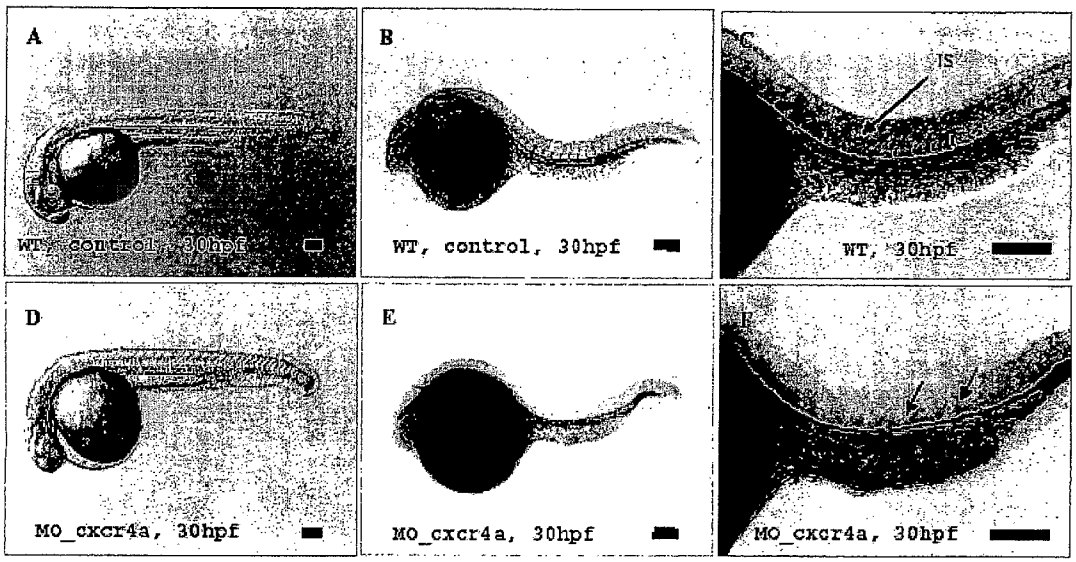
(54) **Title:** GPCRS AS ANGIOGENESIS TARGETS



(57) **Abstract:** The invention provides methods of modulating angiogenesis by increasing or decreasing expression or function of G-Protein Coupled Receptors (GPCRs) namely Lectomedin-3 (LEC3), Endothelin-1 Receptor (EDNRA) and C-X-C Chemokine Receptor 4 (CXCR-4). The invention also provides methods of inhibiting and promoting GPCR-dependent angiogenesis in vertebrates, particular mammals, including humans, for the treatment of angiogenesis-related diseases. The invention also provides methods of identifying compounds that promote or inhibit angiogenesis through their interaction with LEC3, EDNRA and/or CXCR-4. The invention further provides methods of modulating angiogenesis through the modulation of these GPCRs and VEGF.

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GPCRS AS ANGIOGENESIS TARGETS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of U.S. Provisional Application No. 60/772,991 filed February 14, 2006, which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] The invention relates to three G-Protein Coupled Receptor (GPCRs) proteins and their relationship to angiogenesis. Specifically, the application relates to the role of Lectomedin-3 (LEC3), Endothelin-1 Receptor type A (EDNRA) and C-X-C Chemokine Receptor 4 (CXCR4) as drug targets for anti-angiogenesis and pro-angiogenesis related therapies.

BACKGROUND OF THE INVENTION

[0003] Angiogenesis is a process where new blood vessels are formed from pre-existing blood vessels. This involves proliferation, differentiation and migration of endothelial cells and possibly other cell types found in the vasculature, such as smooth muscle cells and fibroblasts. Alteration of this process, either by potentiation or by inhibition, can be beneficial for the treatment of human diseases, such as cancer, macular degeneration, rheumatoid arthritis, Alzheimer's disease, wound healing, atherosclerosis and ischemia.

[0004] Inhibition of angiogenesis represents a powerful new approach to cancer therapy. To fully realize the potential of this avenue for cancer treatment, assays that can rapidly screen compounds for anti-angiogenic activity are essential. Solid tumors require adequate supply of nutrients from the blood to survive, grow, and metastasize (Hanahan, D. & J. Folkman (1996) *Cell* 86:353-364; Li, C.Y. *et al.* (2000) *Cancer Met. Rev.* 19:7-11). New blood vessels that nourish growing tumors by sprouting from existing blood vessels, a process known as angiogenesis. In recent years, angiogenesis has received considerable attention as a novel process to target against cancer. Many drugs already in clinical trials have been shown to have anti-angiogenic activity and new drugs are being developed specifically for their ability to stop such blood vessel growth (Rosen, L. (2000) *Oncologist* 5(suppl. 1):20-27).

[0005] Abnormal angiogenesis in the retina is a leading factor in age-related macular degeneration, diabetic retinopathy, and retinopathy of prematurity which are the primary causes

of vision loss. Thus, treatment of angiogenesis-related disorders is of particular importance in retinal pathology.

[0006] Anti-angiogenic drugs have had mixed success in clinical application. Many new compounds may need to be tested *in vivo* to identify drugs capable of treating a wide range of tumors. Thus, suitable *in vivo* assays for screening potential anti-angiogenic compounds are increasingly important. U.S. Patent Publication No. 20040143865 to Rubinstein *et al.* provides an assay using the zebrafish (*Danio rerio*) that provides the relevance of an *in vivo* environment as well as the potential for high-throughput drug screening. The technique involves generating a transgenic line of zebrafish that expresses a reporter protein, for example, green reef coral fluorescent protein (G-RCFP, Matz, M.V. *et al.* (1999) *Nat. Biotechnol.* 17:969-973) or red fluorescent protein (dsRed2) which are specifically expressed in the blood vessels.

[0007] The zebrafish has become a well-accepted model for studies of vertebrate development. Unlike the mouse, zebrafish embryos develop outside the mother and are transparent, facilitating the observation of differentiating tissues and organs. The vascular system of the zebrafish, in particular, has been well described and shown to be highly conserved from the zebrafish to human (Isogai, S. *et al.* (2001) *Dev. Biol.* 230(2):278-301; Vogel, A.M. & B.M. Weinstein (2000) *Trends Cardiovasc. Med.* 10(8):352-360). Furthermore, zebrafish embryos can live for several days without a significant blood supply, thus facilitating the study of embryos with vascular defects. The intersegmental blood vessels in the zebrafish embryos form by angiogenic sprouting which is very similar to tumor angiogenesis and this process appears to require the same proteins shown to be necessary for blood vessel growth in mammals. For instance, the anti-angiogenic compound PTK787/ZK222584, an inhibitor of vascular endothelial growth factor (VEGF) receptor tyrosine kinases, has been shown to affect the formation of zebrafish blood vessels (Chan, J. *et al.* (2002) *Cancer Cell* 1:257-267).

[0008] Current methods of visualizing blood vessels in the zebrafish include whole mount *in situ* hybridization of vascular endothelial cell marker (Fouquet, B. *et al.* (1997) *Dev. Biol.* 183:37-48.; Liao, W. *et al.* (1997) *Development* 124:381-389), detection of endogenous alkaline phosphatase activity in the vessels and microangiography of the circulating cardiovascular system. The latter technique involves injection of fluorescent beads into the circulation of living zebrafish larvae (Weinstein, B.M. *et al.* (1996) *Nat. Med.* 1:1143-1147) and is useful for visualization of blood vessels in a complete circulatory system. It was found that the formation of intersegmental blood vessels, blocked by application of tyrosine kinase inhibitors that target the VEGF receptor, could be easily visualized in the transgenic fish expressing the fluorescent protein in the vascular system.

[0009] The zebrafish is an excellent model to study angiogenesis. There remains a need in the art to find targets to act upon to promote or to inhibit angiogenesis (depending on the desired outcome) and the zebrafish model can be used to demonstrate the utility of these targets for pro-angiogenesis and anti-angiogenesis therapies.

[0010] Toxicity is a major cause of failure during drug development. Many drugs shown to be safe in cell culture have proven to be toxic in animal studies. Therefore, although zebrafish are useful animal models for initial toxicity screening, further studies are often required in mammalian systems which are believed to more faithfully mimic the genetic and biological basis of human diseases. Therefore, mammalian models of disease are needed as a second stage of screening for compounds useful in angiogenesis related disorders such as retinopathies.

SUMMARY OF THE INVENTION

[0011] The invention provides a method for promoting angiogenesis using the isolated nucleic acid sequences for CXCR4, LEC3 and/or EDNRA. In some embodiments, the nucleic acids encoding CXCR4 have the polynucleotide sequence of SEQ ID NO:1, SEQ ID NO:7, or SEQ ID NO:32. In some embodiments, the nucleic acids encoding LEC3 have the polynucleotide sequences of SEQ ID NO:3, or SEQ ID NO:9. In some embodiments, the nucleic acids encoding EDNRA have the polynucleotide sequences of SEQ ID NO:5, SEQ ID NO:11, SEQ ID NO:19 or SEQ ID NO:35. The invention also provides CXCR4-, LEC3- and/or EDNRA-encoding polynucleotides and portions, homologs and fragments thereof from other vertebrate species for use in the methods of the invention. In some embodiments, the polynucleotides are derived from such species as human, mouse, rat, and cow, for example.

[0012] The invention also provides isolated zebrafish CXCR4, LEC3 and EDNRA polypeptides. In some embodiments, the polypeptides have the amino acid sequence of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6, respectively. The invention also provides CXCR4, LEC3 and EDNRA polypeptides and portions, homologs and fragments thereof from other vertebrate species for use in the methods of the invention. In some embodiments, the polypeptides are derived from such species as human, mouse, cow and rat, for example. In some embodiments the CXCR4 polypeptides have the amino acid sequence of SEQ ID NO:2, SEQ ID NO:8, or SEQ ID NO:33. In some embodiments, the LEC3 polypeptides have the amino acid sequence of SEQ ID NO:4, SEQ ID NO:10, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:27, or SEQ ID NO:28. In some embodiments, the EDNRA polypeptides have the amino acid sequence of SEQ ID NO:6, SEQ ID NO:12, SEQ ID NO:20, SEQ ID NO:22, or SEQ ID NO:23.

[0013] The invention also provides isolated zebrafish peptide ligands, stromal cell-derived factor 1 (SDF-1 or named CXCL12) for CXCR4 receptor and endothelin-1 (ET-1 or named ETA) for EDNRA receptor. In some embodiments, the polypeptides have the amino acid sequence of SEQ ID NO:37 (SDF-1: NP_954637) and SEQ ID NO:39 (ET-1: NP_001946) respectively. The polynucleotides encoding these SDF-1 and ET-1 proteins are SEQ ID NO:38 (NM_199168) and SEQ ID NO:40 (NM_001955), respectively. The invention also provides SDF-1 and ET-1 polypeptides and portions, homologs and fragments thereof from other vertebrate species for use in the methods of the invention. In some embodiments, the polypeptides are derived from such species as human, mouse, cow and rat, for example. In some embodiments, the peptide SDF-1 ligand for CXCR4 have an amino acid sequence of SEQ ID NO:42 (NP_000600 for *Homo sapiens*, human), SEQ ID NO:44 (NP_068350, *Mus musculus*, mouse), SEQ ID NO:46 (NP_071513 for *Rattus norvegicus*, rat), SEQ ID NO:48_(NP_989841 for *Gallus gallus*, chicken), SEQ ID NO:50 (NP_001009580 for *Sus scrofa*, swine). The polynucleotides encoding these CXCR4 proteins are SEQ ID NO:41 (for *Homo sapiens*, human), SEQ ID NO:43 (for *Mus musculus*, mouse), SEQ ID NO:45 (for *Rattus norvegicus*, rat), SEQ ID NO:47 (for *Gallus gallus*, chicken), and SEQ ID NO:49 (for *Sus scrofa*, swine). The invention also provides methods of promoting angiogenesis in an animal. The peptide ET-1 ligand for EDNRA have an amino acid sequence of SEQ ID NO:39 (NP_001946 for *Homo sapiens*, human), SEQ ID NO:52 (NP_034234 for *Mus musculus*, mouse), SEQ ID NO:54 (NP_036680 for *Rattus norvegicus*, rat), SEQ ID NO:56 (NP_851353 for *Bos taurus*, bovine), and SEQ ID NO:58 (NP_999047 for *Sus scrofa*, swine). The polynucleotides encoding these ET-1 ligands are SEQ ID NO:40 (for *Homo sapiens*, human), SEQ ID NO:51 (NP_034234 for *Mus musculus*, mouse), SEQ ID NO:53 (for *Rattus norvegicus*, rat), SEQ ID NO:55 (for *Bos taurus*, bovine), and SEQ ID NO:57 (for *Sus scrofa*, swine). In some embodiments, the method comprises administering to the animal an effective amount of peptide ligand SDF-1 for CXCR4 and ET-1 for EDNRA receptor activation, thereby promoting angiogenesis. In addition, antibodies against the ligands, SDF-1 and ET-1, may be used to block the bio-availability of the endogenous ligands to their receptor CXCR4 and EDNRA, respectively, thereby, can be used to block the activation of the CXCR4 and EDNRA for anti-angiogenesis. This will be a similar scenario for anti-VEGF antibody, Avastin, to block the bio-availability of VEGF to its receptor *Flk-1* in tumor vasculature for anti-angiogenic therapy to treat cancers (See Ferrara, N., Hillan, K.J. & Novotny, W. (2005) "Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy" *Biochem. Biophys. Res. Commun.* 333:328-335).

[0014] The invention also provides method to isolate endogenous and artificial peptide ligands for the orphan GPCR, LEC3 receptor. Since LEC3 is classified as orphan GPCR, there is no known endogenous ligand for this receptor. However, we propose to screen a combinatory peptide phage library to isolate high affinity peptide sequences that will bind to LEC3 receptor. Once the peptides sequences are identified, we can generate and screen for additional variants of the peptide clones with higher affinity, functional blocking or enhancing activities to the LEC3 receptor. This recombinant peptides identified can have either functional blocking or enhancing activities for the LEC3 receptor, therefore can be use to block (anti-angiogenesis) or inducing angiogenesis (pro-angiogenesis), respectively. In some embodiments, the method comprises administering to the animal an effective amount of this recombinant peptide ligand for LEC3 receptor, which can lead to either receptor activation or inactivation depends on the amino acid variation on that peptide ligand. Therefore, the invention also provides methods of anti-angiogenesis or promoting angiogenesis, respectively, in an animal.

[0015] The invention also provides methods of promoting angiogenesis in an animal. In some embodiments, the method comprises administering to the animal an effective amount of a CXCR4, LEC3 and EDNRA polypeptide, alone or in combination. The polypeptide(s) may be conjugated with various effective delivery reagents to facilitate the passage of the cell membrane barrier. The CXCR4, LEC3 and EDNRA polypeptide(s) may be any vertebrate CXCR4, LEC3 and EDNRA polypeptide(s) (*i.e.*, derived from any species). In some embodiments the CXCR4 polypeptides have the amino acid sequence of SEQ ID NO:2, SEQ ID NO:8, or SEQ ID NO:33. In some embodiments, the LEC3 polypeptides have the amino acid sequence of SEQ ID NO:4, SEQ ID NO:10, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:27, or SEQ ID NO:28. In some embodiments, the EDNRA polypeptides have the amino acid sequence of SEQ ID NO:6, SEQ ID NO:12, SEQ ID NO:20, SEQ ID NO:22, or SEQ ID NO:23.

[0016] In other embodiments of the invention, methods for promoting angiogenesis include administering to an animal in need of angiogenesis an effective amount of a polynucleotide encoding a CXCR4, LEC3 or EDNRA polypeptide, alone or in combination. The polynucleotide may encode any CXCR4, LEC3 or EDNRA polypeptide. In some embodiments the polynucleotide comprises the nucleic acid sequence of SEQ ID NO:1. In other embodiments the polynucleotide comprises the nucleic acid sequence of SEQ ID NO:7. In other embodiments the polynucleotide comprises the nucleic acid sequence of SEQ ID NO:32. In other embodiments the polynucleotide comprises the nucleic acid sequence of SEQ ID NO:3. In other embodiments the polynucleotide comprises the nucleic acid sequence of SEQ ID NO:9. In other embodiments the polynucleotide comprises the nucleic acid sequence of SEQ ID NO:5. In other

embodiments the polynucleotide comprises the nucleic acid sequence of SEQ ID NO:11. In other embodiments the polynucleotide comprises the nucleic acid sequence of SEQ ID NO:19. In other embodiments the polynucleotide comprises the nucleic acid sequence of SEQ ID NO:35.

[0017] The invention also provides a method of promoting angiogenesis in an animal in need thereof, comprising administering to an animal an effective amount of a first polynucleotide encoding a CXCR4, LEC3 or EDNRA polypeptide and an effective amount of a second polynucleotide encoding a VEGF polypeptide. In some embodiments, the VEGF polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:60, SEQ ID NO:62, SEQ ID NO:64, SEQ ID NO:66, SEQ ID NO:68, SEQ ID NO:70, SEQ ID NO:72, SEQ ID NO:74 and SEQ ID NO:76.

[0018] The invention also provides methods of inhibiting angiogenesis in an animal. In some embodiments, the method comprises administering to the animal an effective amount of a polynucleotide, such as antisense oligonucleotide, or phosphorodiamidate morpholino oligonucleotide (PMO), that inhibits the expression of a CXCR4, LEC3 and/or EDNRA polypeptide. The CXCR4, LEC3 and/or EDNRA polypeptide may be any vertebrate CXCR4, LEC3 and/or EDNRA polypeptide (including human, mouse, cow, rat, zebrafish and all other vertebrates). To improve efficient uptake of a PMO into cells, a peptide such as an arginine rich peptide may be added to the PMO (Moulton, H.M. *et al.* (2004) Cellular Uptake of Antisense Morpholino Oligomers Conjugated to Arginine-Rich Peptides. *Bioconjugate Chem.* 15:290-299). In some embodiments the CXCR4 polypeptides have the amino acid sequence of SEQ ID NO:2, SEQ ID NO:8, or SEQ ID NO:33. In some embodiments, the LEC3 polypeptides have the amino acid sequence of SEQ ID NO:4, SEQ ID NO:10, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:27, or SEQ ID NO:28. In some embodiments, the EDNRA polypeptides have the amino acid sequence of SEQ ID NO:6, SEQ ID NO:12, SEQ ID NO:20, SEQ ID NO:22, or SEQ ID NO:23. In some embodiments, the nucleic acids encoding CXCR4 have the polynucleotide sequence of SEQ ID NO:1, SEQ ID NO:7, or SEQ ID NO:32. In some embodiments, the nucleic acids encoding LEC3 have the polynucleotide sequences of SEQ ID NO:3, or SEQ ID NO:9.In some embodiments, the nucleic acids encoding EDNRA have the polynucleotide sequences of SEQ ID NO:5, SEQ ID NO:11, SEQ ID NO:19 or SEQ ID NO:35.

[0019] The invention also provides a method of treating an angiogenesis-related disease comprising administering to a patient in need of such treatment a polynucleotide that inhibits the expression of CXCR4, LEC3 and/or EDNRA in an amount sufficient to inhibit angiogenesis, or in an amount that would enhance the efficacy of any combined regimen of therapy, such as anti-VEGF, and chemotherapy. An angiogenesis-related disease includes, but is

not limited to angiogenesis-dependent cancers; benign tumors; rheumatoid arthritis; psoriasis; ocular angiogenesis diseases; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; wound granulation; intestinal adhesions, atherosclerosis, scleroderma, hypertrophic scars, cat scratch disease and *Helicobacter pylori* ulcers.

[0020] As such, the method encompasses treating a patient with an angiogenesis-dependent tumor by administering a polynucleotide, modified polynucleotide or in combination with vehicles that allow effective delivery of polynucleotides into the cells and tissues, and that inhibit the expression of CXCR4, LEC3 and/or EDNRA in an amount sufficient to cause tumor regression or stabilization of the cancer condition, or in an amount that would enhance the efficacy of any combined regimen of therapy. The polynucleotide or modified polynucleotide may be an antisense oligonucleotide, siRNA, a morpholino oligonucleotide, or a ribozyme that specifically hybridizes to a nucleic acid encoding CXCR4, LEC3 and/or EDNRA. The invention also provides a method for treating a subject with cell-permeable peptides to inhibit the interaction of CXCR4, LEC3 and/or EDNRA with their ligands or disrupt downstream effector functions. The methods of the invention may further include cell-permeable peptide inhibition of VEGF activity.

[0021] In some embodiments, the invention provides a method for treating an angiogenesis-related disease comprising administering to a patient in need of such treatment a first reagent that inhibits the expression of CXCR4, LEC3 and/or EDNRA and a second reagent that inhibits the expression of VEGF, wherein the first reagent and the second reagent together act synergistically to inhibit angiogenesis. The first reagent may comprise a polynucleotide (including antisense molecules, such as RNAis, siRNAs, miRNAs, modified nucleic acids, PNAs, or morpholino oligonucleotides or ribozymes, *etc.*) directed against a CXCR4, LEC3 and/or EDNRA-encoding polynucleotide. In some embodiments, the first reagent has a sequence selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26 and combinations thereof.

[0022] It is not as straightforward to define only a small region of the targeted sequence for antisense morpholino or other siRNA. Translational blocking antisense morpholino oligos work anywhere from 5' untranslated region to 25 bp downstream of the ATG start codon. In addition, splicing inhibiting antisense morpholino oligos work between exon/intron boundaries. Thus, any exon/intron boundaries of the CXCR4, LEC3 and/or EDNRA gene are target sequences. Therefore, any region of the CXCR4, LEC3 and/or EDNRA mRNA transcript for translational blocking antisense and any genomic region of the CXCR4, LEC3 and/or

EDNRA gene for splicing inhibition may be used. The second reagent may comprise a polynucleotide (including antisense molecules, RNAis, siRNAs, miRNAs, modified nucleic acids, PNAs, or morpholino oligonucleotides or ribozymes, *etc.*) directed against a VEGF-encoding polynucleotide. In some embodiments, the first reagent has a sequence selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26 and combinations thereof. The human VEGF accession number is NM_003376, Unigene cluster: Hs.73793 (Ensembl gene ID: ENSG00000112715). In some embodiments, the angiogenesis-related disease is related to tumor or cancer progression.

[0023] In order to design oligonucleotides to disrupt the function of CXCR4, LEC3 and/or EDNRA and/or VEGF, one may use the various computer-based algorithms as are known in the art for predicting sequences that are likely to have the desired affect of inhibiting RNA expression. An example of such an algorithm includes, but is not limited to Sfold as is available on the Sfold webserver on the world wide web under the domain address of sfold.wadsworth,org. This algorithm is also described in Ding, Y. *et al.* (2004) *Nucl. Acids Res.* 32:W135-W141.

[0024] In some embodiments, the first reagent is a small molecule, a natural compound or a synthetic compound from combinatorial chemistry that inhibits the expression or function of CXCR4, LEC3 and/or EDNRA, and VEGF. Such compounds may be those known in the art or may be identified using the screening methods described herein. Such compounds may, for example, inhibit the expression of CXCR4, LEC3 and/or EDNRA, the post-translational modification of CXCR4, LEC3 and/or EDNRA, or alter the allosteric conformation of CXCR4, LEC3 and/or EDNRA, or compete with the binding of endogenous ligands to the receptor CXCR4, LEC3 and EDNRA.

[0025] The invention further provides a method of treating an angiogenesis-related disease comprising administering to a patient in need of such treatment a first compound that inhibits the function or expression of CXCR4, LEC3 and/or EDNRA and a second compound that inhibits the expression or function of VEGF signaling, including its receptors and downstream signaling molecules, wherein said first compound and said second compound are provided in sufficient amounts to inhibit angiogenesis. In some embodiments, the compound that inhibits the function of CXCR4 is an antibody that specifically binds CXCR4.

[0026] In a particular embodiments of the invention, the invention provides a method of treating a patient with an angiogenesis-dependent tumor comprising administering to a patient in need of such treatment a first compound that inhibits the expression or function of CXCR4, LEC3 and/or EDNRA and a second compound, (including a natural compound, a synthetic compound or a small molecule from combinatorial chemistry), that inhibits the expression or

function of VEGF, wherein said first compound and said second compound are provided in sufficient amounts to cause tumor regression. In some embodiments, the compound that inhibits the expression or function of CXCR4, LEC3 and/or EDNRA is a polynucleotide, such as an antisense, morpholino oligonucleotide, or ribozyme that specifically hybridizes to a nucleic acid comprising a sequence that encodes a CXCR4, LEC3 and/or EDNRA polypeptide. In other embodiments, the compound that inhibits the expression or function of CXCR4, LEC3 and/or EDNRA is an antibody that specifically binds a CXCR4, LEC3 and/or EDNRA polypeptide. In some embodiments, the compound that inhibits the expression or function of VEGF is a polynucleotide, such as an antisense, morpholino oligonucleotide, or ribozyme that specifically hybridizes to a nucleic acid comprising a sequence that encodes a VEGF polypeptide. In other embodiments, the compound that inhibits the function of VEGF is an antibody that specifically binds a VEGF polypeptide. In some embodiments the compounds that inhibit CXCR4, LEC3 and/or EDNRA and/or VEGF expression and/or function are combinations of polynucleotides and antibodies.

[0027] In another particular method of the invention, a method is provided for preventing or treating retinopathy by administering to the eye of a patient in need of such treatment a polynucleotide of that inhibits the expression of an angiogenesis-related protein in an amount sufficient to inhibit angiogenesis. The protein may be, for example, LEC3, EDNRA and CXCR4. The method may involve administration of a polynucleotide such as an antisense oligonucleotide or a phosphorodiamidate morpholino oligomer. Such an antisense oligonucleotide or PMO may be conjugated to a peptide to promote cellular uptake of the polynucleotide. In other embodiments, the method that inhibits the expression or function of CXCR4, LEC3 and/or EDNRA is an antibody that specifically binds a CXCR4, LEC3 and/or EDNRA polypeptide. These methods are useful for preventing or treating retinopathy such as age-related macular degeneration, diabetic retinopathy, or retinopathy of prematurity. The sequences of the oligonucleotides and PMO may be those as described for inhibiting angiogenesis elsewhere in this specification.

BRIEF DESCRIPTION OF THE DRAWINGS

[0028] **Figure 1** shows the membrane structure of CXCR4 (SEQ ID NO:13). Legend: O, amino acids outside membrane; M, transmembrane amino acids; i, amino acids inside membrane compartment.

[0029] **Figure 2** shows a comparison of conserved amino acids between human (SEQ ID NO:15) and zebrafish (SEQ ID NO:14) CXCR4.

[0030] **Figure 3A and B** shows the membrane structure of human LEC3 (SEQ ID NO:16). Legend: O, amino acids outside membrane; M, transmembrane amino acids; i, amino acids inside membrane compartment.

[0031] **Figure 4** shows a comparison of conserved amino acids between human (SEQ ID NO:18) and zebrafish (SEQ ID NO:17) LEC3.

[0032] **Figure 5** shows an alignment of the nucleic acid (SEQ ID NO:19) and deduced amino acid (SEQ ID NO:20) sequence of zebrafish EDNRA.

[0033] **Figure 6** shows the membrane structure of human EDNRA (SEQ ID NO:21). Legend: O, amino acids outside membrane; M, transmembrane amino acids; i, amino acids inside membrane compartment.

[0034] **Figure 7** shows a comparison of conserved amino acids between human (SEQ ID NO:23) and zebrafish (SEQ ID NO:22) EDNRA.

[0035] **Figure 8** shows CXCR4 expression and its essential function in developing vessels.

[0036] **Figure 9** shows expression of lectomedin-3 (*lec3*) in dorsal aorta of 1 day-postfertilization (dpf) zebrafish embryo. Using *in situ* hybridization and RNA probe against zebrafish *lec3*, we reveal that *lec3* is expressed in the dorsal aorta where the intersomitic vessels will be formed by angiogenic sprouting in 24- and 28-hour-postfertilization (hpf) zebrafish embryo. (scale bars are 100um).

[0037] **Figure 10** shows expression of C-X-C chemokine receptor type 4 (*cxcr4a*) in developing blood vessels at 1dpf. Using *in situ* hybridization and RNA probe against zebrafish *cxcr4a*, we reveal that *cxcr4a* is expressed in the dorsal aorta, intersomitic vessels and cranial vessels in 28-hour-postfertilization zebrafish embryo. Legend: d.a., dorsal aorta; c.v., cranial vessels; s.v., sprouting vessels. (scale bars are 100um).

[0038] **Figure 11** shows expression of endothelin-1 receptor (*ednra*) in axial vasculature and developing vessel. Using *in situ* hybridization and RNA probe against zebrafish *ednra*, we reveal that *ednra* is expressed in the dorsal aorta where the intersomitic vessels will be formed by angiogenic sprouting in 24- and 28-hour-postfertilization zebrafish embryo. (scale bars are 100um). Legend: c.v., cranial vessels; a.v., axial vasculature.

[0039] **Figure 12** shows targeted knockdown of *lec3* perturbs angiogenic sprouting of intersomitic vessels using *flk-1* marker. Targeted knockdown of *lec3*, using morpholino antisense oligo in zebrafish embryos, perturbs angiogenic sprouting of intersomitic vessels as revealed by vascular endothelial-specific marker *flk-1*. Legend: IS, intersomitic vessel. (scale bars are 100um).

[0040] **Figure 13** shows targeted knockdown of *cxcr4a* specifically blocks *flk-1* expression in the sprouting vessels. Targeted knockdown of *cxcr4a*, using morpholino antisense oligo in zebrafish embryos, perturbs angiogenic sprouting of intersomitic vessels as revealed by vascular endothelial specific marker *flk-1*. Legend: IS, intersomitic vessel. (scale bars are 100um).

[0041] **Figure 14** shows targeted knockdown of *ednra* perturbs angiogenic sprouting of intersomitic vessels using *flk-1* marker. Targeted knockdown of *ednra*, using morpholino antisense oligo in zebrafish embryos, perturbs angiogenic sprouting of intersomitic vessels as revealed by vascular endothelial specific marker *flk-1*. Legend: IS, intersomitic vessel. (scale bars are 100um).

[0042] **Figure 15** shows synergistic inhibition by CXCR4- and VEGF-dependent pathways for angiogenesis using *flk-1* marker. **A** and **B**: wildtype control (**B**, high magnification); **C** and **D**: targeted knockdown of *cxcr4a* (**D**, high magnification) at sub-effective dosage exhibited no effect on sprouting of intersomitic vessels (IS); **E** and **F**: targeted knockdown of *vegf* (**F**, high magnification) at sub-effective dosage exhibited no effect on sprouting of intersomitic vessels (IS); **G** and **H**: co-targeting both *cxcr4a* and *vegf* (**H**, high magnification) by morpholino antisense oligos in zebrafish embryos exhibited synergistic inhibition on angiogenic sprouting of intersomitic vessels as revealed by vascular endothelial specific marker *flk-1*. Scale bars are 100um.

[0043] **Figure 16** shows a sequence comparison of various GPCR regions homologous to hamster adrenergic receptor (α1B-AR) which shows a dominant-negative effect when phenylalanine is substituted with glycine (G) or asparagine (N) (Chen S. *et al.* (2000) *EMBO J.* 19(16):4265-4271). Shown are the homologous regions from human CXCR4 (SEQ ID NO:80); human LEC3 (SEQ ID NO:81); human EDNRA (SEQ ID NO:82); hamster α1B-AR (SEQ ID NO:83); hamster α2C2-AR (SEQ ID NO:84); hamster β2-AR (SEQ ID NO:85); hamster H1 (SEQ ID NO:86); hamster D2 (SEQ ID NO:87); hamster M3 (SEQ ID NO:88); hamster AT1 (SEQ ID NO:89); hamster rhodopsin (SEQ ID NO:90). Conserved phenylalanine (Phe303 of hamster α1B-AR) is boxed.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0044] Three drugable targets have been identified that are essential for the formation of new blood vessels (*i.e.*, angiogenesis) which is a crucial step during tumor pathogenesis. Inhibition of any one of these genes prohibits angiogenesis in the vertebrate model organism, zebrafish. The tiny, transparent and fast-growing zebrafish offers the promise of becoming a

leading vertebrate model for studying human diseases related to angiogenesis. Results from this *in vivo* study of angiogenesis using the zebrafish model reveals three G protein coupled receptors (GPCRs) with important roles in angiogenesis, and provides methods for anti-cancer therapy.

[0045] GPCRs accounted for about 50% of all drug targets on the market over the past 20 years. More than 30% of the top 50 best-selling drug target GPCRs, include well-known medications for allergy, schizophrenia and bipolar disorder, heartburn and hypertension. Thus, the discovery of the GPCRs essential for angiogenesis allows us to tap into the drugable targets for modulating pathological angiogenesis, and facilitates the drug discovery process to fight cancer. Three important molecules have been identified whose functions are indispensable for angiogenesis. These molecules are C-X-C chemokine receptor type 4 (CXCR4), Lectomedin-3 (LEC3) and Endothelin-1 receptor (EDNRA). First, using zebrafish as *in vivo* vertebrate model, it is demonstrated that these genes are expressed in human vascular endothelial cells and zebrafish vasculatures. Second, using reverse genetic tools, targeted knock down of each of these genes inhibited angiogenesis in an animal model.

[0046] The present invention provides polynucleotides and polypeptides for zebrafish (and other vertebrate species, particularly human) CXCR4, LEC3 and EDNRA as well as methods of promoting and inhibiting angiogenesis in animal using CXCR4, LEC3 and EDNRA sense and antisense polynucleotides.

[0047] The reference works, patents, patent applications, and scientific literature, including accession numbers to GenBank database sequences that are referred to herein establish the knowledge of those with skill in the art and are hereby incorporated by reference in their entirety to the same extent as if each was specifically and individually indicated to be incorporated by reference. Any conflict between any reference cited herein and the specific teachings of this specification shall be resolved in favor of the latter.

[0048] Various definitions are made throughout this document. Most words have the meaning that would be attributed to those words by one skilled in the art. Words specifically defined either below or elsewhere in this document have the meaning provided in the context of the present invention as a whole and as are typically understood by those skilled in the art. Any conflict between an art-understood definition of a word or phrase and a definition of the word or phrase as specifically taught in this specification shall be resolved in favor of the latter. Headings used herein are for convenience and are not to be construed as limiting.

[0049] Standard reference works setting forth the general principles of recombinant DNA technology known to those of skill in the art include Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, 1998; Sambrook *et al.*, MOLECULAR

CLONING: A LABORATORY MANUAL, 2D ED., Cold Spring Harbor Laboratory Press, Plainview, New York, 1989; Kaufman *et al.*, Eds., HANDBOOK OF MOLECULAR AND CELLULAR METHODS IN BIOLOGY AND MEDICINE, CRC Press, Boca Raton, 1995; McPherson, Ed., DIRECTED MUTAGENESIS: A PRACTICAL APPROACH, IRL Press, Oxford, 1991. Standard works setting forth general principles and protocols for working with zebrafish known to those of skill in the art include, but are not limited to THE ZEBRAFISH BOOK: A GUIDE FOR THE LABORATORY USE OF ZEBRAFISH (*DANIO RERIO*), Westerfield, M., 4th ed., Univ. of Oregon Press, Eugene, 2000.

[0050] As used herein "isolated" means that the material is removed from its original environment (*e.g.*, the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

[0051] A "purified" or "substantially purified" polynucleotide or polypeptide is substantially separated from other cellular components that naturally accompany a native (or wild-type) nucleic acid or polypeptide and/or from other impurities (*e.g.*, agarose gel). A purified polypeptide or protein will comprise about 60% to more than 99% w/w of a sample, and may be about 90%, about 95%, or about 98% pure.

[0052] "About" as used herein refers to +/- 10% of the reference value.

[0053] As used herein, "variant" nucleotide or amino acid sequences refer to homologs, including, for example, isoforms, species variants, allelic variants, and fragments of the sequence of interest. "Homologous nucleotide sequence" or "homologous amino acid sequence," or variations thereof, refers to sequences characterized by a percentage identity (if referring to polynucleotides) or homology (if referring to polypeptides) of at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, preferably at least about 90%, at least about 95%, at least about 98%, or at least about 99%, and more preferably 100%, with respect to a reference sequence, or portion or fragment thereof encoding or having a functional domain.

[0054] The term "therapeutically effective amount" of a CXCR4, LEC3 and EDNRA antagonist, such as a CXCR4, LEC3 or EDNRA antisense oligonucleotide, means an amount calculated to achieve and maintain a therapeutically effective level in the disease state (*e.g.*, a tumor, if applied to a tumor), or in the plasma, if administered systematically, so as to inhibit angiogenesis (and, if applied to cancer, to inhibit the proliferation of cancer cells). By way of

example, the therapeutic amount sufficient to inhibit proliferation of more than about 50 percent of cancer cells, such as KS cells, *in vitro*. Of course, the therapeutic dose will vary with the potency of each CXCR4, LEC3 and EDNRA antagonist in inhibiting cancer cell growth *in vitro*, and the rate of elimination or metabolism of the CXCR4, LEC3 and EDNRA antagonist by the body in the tumor tissue and/or in the plasma. The therapeutic dose also applies to an amount of CXCR4, LEC3 and EDNRA antagonist that would enhance the efficacy of any combined regimen of therapy, such as anti-VEGF, and chemotherapy.

1. Polynucleotides

A. Vertebrate CXCR4, LEC3 and EDNRA

[0055] The invention provides polynucleotides encoding the zebrafish CXCR4, LEC3 and EDNRA polypeptides and also provides polynucleotides encoding CXCR4, LEC3 and EDNRA derived from other vertebrates for use in the methods of the invention. As used herein "polynucleotide" refers to a nucleic acid molecule and includes genomic DNA, cDNA, RNA, mRNA, mixed polymers, recombinant nucleic acids, fragments and variants thereof, and the like. Polynucleotide fragments useful in the invention comprise at least 10, and preferably at least 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 75, or 100 consecutive nucleotides of a reference polynucleotide. The polynucleotides of the invention include sense and antisense strands. The polynucleotides of the invention may be naturally occurring or non-naturally occurring polynucleotides. A "synthesized polynucleotide" as used herein refers to polynucleotides produced by purely chemical, as opposed to enzymatic, methods. "Wholly" synthesized DNA sequences are therefore produced entirely by chemical means, and "partially" synthesized DNAs embrace those wherein only portions of the resulting DNA were produced by chemical means. The polynucleotides of the invention may be single- or double-stranded. The polynucleotides of the invention may be chemically modified and may contain non-natural or derivatized nucleotide bases as will be readily appreciated by those skilled in the art. Such modifications include, for example, labels, methylation, substitution of one or more nucleotides with an analog, internucleotide modifications such as uncharged linkages (*e.g.*, methyl phosphonates, phosphotriesters, phosphoramidates, carbamates, *etc.*), charged linkages (*e.g.*, phosphorothioates, phosphorodithioates, *etc.*), pendent moieties (*e.g.*, polypeptides, *etc.*), intercalators (*e.g.*, acridine, psoralen, *etc.*), chelators, alkylators, and modified linkages (*e.g.*, alpha anomeric nucleic acids, *etc.*). Also included are synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence via hydrogen bonding and other chemical interactions. Such molecules

are known in the art and include, for example, those in which peptide linkages substitute for phosphate linkages in the backbone of the molecule.

[0056] The polynucleotides of the invention include those that encode the polypeptide sequence of SEQ ID NO:2 (human CXCR4), SEQ ID NO:4 (human LEC3) and SEQ ID NO:6 (human EDNRA). In some embodiments, the polynucleotides comprise the nucleic acid sequences of SEQ ID NO:1 (human CXCR4), SEQ ID NO:3 (human LEC3) and SEQ ID NO:5 (human EDNRA). The polynucleotides may contain mutations that result in amino acid changes that are either conservative or non-conservative. Mutations can be introduced into a nucleic acid sequence of the invention by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions may be made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (for example, lysine, arginine, and histidine), acidic side chains (for example, aspartic acid, glutamic acid), uncharged polar side chains (for example, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (for example, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (for example, threonine, valine, isoleucine), and aromatic side chains (for example, tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue is replaced with another amino acid residue from the same side chain family. Alternatively, mutations can be introduced randomly along all or part of a coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that retain activity. Following mutagenesis, the encoded protein may be expressed by any recombinant technology known in the art and the activity of the protein can be determined.

[0057] The nucleotide sequences are presented by single strands only, in the 5' to 3' direction, from left to right. Nucleotides are represented in the manner recommended by the IUPAC-IUB Biochemical Nomenclature Commission.

[0058] In some embodiments of the invention the mutations will not significantly alter the post-translational modifications or biological activity of the CXCR4, LEC3 and EDNRA proteins.

B. Expression Vectors

[0059] Another aspect of the invention pertains to vectors, preferably expression vectors, containing a nucleic acid encoding CXCR4, LEC3 and EDNRA polypeptides, or derivatives, fragments, analogs or homologs thereof. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid," which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*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. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors." In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

[0060] The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, that is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner that allows for expression of the nucleotide sequence (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990). Regulatory sequences include those that direct constitutive expression of a nucleotide sequence in many types of host cell and those that direct expression of the nucleotide sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression

vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, *etc.* The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (*e.g.*, CXCR4, LEC3 and EDNRA polypeptides, mutant forms of CXCR4, LEC3 and EDNRA, fusion proteins, *etc.*) from any vertebrate species. In some embodiments, the CXCR4, LEC3 or EDNRA is derived from mammals. In some embodiments, the CXCR4, LEC3 or EDNRA is derived from mice or rats. In other embodiments, the CXCR4, LEC3 or EDNRA is derived from humans. In other embodiments, the CXCR4, LEC3 or EDNRA is derived from zebrafish. A comparison of the amino acid sequences of human and zebrafish CXCR4, LEC3 and EDNRA is shown in **Figs. 2, 4 and 7**, respectively.

[0061] The recombinant expression vectors of the invention can be designed for expression of vertebrate CXCR4, LEC3 and EDNRA in prokaryotic or eukaryotic cells. For example, zebrafish CXCR4, LEC3 and EDNRA can be expressed in bacterial cells such as *Escherichia coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990). Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

[0062] Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: (1) to increase expression of recombinant protein; (2) to increase the solubility of the recombinant protein; and (3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc.; Smith and Johnson (1988) Gene 67:31-40), pMAL (New England Biolabs, Beverly, MA.) and pRIT5 (Pharmacia, Piscataway, NJ) that fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

[0063] Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amrann *et al.*, (1988) Gene 69:301-315) and pET 11d (Studier *et al.*, GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990) pp. 60-89).

[0064] One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacterium with an impaired capacity to proteolytically cleave the recombinant protein. See, Gottesman, GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990) 119-128. Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada *et al.*, (1992) *Nucleic Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

[0065] In another embodiment, the vertebrate CXCR4, LEC3 and EDNRA expression vector is a yeast expression vector. Examples of vectors for expression in yeast *Saccharomyces cerevisiae* include pYepSec1 (Baldari, *et al.*, (1987) *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz *et al.*, (1987) *Gene* 54:113-123), pYES2 (Invitrogen Corporation, San Diego, CA), and picZ (InVitrogen Corp, San Diego, CA).

[0066] Alternatively, the vertebrate CXCR4, LEC3 and EDNRA can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (*e.g.*, *Spodoptera frugiperda* SF9 cells) include the pAc series (Smith *et al.* (1983) *Mol. Cell. Biol.* 3:2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology* 170:31-39).

[0067] In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed (1987) *Nature* 329:840) and pMT2PC (Kaufman *et al.* (1987) *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. Representative examples of promoters include, but are not limited to LTR or SV40 promoter, the *E. coli*, *lac* or *trp*, the phage lambda P_L promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. For other suitable expression systems for both prokaryotic and eukaryotic cells, see, *e.g.*, Chapters 16 and 17 of Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL 2nd ed., Cold Spring Harbor Laboratory, Cold Spring

Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example; Bacterial:pQE70, pQE60, pQE-9 (Qiagen), pBS, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRITS (Pharmacia); Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host. In addition, a complete mammalian transcription unit and a selectable marker can be inserted into a prokaryotic plasmid. The resulting vector is then amplified in bacteria before being transfected into cultured mammalian cells. Examples of vectors of this type include pTK2, pHyg and pRSVneo.

[0068] In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (*e.g.*, tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert *et al.* (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji *et al.* (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (*e.g.*, the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477), pancreas-specific promoters (Edlund *et al.* (1985) *Science* 230:912-916), and mammary gland-specific promoters (*e.g.*, milk whey promoter; U.S. Pat. No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, *e.g.*, the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the α-fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546). Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

[0069] The expression vector may also contain a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression. In addition, in some embodiments, the expression vectors contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase (DHFR) or neomycin (NEO) resistance for eukaryotic cell culture, or such as tetracycline (TET) or ampicillin (AMP) resistance in *E. coli.*

[0070] The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner that allows for expression (by transcription of the DNA molecule) of an RNA molecule that is antisense to vertebrate CXCR4, LEC3 and EDNRA mRNA. Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen that direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen that direct constitutive, tissue specific or cell type specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub *et al.*, (1986) "Antisense RNA as a molecular tool for genetic analysis," *Reviews -- Trends in Genetics*, Vol. 1(1):22-25.

C. Antisense Oligonucleotides

[0071] Antisense oligonucleotides may be used as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotide drugs, including ribozymes, have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

[0072] In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

[0073] While antisense oligonucleotides are contemplated by the present invention, other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below, are also included. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 50 nucleobases (*i.e.* from about 8 to about 50 linked nucleosides). In some embodiments, the antisense oligonucleotides comprise from

about 8 to about 15 nucleobases. In other embodiments, the antisense oligonucleotides comprise from about 16 to about 25 nucleobases. In other embodiments, the antisense oligonucleotides comprise from about 20 to about 30 nucleobases. In other embodiments, the antisense oligonucleotides comprise from about 25 to about 35 nucleobases. In other embodiments, the antisense oligonucleotides comprise from about 30 to about 45 nucleobases. In other embodiments, the antisense oligonucleotides comprise from about 40 to about 50 nucleobases. Antisense compounds include ribozymes, external guide sequence (EGS) oligonucleotides (oligozymes), and other short catalytic RNAs or catalytic oligonucleotides which hybridize to the target nucleic acid and modulate its expression.

[0074] As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

[0075] Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

[0076] In some embodiments, the modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters,

selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage *i.e.*, a single inverted nucleoside residue which may be a basic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

[0077] In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, *i.e.*, the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen *et al.* (1991) *Science* 254:1497-1500.

[0078] Modified oligonucleotides may also contain one or more substituted sugar moieties.

[0079] A further modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene $(-CH_2-)_n$ group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226.

[0080] Other modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$), 2'-allyl (2'-CH_2-CH=CH_2), 2'-O-allyl (2'-O-CH_2-CH=CH_2) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar.

[0081] Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl (-C=C-CH_3) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine(1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (*e.g.* 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in THE CONCISE ENCYCLOPEDIA OF POLYMER SCIENCE AND ENGINEERING, pages 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, those disclosed by Englisch *et al.* Angewandte Chemie, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y. S., Chapter 15, ANTISENSE RESEARCH AND APPLICATIONS, pages 289-302, Crooke, S. T. and Lebleu, B. ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C. (Sanghvi, Y. S., Crooke, S. T. and Lebleu, B., eds., ANTISENSE RESEARCH AND APPLICATIONS, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

[0082] Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. The compounds of the invention can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugates groups include cholesterols, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or strengthen sequence-specific hybridization with RNA. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve oligomer uptake, distribution, metabolism or excretion. Conjugate moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:6553-6556), cholic acid (Manoharan *et al.* (1994) *Bioorg. Med. Chem. Let.* 4:1053-1060), a thioether, *e.g.*, hexyl-S-tritylthiol (Manoharan *et al.* (1992) *Ann. N.Y. Acad. Sci.* 660:306-309; Manoharan *et al.* (1993) *Bioorg. Med. Chem. Let.* 3:2765-2770), a thiocholesterol (Oberhauser *et al.* (1992) *Nucl. Acids Res.* 20:533-538), an aliphatic chain, *e.g.*, dodecandiol or undecyl residues (Saison-Behmoaras *et al.* (1991) *EMBO J.* 10:1111-1118; Kabanov *et al.* (1990) *FEBS Lett.* 259:327-330; Svinarchuk *et al.* (1993) *Biochimie* 75:49-54), a phospholipid, *e.g.*, di-hexadecyl-rac-glycerol or triethyl-ammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan *et al.* (1995) *Tetrahedron Lett.* 36:3651-3654; Shea *et al.* (1990) *Nucl. Acids Res.* 18:3777-3783), a polyamine or a polyethylene glycol chain (Manoharan *et al.* (1995) *Nucleosides & Nucleotides* 14:969-973), or adamantane acetic acid (Manoharan *et al.* (1995) *Tetrahedron Lett.* 36:3651-3654), a palmityl moiety (Mishra *et al.* (1995) *Biochim. Biophys. Acta* 1264:229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke *et al.* (1996) *J. Pharmacol Exp. Ther.* 277:923-937. Oligonucleotides of the invention may also be conjugated to active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (S)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethicin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic.

[0083] It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds which are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, *i.e.*, a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

[0084] Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers.

[0085] The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, Calif.). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

[0086] The antisense compounds of the invention are synthesized *in vitro* and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the *in vivo* synthesis of antisense molecules. The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule

structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption.

D. Morpholino-Modified Oligonucleotides

[0087] A particular form of antisense technology are morpholino oligonucleotides (Summerton, J. and D. Weller (1997) *Antisense Nucl. Acid Drug Dev:* 7:187-195; Nasevicius, A. and S.C. Ekker (2000) *Nat. Genet.* 26:216-220; Yan, Y-K. *et al.* (2002) *Development* 129:5065-5079). Morpholino oligonucleotides are nonionic DNA analogs with altered backbone linkages compared with DNA or RNA, but follow Watson-Crick base-pairing with complementary sequences. Typically, morpholinos are at least about 18-25 nucleobases in length, in some embodiments, the morpholinos are at least about 25-30 nucleobases in length, in still more embodiments, the morpholinos are at least about 30-35 nucleobases in length or more. The strengths of morpholinos as tools for investigating vertebrate development are well described in a recent review by Ekker S.C. (2000) *Yeast* 17:302-306, the disclosure of which is hereby incorporated by reference.

[0088] Morpholinos form RNA-morpholino hybrids that are not substrates for RNase H, and are not degraded. Ekker and colleagues report that fluorescently labeled morpholino oligonucleotides can be injected into sphere-stage zebrafish embryos and achieve uniform distribution. Morpholino oligomers targeted to the start codon for green fluorescent protein (GFP) blocked GFP expression, whereas control oligomers that are complementary to GFP did not. This established the ability of morpholino oligomers to unambiguously block gene expression in a sequence-specific manner. Ekker and colleagues also reported inhibition of several endogenous zebrafish genes.

[0089] The present invention provides morpholino-modified oligonucleotides targeting the 5' untranslated region of a CXCR4, LEC3 or EDNRA polynucleotide or a splice site of a CXCR4, LEC3 or EDNRA polynucleotide. Such morpholino-modified oligonucleotides are effective in inhibiting the expression of CXCR4, LEC3 or EDNRA and interfere with angiogenesis in an animal treated with these morpholino-modified oligonucleotides.

[0090] Morpholinos are highly non-polar. Thus, modified or unmodified morpholino oligos, may be administered in combination with any known delivery vehicle/vector that facilitates delivery of morpholino oligos into cells/tissues.

[0091] Morpholinos (and other oligonucleotides) may also be conjugated to arginine rich peptides to promote cellular uptake as described by Moulton, H.M. *et al.* (2004) *Bioconjugate Chem.* 15:290-299 which is incorporated herein by reference in its entirety.

E. Delivery of nucleic acids to cells:

[0092] The nucleic acid constructs of the invention may be delivered by any means known in the art. In some embodiments, nucleic acid is delivered into a cell using *ex vivo* strategies. In other embodiments, nucleic acid is delivered into a cell using *in vivo* strategies.

[0093] In *ex vivo* gene therapy methods, the cells are removed from the host organism, such as a human, prior to experimental manipulation. These cells are then transfected with a nucleic acid *in vitro* using methods well known in the art. These genetically manipulated cells are then reintroduced into the host organism. Alternatively, *in vivo* gene therapy approaches do not require removal of the target cells from the host organism. Rather, the nucleic acid may be complexed with reagents, such as liposomes or retroviruses, and subsequently administered to target cells within the organism using known methods. See, *e.g.*, Morgan *et al.* (1987) *Science* 237:1476, 1987; Gerrard *et al.* (1993) *Nat. Genet.* 3:180.

[0094] Several different methods for transfecting cells can be used for either *ex vivo* or *in vivo* gene therapy approaches. Known transfection methods may be classified according to the agent used to deliver a select nucleic acid into the target cell. These transfection agents include virus dependent, lipid dependent, peptide dependent, and direct transfection ("naked DNA") approaches. Other approaches used for transfection include calcium co-precipitation and electroporation.

[0095] Viral approaches use a genetically engineered virus to infect a host cell, thereby "transfecting" the cell with an exogenous nucleic acid. Among known viral vectors are recombinant viruses, of which examples have been disclosed, including poxviruses, herpesviruses, adenoviruses, and retroviruses. Such recombinants can carry heterologous genes under the control of promoters or enhancer elements, and are able to cause their expression in vector-infected host cells. Recombinant viruses of the vaccinia and other types are reviewed by Mackett *et al.* (1994) *J. Virol.* 49:3; also see Kotani *et al.* (1994) *Hum. Gene Ther.* 5:19.

[0096] Non-viral vectors, such as liposomes, may also be used as vehicles for nucleic acid delivery in gene therapy. In comparison to viral vectors, liposomes are safer, have higher capacity, are less toxic, can deliver a variety of nucleic acid-based molecules, and are relatively nonimmunogenic. See Felgner, P. L. and Ringold, G. M., (1989) *Nature* 337:387-388. Among these vectors, cationic liposomes are the most studied due to their effectiveness in mediating mammalian cell transfection *in vitro*. One technique, known as lipofection, uses a lipoplex made of a nucleic acid and a cationic lipid that facilitates transfection into cells. The lipid/nucleic acid complex fuses or otherwise disrupts the plasma or endosomal membranes and transfers the

nucleic acid into cells. Lipofection is typically more efficient in introducing DNA into cells than calcium phosphate transfection methods. Chang *et al.* (1988) *Focus* 10:66.

[0097] One known protein dependent approach involves the use of polylysine mixed with a nucleic acid. The polysine/nucleic acid complex is then exposed to target cells for entry. See, *e.g.*, Verma and Somia (1997) *Nature* 389:239; Wolff *et al.* (1990) *Science* 247:1465.

[0098] "Naked DNA" transfection approaches involve methods where nucleic acids are administered directly *in vivo*. See U.S. Pat. No. 5,837,693 to German *et al.* Administration of the nucleic acid could be by injection into the interstitial space of tissues in organs, such as muscle or skin, introduction directly into the bloodstream, into desirable body cavities, or, alternatively, by inhalation. In these so called "naked DNA" approaches, the nucleic acid is injected or otherwise contacted with the animal without any adjuvants. It has been reported that injection of free ("naked") plasmid DNA directly into body tissues, such as skeletal muscle or skin, can lead to protein expression. See Ulmer *et al.* (1993) *Science* 259:1745-1749; Wang *et al.* (1993) *Proc. Nat. Acad. Sci. USA* 90:4157-4160; Raz *et al.* (1994) *Proc. Nat. Acad. Sci. USA* 91:9519-9523.

[0099] Electroporation is another transfection method. See U.S. Pat. No. 4,394,448 to Szoka, Jr. *et al.* and U.S. Pat. No. 4,619,794 to Hauser. The application of brief, high-voltage electric pulses to a variety of animal and plant cells leads to the formation of nanometer-sized pores in the plasma membrane. DNA can enter directly into the cell cytoplasm either through these small pores or as a consequence of the redistribution of membrane components that accompanies closure of the pores.

2. Host Cells

[0100] Another aspect of the invention pertains to host cells into which a recombinant expression vector of the invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but 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 as used herein.

[0101] A host cell can be any prokaryotic or eukaryotic cell. For example, vertebrate CXCR4, LEC3 or EDNRA polypeptide can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

[0102] Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, *et al.* (MOLECULAR CLONING: A LABORATORY MANUAL 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), and other laboratory manuals.

[0103] For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Various selectable markers include those that confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding vertebrate CXCR4, LEC3 or EDNRA or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

[0104] A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (*i.e.*, express) vertebrate CXCR4, LEC3 or EDNRA polypeptides. Accordingly, the invention further provides methods for producing vertebrate CXCR4, LEC3 and EDNRA polypeptides using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of invention (into which a recombinant expression vector encoding vertebrate CXCR4, LEC3 or EDNRA has been introduced) in a suitable medium such that vertebrate CXCR4, LEC3 or EDNRA polypeptide is produced. In another embodiment, the method further comprises isolating vertebrate CXCR4, LEC3 or EDNRA from the medium or the host cell.

3. Transgenic Animals:

[0105] The host cells of the invention can also be used to produce nonhuman transgenic animals. For example, in one embodiment, a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which vertebrate CXCR4, LEC3 or EDNRA-encoding sequences have been introduced. Such host cells can then be used to create non-human

transgenic animals in which exogenous vertebrate CXCR4, LEC3 or EDNRA sequences have been introduced into their genome or homologous recombinant animals in which endogenous vertebrate CXCR4, LEC3 or EDNRA sequences have been altered. Such animals are useful for studying the function and/or activity of vertebrate CXCR4, LEC3 or EDNRA and for identifying and/or evaluating modulators of vertebrate CXCR4, LEC3 or EDNRA activity. As used herein, a "transgenic animal" is a non-human animal, in some embodiments, the animal is a fish, in other embodiments, the animal is a mammal (*e.g.*, a rodent such as a rat or mouse), in which one or more of the cells of the animal includes a transgene. Other examples of transgenic animals include non-human 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, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. As used herein, a "homologous recombinant animal" is a non-human animal, such as a mammal (*e.g.*, mouse) in which an endogenous CXCR4, LEC3 or EDNRA gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, *e.g.*, an embryonic cell of the animal, prior to development of the animal.

[0106] A transgenic animal of the invention can be created by introducing vertebrate CXCR4, LEC3 or EDNRA-encoding nucleic acid 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. The vertebrate CXCR4, LEC3 or EDNRA cDNA sequence of SEQ ID NO:1 (human CXCR4), SEQ ID NO:3 (human LEC3), SEQ ID NO:5 (human EDNRA), SEQ ID NO:7 (*Danio* CXCR4), SEQ ID NO:9 (*Danio* LEC3), SEQ ID NO:11(*Danio* EDNRA) or an artificial construct, for example, can be introduced as a transgene into the genome of a non-human animal. In some embodiments, variants of the vertebrate CXCR4, LEC3 or EDNRA are introduced in which the vertebrate CXCR4, LEC3 or EDNRA lacks one or more domains, or has substitutions of wild-type sequences with homologs. Alternatively, a homologue of the vertebrate CXCR4, LEC3 or EDNRA gene, can be isolated based on hybridization to the vertebrate CXCR4, LEC3 or EDNRA cDNA (described further above) and used as a transgene. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to the vertebrate CXCR4, LEC3 or EDNRA transgene to direct expression of vertebrate CXCR4, LEC3 or EDNRA polypeptide to particular cells. Methods for generating transgenic animals via embryo manipulation and

microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Pat. Nos. 4,736,866; 4,870,009; and 4,873,191; and Hogan 1986, In: MANIPULATING THE MOUSE EMBRYO, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified based upon the presence of the vertebrate CXCR4, LEC3 or EDNRA transgene in its genome and/or expression of vertebrate CXCR4, LEC3 or EDNRA mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene encoding vertebrate CXCR4, LEC3 or EDNRA can further be bred to other transgenic animals carrying other transgenes.

[0107] To create a homologous recombinant animal, a vector is prepared which contains at least a portion of a vertebrate CXCR4, LEC3 or EDNRA gene into which a deletion, addition or substitution has been introduced to thereby alter, *e.g.*, functionally disrupt, the vertebrate CXCR4, LEC3 or EDNRA gene. The vertebrate CXCR4, LEC3 or EDNRA gene can be the cDNA of SEQ ID NO:1 (human CXCR4), SEQ ID NO:3 (human LEC3), SEQ ID NO:5 (human EDNRA), SEQ ID NO:7 (*Danio* CXCR4), SEQ ID NO:9 (*Danio* LEC3), SEQ ID NO:11(*Danio* EDNRA). The vertebrate CXCR4, LEC3 or EDNRA may then be introduced into the genome of a mammal, or other vertebrate. In one embodiment, the vector is designed such that, upon homologous recombination, the endogenous vertebrate CXCR4, LEC3 or EDNRA gene is functionally disrupted (*i.e.*, no longer encodes a functional protein; also referred to as a "knock out" vector).

[0108] Alternatively, the vector can be designed such that, upon homologous recombination, the endogenous vertebrate CXCR4, LEC3 or EDNRA gene is mutated or otherwise altered but still encodes functional protein (*e.g.*, the upstream regulatory region can be altered to thereby alter the expression of the endogenous vertebrate CXCR4, LEC3 or EDNRA polypeptide). In the homologous recombination vector, the altered portion of the vertebrate CXCR4, LEC3 or EDNRA gene is flanked at its 5' and 3' ends by additional nucleic acid of the vertebrate CXCR4, LEC3 or EDNRA gene to allow for homologous recombination to occur between the exogenous vertebrate CXCR4, LEC3 or EDNRA gene carried by the vector and an endogenous vertebrate CXCR4, LEC3 or EDNRA gene in an embryonic stem cell. The additional flanking vertebrate CXCR4, LEC3 or EDNRA nucleic acid is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector. See *e.g.*, Thomas *et al.* (1987) *Cell* 51:503 for a description of homologous recombination vectors. The vector is

introduced into an embryonic stem cell line (*e.g.*, by electroporation) and cells in which the introduced vertebrate CXCR4, LEC3 or EDNRA gene has homologously recombined with the endogenous vertebrate CXCR4, LEC3 or EDNRA gene are selected (see *e.g.*, Li *et al.* (1992) *Cell* 69:915). Methods of making transgenic non-human animals are well-known in the art (for mice see Brinster *et al.* (1985) *Proc. Nat. Acad. Sci. USA* 82:4438-42; U.S. Pat. Nos. 4,736,866, 4,870,009, 4,873,191, 6,127,598; Hogan, B., MANIPULATING THE MOUSE EMBRYO, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1986); for homologous recombination see Capecchi (1989) *Science* 244:1288-1292; Joyner *et al.* (1989) *Nature* 338:153-156; for particle bombardment see U.S. Pat. No., 4,945,050; for Drosophila see Rubin and Spradling (1982) *Science* 218:348-53, U.S. Pat. No. 4,670,388; for transgenic insects see Berghammer A.J. *et al.* (1999) *Nature* 402:370-371; for zebrafish see Lin S. (2000) *Methods Mol. Biol.* 136:375-3830; for fish, amphibians and birds see Houdebine and Chourrout, (1991) *Experientia* 47:897-905; for rats see Hammer *et al.* (1990) *Cell* 63:1099-1112; for embryonic stem (ES) cells see TERATOCARCINOMAS AND EMBRYONIC STEM CELLS, A PRACTICAL APPROACH, E. J. Robertson, ed., IRL Press (1987); for livestock see Pursel *et al.* (1989) *Science* 244:1281-1288; for nonhuman animal clones see Wilmut, I. *et al.* (1997) *Nature* 385:810-813, PCT Publication Nos. WO 97/07668 and WO 97/07669; for recombinase systems for regulated transgene expression see, Lakso *et al.* (1992) *Proc. Natl. Acad. Sci.* 89:6232-6236; U.S. Pat. No. 4,959,317 (for cre.loxP) and O'Gorman *et al.* (1991) *Science* 251:1351-1355; U.S. Pat. No. 5,654,182 (for FLP/FRT). 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 provided through the construction of "double" transgenic animals, *e.g.*, by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase.

[0109] The invention also provides dominant-negative and constitutively active mutant forms of CXCR4, LEC3 and EDNRA transgenes. These mutant transgenes permit the study of these GPCRs and their therapeutic potentials. In some embodiments, expression vectors comprising the nucleic acid sequences encoding dominant-negative mutant forms of CXCR4, LEC3 or EDNRA are introduced into the non-human embryos or cells and the resulting transgenic animals or cells have dominant-negative expression of the subject GPCR.

[0110] Dominant negative mutations may be introduced, for example by site directed mutagenesis (by any means known in the art) to a region of the protein sequence that would confer the dominant-negative effect. For example, but not by way of limitation, it has been shown that phenylalanine at amino acid position 303 (F303G and F303N) in hamster α_1-

adenergic receptor was associated with conferring a dominant-negative effect of this GPCR (Chen S. *et al.* (2000) *EMBO J.* 19(16):4265-4271). This region of the GPCR protein is highly conserved, and it is therefore likely to create a similar dominant negative receptors for GPCRs with conserved sequences (Chen S. *et al.* (2000) *EMBO J.* 19(16):4265 at page 4270). This region is in fact conserved among, for example, CXCR4, LEC3, EDNRA, and hamster α_1-adenergic receptors, histamine (H_1), Dopamine (D_2), muscarinic (M_3), and angiotensin II (AT_1). Thus, mutations to this region particularly to the phenylalanine shown boxed in **Fig. 16**, are useful in creating dominant-negative mutants. Further mutations in the extracellular domains of the GPCRs close to the carboxyl-terminal areas of the extracellular domains of the GPCR as found by Dosil M. *et al.* (1998) *Mol. Cell. Biol.* 18(10):5981-5991 or in regions analogous to those reported by Leavitt L.M. *et al.* (1999) *Mol. Gen. Genet.* 261(6):917-932.

[0111] In some embodiments, expression vectors comprising the nucleic acid sequences encoding CXCR4, LEC3 or EDNRA are operably linked to promoters that drive constitutive expression of the GPCR protein. These sequences are introduced into the non-human embryos and the resulting transgenic animals have constitutive expression of the subject GPCR. Constitutive promoters are well known in the art and include, without limitation, the SV40, cytomegalovirus promoters (*e.g.*, CMV5), human CMV enhancer/chicken b-actin promoter, mouse *pgk* promoter, and human ubiquitin-C promoter.

[0112] In other embodiments, the CXCR4, LEC3 or EDNRA proteins are altered by site directed mutagenesis to have substitutions in the amino acid sequence that lead to a constitutively active protein by analogy to those described in the literature for other GPCRs. Examples include, but are not limited to Vischer H.F. *et al.* (2006) *Trends Pharmacol. Sci.* 27(1):56-63; Miura S. *et al.* (2005) *J. Biol. Chem.* 280(18):18237-18244; Ladds G. *et al.* (2005) *Mol. Microbiol.* 55(2):482-497; Zhang M. *et al.* (2005) *Mol. Biol. Cell.* 16(2):562-572; Cotecchia S. *et al.* (2003) *Assay Drug Dev. Technol.* 1(2):311-316; Behan D.P. and D.T. Chalmers. (2001) *Curr. Opin. Drug Discov. Devel.* 4(5):548-560; Parnot C. *et al.* (2002) *Trends Endocrinol. Metab.* 13(8):336-343; and Egan C. *et al.* (1998) *Ann. NY Acad. Sci.* 861:136-139.

[0113] In some embodiments, a promoter used that drives expression of CXCR4, LEC3 or EDNRA may be tissue-specific. This allows constitutive expression of the GPCR in selected tissues of the animals. Such constitutively expressed GPCR are useful in screening for compounds that modulate the GPCR activities for evaluation of therapeutic potential. Tissue-specific promoters are also well known in the art and include, without limitation, neuron-specific enolase (NSE) which promotes high expression in neurones; tubulin α1 (T- α1) which promotes high expression in neurones; glial-fibrillary acidic protein (GFAP) which promotes high

expression in astrocytes; myosin light chain-2 (MLC2) which promotes high expression in cardiomyocytes; preproendothelin-1 (ET-1) which promotes high expression in endothelial cells; tie (tie) which promotes high expression in endothelial cells; SM22a (SM22a) which promotes high expression in vascular smooth muscle cells; α1-antitrypsin (α1-AT) which promotes high expression in hepatocytes; albumin (ALB) which promotes high expression in hepatocytes; side-chain-cleavage enzyme (SCC) which promotes high expression in steroidogenic cells; and kidney-androgen responsive protein (KAP) which promotes high expression in renal proximal tubular cells.

[0114] The invention also provides CXCR4, LEC3, and EDNRA2, fusion proteins wherein the fusion protein partner may be visualized grossly or histologically. Briefly an expression construct is produced that has an in-frame joinder of CXCR4, LEC3 or EDNRA with a protein that may be visualized or easily identified. Such fusion proteins include, for example, green fluorescent protein (GFP) and others that are well-known in the art. Such constructs introduced into animals (by homologous recombination, for example) or cells (through various transfection technologies) and are useful in order to study the molecular interaction with other signaling molecules, such as G proteins, subcellular localization, physiological, pathophysiological and metabolic changes of these GPCR targets.

[0115] The invention also provides a GFP fluorescent reporter constructs driven by the CXCR4, LEC3, or EDNRA promoters. Such constructs are generated by operably linking the promoters of CXCR4, LEC3 or EDNRA to a reporter, such as, for example, GFP. Such constructs may be introduced into animals (by homologous recombination, for example) or cells (through various transfection technologies) in order to facilitate the study of pharmacological or genetic regulation on the expression of these GPCRs. For example, a transgenic zebrafish expressing GFP under the control of the promoter for CXCR4, LEC3 or EDNRA are useful in facilitating large-scale drug screening or genetic screening to identify therapeutic pharmaceuticals or genetic modifiers that can regulate the expression level of these GPCRs. Promoters for CXCR4 are known in the art and may be found for example in Haviv Y.S. *et al.* (2004) *Mol. Cancer Ther.* 3(6):687-691 ; Moriuchi M. *et al.* (1999) *AIDS Res. Hum. Retroviruses* 15(9):821-827 ; Moriuchi M. *et al.* (1999) *J. Immunol.* 162(10):5986-5992; and Caruz A. *et al.* (1998) *FEBS Lett.* 426(2):271-278; Moriuchi M. *et al.* (1997) *J. Immunol.* 159(9):4322-4329. Promoters for EDNRA are also known in the art and are described, for example in Yamashita J. *et al.* (1998) *J. Biol. Chem.* 273(26):15993-15999 and Yamashita J. *et al.* (1995) *J. Cardiovasc. Pharmacol.* 26 Suppl 3:S26-28.

4. Vertebrate Polypeptides

[0116] The invention also provides vertebrate CXCR4, LEC3 and EDNRA polypeptides. The CXCR4, LEC3 and EDNRA polypeptides, variants, fragments and antigenic portions thereof may be derived from any vertebrate species. In some embodiments, the CXCR4, LEC3 and EDNRA polypeptides are derived from mammals. In some embodiments, the CXCR4, LEC3 and EDNRA polypeptides are derived from mice or rats. In other embodiments, the CXCR4, LEC3 and EDNRA polypeptides are derived from humans. In other embodiments, the CXCR4, LEC3 and EDNRA polypeptides are derived from fish, such as the zebrafish. In some embodiments the CXCR4 polypeptides have the amino acid sequence of SEQ ID NO:2, SEQ ID NO:8, or SEQ ID NO:33. In some embodiments, the LEC3 polypeptides have the amino acid sequence of SEQ ID NO:4, SEQ ID NO:10, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:27, or SEQ ID NO:28. In some embodiments, the EDNRA polypeptides have the amino acid sequence of SEQ ID NO:6, SEQ ID NO:12, SEQ ID NO:20, SEQ ID NO:22, or SEQ ID NO:23.

[0117] The terms "polypeptide," "peptide," and "protein" are used interchangeably herein. "Polypeptide" refers to a polymer of amino acids without referring to a specific length. Polypeptides of the invention include peptide fragments, derivatives, and fusion proteins. Peptide fragments preferably have at least about 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, or 100 amino acids. Some peptide fragments of the invention are biologically active. Biological activities include immunogenicity, ligand binding, and activity associated with the reference peptide. Immunogenic peptides and fragments of the invention generate an epitope-specific immune response, wherein "epitope" refers to an immunogenic determinant of a peptide and preferably contains at least three, five, eight, nine, ten, fifteen, twenty, thirty, forty, forty-five, or fifty amino acids. Some immunogenic peptides of the invention generate an immune response specific to that peptide. Polypeptides of the invention include naturally occurring and non-naturally occurring peptides. The term includes modified polypeptides (wherein examples of such modifications include glycosylation, acetylation, phosphorylation, carboxylation, ubiquitination, labeling, *etc.*), analogs (such as non-naturally occurring amino acids, substituted linkages, *etc.*), and functional mimetics. A variety of methods for labeling polypeptides are well known in the art and include radioactive isotopes such as ^{32}P or ^{35}S, ligands that bind to labeled antiligands (*e.g.*, antibodies), fluorophores, chemiluminescent agents, enzymes, and antiligands.

[0118] As used herein, the term "amino acid" denotes a molecule containing both an amino group and a carboxyl group. In some embodiments, the amino acids are α-, β-, γ- or δ-amino acids, including their stereoisomers and racemates. As used herein the term "L-amino

acid" denotes an α-amino acid having the L configuration around the α-carbon, that is, a carboxylic acid of general formula CH(COOH)(NH_2)-(side chain), having the L-configuration. The term "D-amino acid" similarly denotes a carboxylic acid of general formula CH(COOH)(NH_2)-(side chain), having the D-configuration around the α-carbon. Side chains of L-amino acids include naturally occurring and non-naturally occurring moieties. Non-naturally occurring (*i.e.*, unnatural) amino acid side chains are moieties that are used in place of naturally occurring amino acid side chains in, for example, amino acid analogs. Amino acid substituents may be attached, for example, through their carbonyl groups through the oxygen or carbonyl carbon thereof, or through their amino groups, or through functionalities residing on their side chain portions.

[0119] The amino acid sequences are presented in the amino (N) to carboxy (C) direction, from left to right. The N-terminal α-amino group and the C-terminal β-carboxy groups are not depicted in the sequence. Amino acids are represented in the manner recommended by the IUPAC-IUB Biochemical Nomenclature Commission, or amino acids are represented by their three letters code designations.

[0120] The invention also contemplates the use of cell-permeable peptides. Cell permeable peptides have been designed to inhibit the function of various pathways including neuronal degeneration (Borsello T, and C. Bonny (2004) "Use of cell-permeable peptides to prevent neuronal degeneration" Trends Mol. Med. 10(5):239-44 and Abeta1-40 fibrillogenesis (Gordon D.J. *et al.* (2002) "Design and characterization of a membrane permeable N-methyl amino acid-containing peptide that inhibits Abeta1-40 fibrillogenesis" *J. Pept. Res.* 60(1):37-55). Methods for designing cell-permeable peptides are known in the art and are described for example in Du C. *et al.* (1998) "Conformational and topological requirements of cell-permeable peptide function" *J. Pept. Res.* 51(3):235-243. Cell permeable peptides derived from CXCR4, LEC3 or EDNRA and/or VEGF may also be used in the methods of the invention to inhibit angiogenesis in a subject and to treat angiogenesis-related diseases and angiogenesis-dependent tumors. In order to determine which peptides are useful for such purpose, the screening procedures described herein may be used to routinely screen for functional cell-permeable peptides.

5. Different antibodies that specifically recognize vertebrate CXCR4, LEC3 or EDNRA

[0121] The invention provides antibodies (*e.g.*, monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies, bifunctional/bispecific antibodies, humanized antibodies, human antibodies, and complementary determining region (CDR)-grafted

antibodies), including compounds which include CDR sequences which specifically recognize vertebrate CXCR4, LEC3 or EDNRA, or fragments of vertebrate CXCR4, LEC3 or EDNRA wherein the epitope comprises at least a portion of the amino acid sequence of the amino acid sequence of SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:33, SEQ ID NO:4, SEQ ID NO:10, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:6, SEQ ID NO:12, SEQ ID NO:20, SEQ ID NO:22, or SEQ ID NO:23.

[0122] Antibody fragments, including Fab, Fab', $F(ab')_2$, and F_v, are also provided by the invention. The term "specific for," when used to describe antibodies of the invention, indicates that the variable regions of the antibodies of the invention recognize and bind vertebrate CXCR4, LEC3 or EDNRA exclusively (*i.e.*, are able to distinguish vertebrate CXCR4, LEC3 or EDNRA from other different GPCRs by virtue of measurable differences in binding affinity, despite the possible existence of localized sequence identity, homology, or similarity between vertebrate CXCR4, LEC3 or EDNRA and other GPCR family members). "High binding affinity" refers to binding affinities of greater than about 5×10^{-8} M, preferably between about 5×10^{-8} M and about 5×10^{-12} M, in some embodiments the binding affinity is about 5×10^{-9} M to about 5×10^{-11} M, in some embodiments the binding affinity is about 5×10^{-7} M to about 5×10^{-8} M, in some embodiments the binding affinity is about 5×10^{-8} M to about 5×10^{-9} M, in some embodiments the binding affinity is about 5×10^{-9} M to about 5×10^{-10} M, in some embodiments the binding affinity is about 5×10^{-10} M to about 5×10^{-11} M.

[0123] It will be understood that specific antibodies may also interact with other proteins (for example, *Staphylococcus aureus* protein A or other antibodies in ELISA techniques) through interactions with sequences outside the variable region of the antibodies, and, in particular, in the constant region of the molecule. Screening assays to determine binding specificity of an antibody of the invention are well known and routinely practiced in the art. For a comprehensive discussion of such assays, see Harlow *et al.* (Eds.), ANTIBODIES: A LABORATORY MANUAL; Cold Spring Harbor Laboratory; Cold Spring Harbor, N.Y., 1988, Chapter 6. Antibodies that recognize and bind fragments of vertebrate CXCR4, LEC3 or EDNRA are also contemplated, provided that the antibodies are specific for vertebrate CXCR4, LEC3 or EDNRA. Antibodies of the invention can be produced using any method well known and routinely practiced in the art.

[0124] For the production of polyclonal antibodies, various suitable host animals (*e.g.*, rabbit, goat, mouse or other mammal) may be immunized by injection with the native polypeptide, or a synthetic variant thereof, or a derivative of the foregoing. An appropriate immunogenic preparation can contain, for example, recombinantly expressed vertebrate CXCR4,

LEC3 or EDNRA polypeptide or a chemically synthesized vertebrate CXCR4, LEC3 or EDNRA polypeptide. The preparation can further include an adjuvant. Various adjuvants used to increase the immunological response include, but are not limited to, Freund's (complete and incomplete), mineral gels (*e.g.*, aluminum hydroxide), surface active substances (*e.g.*, lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, dinitrophenol, *etc.*), human adjuvants such as Bacille Calmette-Guerin and *Corynebacterium parvum*, or similar immunostimulatory agents. If desired, the antibody molecules directed against vertebrate CXCR4, LEC3 or EDNRA can be isolated from the mammal (*e.g.*, from the blood) and further purified by well known techniques, such as protein A chromatography to obtain the IgG fraction.

[0125] The term "monoclonal antibody" or "monoclonal antibody composition," as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of vertebrate CXCR4, LEC3 or EDNRA. A monoclonal antibody composition thus typically displays a single binding affinity for a particular vertebrate CXCR4, LEC3 or EDNRA polypeptide with which it immunoreacts. For preparation of monoclonal antibodies directed towards a particular CXCR4, LEC3 or EDNRA polypeptide, or derivatives, fragments, analogs or homologs thereof, any technique that provides for the production of antibody molecules by continuous cell line culture may be utilized. Such techniques include, but are not limited to, the hybridoma technique (see Kohler & Milstein, 1975 Nature 256: 495-497); the trioma technique; the human B-cell hybridoma technique (see Kozbor, *et al.*, (1983) *Immunol Today* 4: 72) and the EBV hybridoma technique to produce human monoclonal antibodies (see Cole, *et al.*, 1985 In: MONOCLONAL ANTIBODIES AND CANCER THERAPY, Alan R. Liss, Inc., pp. 77-96). Human monoclonal antibodies may be utilized in the practice of the present invention and may be produced by using human hybridomas (see Cote, *et al.* (1983) *Proc Natl Acad Sci USA* 80:2026-2030) or by transforming human B-cells with Epstein Barr Virus *in vitro* (see Cole, *et al.* (1985) In: MONOCLONAL ANTIBODIES AND CANCER THERAPY, Alan R. Liss, Inc., pp. 77-96).

[0126] According to the invention, techniques can be adapted for the production of single-chain antibodies specific to a vertebrate LEC3, EDNRA or CXCR-4 polypeptide (see *e.g.*, U.S. Pat. No. 4,946,778). In addition, methodologies can be adapted for the construction of Fab expression libraries (see *e.g.*, Huse, *et al.* (1989) *Science* 246:1275-1281) to allow rapid and effective identification of monoclonal Fab fragments with the desired specificity for a LEC3, EDNRA or CXCR-4 polypeptide or derivatives, fragments, analogs or homologs thereof. Non-human antibodies can be "humanized" by techniques well known in the art. See *e.g.*, U.S. Pat. No. 5,225,539. Antibody fragments that contain the idiotypes to a CXCR4, LEC3 or EDNRA

polypeptide may be produced by techniques known in the art including, but not limited to: (i) an $F(ab')_2$ fragment produced by pepsin digestion of an antibody molecule; (ii) an Fab fragment generated by reducing the disulfide bridges of an $F(ab')_2$ fragment; (iii) an Fab fragment generated by the treatment of the antibody molecule with papain and a reducing agent and (iv) Fv fragments.

[0127] Additionally, recombinant anti-vertebrate CXCR4, LEC3 or EDNRA antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in International Application No. PCT/US86/02269; European Patent Application No. 184,187; European Patent Application No. 171,496; European Patent Application No. 173,494; PCT International Publication No. WO 86/01533; U.S. Pat. No. 4,816,567; U.S. Pat. No. 5,225,539; European Patent Application No. 125,023; Better *et al.* (1988) *Science* 240:1041-1043; Liu *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:3439-3443; Liu *et al.* (1987) *J. Immunol.* 139:3521-3526; Sun *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:214-218; Nishimura *et al.* (1987) *Cancer Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; Shaw *et al.* (1988) *J. Natl. Cancer Inst.* 80:1553-1559); Morrison (1985) *Science* 229:1202-1207; Oi *et al.* (1986) *BioTechniques* 4:214; Jones *et al.* (1986) *Nature* 321:552-525; Verhoeyan *et al.* (1988) *Science* 239:1534; and Beidler *et al.* (1988) *J. Immunol.* 141:4053-4060.

[0128] In one embodiment, methodologies for the screening of antibodies that possess the desired specificity include, but are not limited to, enzyme-linked immunosorbent assay (ELISA) and other immunologically-mediated techniques known within the art. In a specific embodiment, selection of antibodies that are specific to a particular domain of a zebrafish CXCR4, LEC3 or EDNRA polypeptide is facilitated by generation of hybridomas that bind to the fragment of a vertebrate CXCR4, LEC3 or EDNRA polypeptide possessing such a domain. Antibodies that are specific for an Ig-like domain within a vertebrate CXCR4, LEC3 or EDNRA polypeptide, or derivatives, fragments, analogs or homologs thereof, are also provided herein.

[0129] Anti-CXCR4, anti-LEC3 or anti-EDNRA antibodies may be used in methods known within the art relating to the localization and/or quantitation of a vertebrate CXCR4, LEC3 or EDNRA polypeptide (*e.g.*, for use in measuring levels of the vertebrate CXCR4, LEC3 or EDNRA polypeptide within appropriate physiological samples, for use in diagnostic methods, for use in imaging the polypeptide, and the like). In a given embodiment, antibodies for

vertebrate CXCR4, LEC3 or EDNRA polypeptides, or derivatives, fragments, analogs or homologs thereof, that contain the antibody derived binding domain, are utilized as pharmacologically-active compounds (hereinafter "therapeutics").

[0130] An anti-CXCR4, anti-LEC3 or anti-EDNRA antibody (*e.g.*, monoclonal antibody) can be used to isolate zebrafish CXCR4, LEC3 or EDNRA by standard techniques, such as affinity chromatography or immunoprecipitation. An anti-CXCR4, anti-LEC3 or anti-EDNRA antibody can facilitate the purification of natural CXCR4, LEC3 or EDNRA from cells and of recombinantly produced vertebrate CXCR4, LEC3 or EDNRA expressed in host cells. Moreover, an anti-CXCR4, anti-LEC3 or anti-EDNRA antibody can be used to detect vertebrate CXCR4, LEC3 or EDNRA polypeptide (*e.g.*, in a cellular lysate) in order to evaluate the abundance and pattern of expression of the vertebrate CXCR4, LEC3 or EDNRA polypeptide. Anti-CXCR4, anti-LEC3 or anti-EDNRA antibodies can be used diagnostically to monitor protein levels in tissue as part of a clinical testing procedure, *e.g.*, to, for example, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling (*i.e.*, physically linking) the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include Iodine-125, Iodine-131, Sulfur-35 or tritium. In addition, the antibodies of the present invention may be conjugated to toxins such as radioisotopes, protein toxins and chemical toxins. Such toxins include, but are not limited to Lead-212, Bismuth-212, Astatine-211, Iodine-131, Scandium-47, Rhenium-186, Rhenium-188, Yttrium-90, Iodine-123, Iodine-125, Bromine-77, Indium-111, Boron-10, Actinide, ricin, adriamycin, calicheamicin, and 5-fluorouracil.

[0131] Antibodies against LEC3 may have particular utility as LEC3 is a cell surface receptor. Therefore, antibodies may act on external loops of LEC3. Antibodies may be raised against isolated peptides derived from the deduced amino acid sequences that are predicted to be on the external surface of the cell (see **Fig. 3A-B**). The portions of the LEC3 polypeptide sequence predicted to be on the external surface of the cell include amino acids 1-806 of SEQ ID

NO:16; amino acids 861-868 of SEQ ID NO:16; amino acids 930-947 of SEQ ID NO:16; and amino acids 1026-1028 of SEQ ID NO:16.

6. Pharmaceutical Compositions

[0132] The CXCR4, LEC3 and EDNRA nucleic acid molecules, CXCR4, LEC3 and EDNRA polypeptides (including cell permeable modified versions of the protein), and anti-CXCR4, anti-LEC3 and anti-EDNRA antibodies (also referred to herein as "active ingredients") of the invention, and derivatives, fragments, analogs and homologs thereof, can be incorporated into pharmaceutical compositions in therapeutically effective amounts suitable for administration. Such compositions typically comprise the nucleic acid molecule, protein, or antibody and a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Suitable carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, a standard reference text in the field, which is incorporated herein by reference. Preferred examples of such carriers or diluents include, but are not limited to, water, saline, ringer's solutions, dextrose solution, and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils may also be used. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

[0133] A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerin, 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; buffers such as acetates, citrates or phosphates, and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH 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.

[0134] Pharmaceutical compositions suitable for injectable use 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 to the extent that easy syringeability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, and sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

[0135] Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*, a CXCR4, LEC3 or EDNRA polypeptide or anti-CXCR4, anti-LEC3 or anti-EDNRA antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0136] 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 and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The 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.

[0137] For administration by inhalation, the compounds are delivered in the form of an aerosol spray from a pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

[0138] Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

[0139] The compounds can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

[0140] In one embodiment, the active compounds are prepared with carriers that will 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. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811.

[0141] It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The

specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

[0142] 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 (see U.S. Pat. No. 5,328,470) or by stereotactic injection (see *e.g.*, Chen *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in 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.

[0143] The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

7. Methods of Modulating Angiogenesis

[0144] Angiogenesis-related diseases may be diagnosed and treated using the vertebrate CXCR4, LEC3 or EDNRA protein of the present invention as drug target for development of therapeutics. Angiogenesis-related diseases include, but are not limited to, angiogenesis-dependent cancer, including, for example, solid tumors, blood born tumors such as leukemias, and tumor metastases; benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; rheumatoid arthritis; psoriasis; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; and wound granulation. In some embodiments, a goal is to promote angiogenesis in the subject. Angiogenesis is important, for example, in wound healing. Thus, CXCR4, LEC3 or EDNRA or their products can be used, without limitation, to promote wound healing, to promote endothelialization in vascular graft surgery, and to promote endothelialization to heal vascular damage following myocardial infarction.

[0145] The invention provides methods of modulating angiogenesis in an animal. In some embodiments, the method promotes angiogenesis. In some embodiments, the method inhibits angiogenesis.

A. Pro-Angiogenesis

[0146] In some embodiments of the invention, angiogenesis is promoted in cells by increasing the expression of CXCR4, LEC3 or EDNRA. In the method of the invention, nucleic acid sequences encoding CXCR4, LEC3 or EDNRA are administered to cells to promote increased expression of CXCR4, LEC3 or EDNRA. The nucleic acid sequences may be any CXCR4, LEC3 or EDNRA (*i.e.*, may be derived from any species) provided the CXCR4, LEC3 or EDNRA nucleic acid molecules encode CXCR4, LEC3 or EDNRA with biological activity. The polynucleotides encoding CXCR4, LEC3 or EDNRA may be part of an expression vector as described herein. Methods of introducing nucleic acids into cells and animals are known in the art. The polynucleotides that may be used that encode CXCR4, LEC3 or EDNRA include, but are not limited to SEQ ID NO:1, SEQ ID NO:7, SEQ ID NO:32, SEQ ID NO:3, SEQ ID NO:9, SEQ ID NO:5, SEQ ID NO:11, SEQ ID NO:19 or SEQ ID NO:35. The amount of CXCR4, LEC3 or EDNRA expression may be driven by the strength of the promoter, and tissue specific factors as selected for the appropriate system. Generally, the amount of expression should be such that angiogenesis is stimulated. In some embodiments, CXCR4, LEC3 or EDNRA alone is sufficient, however, in other embodiments, more than one of CXCR4, LEC3 or EDNRA is used to activate a biological pathway. Thus, the invention also comprises the co-expression of more than one of CXCR4, LEC3 and EDNRA for the promotion of angiogenesis. Co-expression may be achieved by any means known in the art. Nucleic acids encoding the two polypeptides may be incorporated into the same expression vector or may be present on separate expression vectors. Transfection of the vectors into cells may be simultaneous or sequential. Expression of the two polypeptides may be under the control of the same regulatory elements or different regulatory elements to allow controlled expression of one or both polypeptides. Alternatively, the expression of either or both polypeptides may be made to be constitutive.

B. Anti-Angiogenesis

[0147] In some embodiments of the invention, angiogenesis is inhibited in cells by decreasing the expression of CXCR4, LEC3 or EDNRA, or by inhibiting the function of CXCR4, LEC3 or EDNRA. Angiogenesis may be inhibited through the use of compounds targeted against CXCR4, LEC3 or EDNRA, such as chemical compounds including natural or synthetic small molecules, antisense CXCR4, LEC3 or EDNRA molecules or antibodies against CXCR4, LEC3 or EDNRA. The polynucleotides that inhibit angiogenesis are those that bind to CXCR4, LEC3 or EDNRA polynucleotides and inhibit the expression of CXCR4, LEC3 or EDNRA or the correct splicing of CXCR4, LEC3 or EDNRA RNA. Examples of antisense

molecules that inhibit CXCR4, LEC3 or EDNRA expression include, but are not limited to 5'-cgcccgatctcatctacagaaagag-3' (SEQ ID NO:24)(MO-LEC3 against zebrafish gene (antisense for splicing inhibition)); 5'-taagccatctctaaaagacttctcc-3' (SEQ ID NO:25) (MO-CXCR4a against zebrafish gene (antisense for translational blocking); 5'-gccattgcagaacactggccgctct-3' (SEQ ID NO:26) (MO-EDNRA against zebrafish gene (antisense for translational blocking).

[0148] The compounds that may be administered to inhibit the function of CXCR4, LEC3 or EDNRA include chemical compounds including natural or synthetic small molecules, polyclonal and monoclonal antibodies that specifically bind CXCR4, LEC3 or EDNRA. The antibodies may be those that bind any known CXCR4, LEC3 or EDNRA provided that when administered to the subject, the antibodies specifically bind the CXCR4, LEC3 or EDNRA produced by the cells of the subject.

C. Anti-Angiogenesis Combination Therapy

[0149] In other embodiments of the invention, angiogenesis is modulated by manipulating the expression and/or function of CXCR4, LEC3 or EDNRA in combination with modulating the expression and/or function of vascular endothelial growth factor (VEGF). In some embodiments, angiogenesis is stimulated by administering CXCR4, LEC3 or EDNRA-encoding nucleic acid in combination with VEGF-encoding nucleic acid. The CXCR4, LEC3 or EDNRA and VEGF nucleic acids may be present in a single expression vector or on separate expression vectors. The VEGF and CXCR4, LEC3 or EDNRA nucleic acids may be administered simultaneously or separately. In other embodiments, angiogenesis is inhibited by administering a compound that decreases expression or function of CXCR4, LEC3 or EDNRA and a compound that decreases the expression or function of VEGF. In some embodiments, the compound that decreases the expression of CXCR4, LEC3 or EDNRA is an antisense oligonucleotide. In other embodiments, the compound that decreases the expression of CXCR4, LEC3 or EDNRA is a morpholino directed against CXCR4, LEC3 or EDNRA. In some embodiments, the compound that decreases the expression of VEGF is an antisense oligonucleotide. In other embodiments, the compound that decreases the expression of VEGF is a morpholino directed against VEGF. In some embodiments, the compound that inhibits the function of CXCR4, LEC3 or EDNRA is an anti-CXCR4, anti-LEC3 or anti-EDNRA polyclonal or monoclonal antibody. In some embodiments, the compound that inhibits the function of VEGF is an anti-VEGF polyclonal or monoclonal antibody (See, for example, Ferrara, N., Hillan, K.J. & Novotny, W. (2005) "Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy" *Biochem. Biophys. Res. Commun.* 333:328-335).

[0150] VEGF has been cloned and sequenced and many different VEGFs are known, including VEGF-A, VEGF-B, VEGF-C and VEGF-D (for a review of VEGF biology, see Tammela, T., Enholm, B., Alitalo, K., Paavonen, K. (2005) "The biology of vascular endothelial growth factors" *Cardiovasc. Res.* 65:550-563. Any VEGF-encoding nucleic acid that produces a biologically active VEGF may be used. For example, but not by way of limitation, the VEGF-encoding nucleic acids that may be used include that for human VEGFs (SEQ ID NO:59) (protein: SEQ ID NO:60), human VEGF-B (SEQ ID NO:61)(protein: SEQ ID NO:62), human VEGF-C (SEQ ID NO:69)(protein: SEQ ID NO:70), human VEGF-D (SEQ ID NO:71)(protein: SEQ ID NO:72); mouse VEGF-A (SEQ ID NO:65)(protein: SEQ ID NO:66), mouse VEGF-B (SEQ ID NO:63)(protein: SEQ ID NO:64), mouse VEGF-C (SEQ ID NO:73)(protein: SEQ ID NO: 74), mouse VEGF-D (SEQ ID NO:75)(protein: SEQ ID NO:76); zebrafish VEGF (SEQ ID NO:67)(protein: SEQ ID NO:68), and the like.

[0151] In some embodiments, VEGF expression and/or function is inhibited by administering antisense sequences. The antisense sequences have the general properties described as for CXCR4, LEC3 or EDNRA antisense sequences. In specific embodiments, for example, the antisense sequences have the sequence of SEQ ID NO:77 and SEQ ID NO:79 antisense sequences target zebrafish VEGF. The human VEGF sequence is found under accession number: NM_003376, Unigene cluster: Hs.73793 (Ensembl gene ID: ENSG00000112715).

[0152] In some embodiments, VEGF function is inhibited by the administration of anti-VEGF polyclonal or monoclonal antibodies. Examples of anti-VEGF monoclonal antibodies include, AVASTIN™ (Genentech), and BioCarta Catalog Nos. 09-06-16460 and 09-06-11335. The VEGF signaling pathway may also be inhibited by any tyrosine kinase inhibitor known in the art to disrupt VEGF signaling, or any other type of inhibitor for VEGF known in the art. These inhibitors may be used in combination with strategies described herein to modulate expression or activity of CXCR4, LEC3 or EDNRA.

[0153] In embodiments to inhibit the function of CXCR4, LEC3 or EDNRA and VEGF, the compounds that are used to inhibit the function, or biological activity, include, small molecule compounds, polynucleotides (*e.g.*, antisense and morpholinos, ribozymes, and the like); antibodies against VEGF and CXCR4, LEC3 or EDNRA; cell permeable peptides that block the interaction of CXCR4, LEC3 or EDNRA with other receptors (upstream receptors), G protein partners, and downstream effectors; and combinations of these approaches.

8. Screening for compounds that promote or inhibit angiogenesis:

[0154] Another aspect of the present invention is directed to methods of identifying compounds that bind to either CXCR4, LEC3 or EDNRA or nucleic acid molecules encoding CXCR4, LEC3 or EDNRA, comprising contacting CXCR4, LEC3 or EDNRA, or a nucleic acid molecule encoding CXCR4, LEC3 or EDNRA, with a test compound, and determining whether the test compound binds CXCR4, LEC3 or EDNRA or a nucleic acid molecule encoding CXCR4, LEC3 or EDNRA.

[0155] Binding can be determined by any binding assays known in the art, including but not limited to, gel-shift assays, Western blots, radiolabeled competition assay, phage-based expression cloning, co-fractionation by chromatography, co-precipitation, cross-linking, interaction trap/two-hybrid analysis, southwestern analysis, ELISA, and the like, which are described in, for example, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, 1999, John Wiley & Sons, NY. The CXCR4, LEC3 or EDNRA polypeptide or polynucleotide employed in such a test may either be free in solution, attached to a solid support, or located intracellularly, or associated with a cell fraction. In one embodiment of the invention, high throughput screening ("HTS") for compounds having suitable binding affinity to CXCR4, LEC3 or EDNRA is employed. Large numbers of test compounds may be exposed to immobilized CXCR4, LEC3 or EDNRA. Bound CXCR4, LEC3 or EDNRA is then detected by methods well known in the art.

[0156] Another method for identifying ligands of a target protein is described in Wieboldt *et al.*, *Anal. Chem.*, 69:1683-1691 (1997). This technique screens combinatorial libraries of 20-30 agents at a time in solution phase for binding to the target protein. Agents that specifically bind to the target protein which are retained on the filter are subsequently liberated from the target protein and analyzed by HPLC and pneumatically assisted electrospray (ion spray) ionization mass spectroscopy. This procedure selects library components with the greatest affinity for the target protein, and is particularly useful for small molecule libraries.

[0157] Other embodiments of the invention comprise using antibody-based competitive screening assays. In one embodiment, specific, neutralizing antibodies that bind CXCR4, LEC3 or EDNRA specifically compete with a test compound for binding to CXCR4, LEC3 or EDNRA. In this manner, the antibodies can be used to detect the presence of any peptide that shares one or more antigenic determinants with CXCR4, LEC3 or EDNRA. Such binding studies may use labeled antibodies and/or labeled test compounds. An examples of such a procedure can be found in, for example, Lin, A.H. *et al.* (1997) *Antimicrobial Agents and Chemotherapy* 41(10):2127-2131.

[0158] Another aspect of the present invention is directed to methods of identifying compounds that modulate (*i.e.*, increase or decrease) the biological activity of CXCR4, LEC3 or

EDNRA. Such methods comprise contacting CXCR4, LEC3 or EDNRA with a test compound, and determining whether the compound affects the biological activity of CXCR4, LEC3 or EDNRA in a positive (agonist) or negative (antagonist) way as compared to the activity of CXCR4, LEC3 or EDNRA in the absence of the test compound.

[0159] In some embodiments, the compounds that modulate CXCR4, LEC3 or EDNRA expression or biological activity may be identified in an *in vitro* cell assay in which a test compounds is incubated with a cell expressing a CXCR4, LEC3 or EDNRA polypeptide or having a CXCR4, LEC3 or EDNRA polynucleotide and determining the effect the test compound has on CXCR4, LEC3 or EDNRA expression or biological activity. Modulators of CXCR4, LEC3 or EDNRA activity will be therapeutically useful in treatment of diseases involving angiogenesis. Compounds identified as modulating CXCR4, LEC3 or EDNRA expression of biological activity *in vitro* may be further tested *vivo* to confirm relevant and effective activity.

[0160] Test compounds contemplated by the invention include compounds from chemical libraries, including natural products and/or synthetic products from combinatorial chemical synthesis. Such compounds may include random peptides, oligonucleotides, or organic molecules.

[0161] In some embodiments, transgenic zebrafish carrying a CXCR4, LEC3 or EDNRA promoter driven-GFP transgene are used to detect whether a compound, small molecule or gene product has regulatory activity on the promoter of the CXCR4, LEC3 or EDNRA gene, resulting in altered expression of the GFP. Increased or decreased activity will be detected by the fluorescent signal generated within the living zebrafish, thus provide an *in vivo* model for screening biologically active drugs, small molecules and gene products.

[0162] The compounds identified by the screening methods may be used in the methods of the invention to promote or inhibit angiogenesis, alone or in combination with the compounds that promote or inhibit VEGF.

[0163] The following examples are merely illustrative of the invention and are not to be construed as limiting the invention in any way.

EXAMPLES

Example 1

[0164] **Fish Stocks:** *Danio rerio*: Florida wildtype strain (Lancaster, Pennsylvania) and Longfin strain (Scientific Hatcheries, Huntington Beach, California).

[0165] Zebrafish antisense CXCR4, LEC3 and EDNRA morpholino oligonucleotides (synthesized from GeneTools, LLC, Philomath, Oregon) had the following sequences: CXCR4 translation inhibition antisense morpholino: 5'-taagccatctctaaaagacttctcc-3' (SEQ ID NO:25); LEC3 Splicing antisense morpholino: 5'-cgcccgatctcatctacagaaagag-3' (SEQ ID NO:24); EDNRA translation inhibition antisense morpholino: 5'-gccattgcagaacactggccgctct-3' (SEQ ID NO:26).

[0166] For LEC3, the portion of the human Lec3 protein that spans the splice junction was identified as shown below:

Human Lec3 (or named Latrophilin 3) protein, NP_056051, 1240 aa (SEQ ID NO:4 (the boldface amino acids and underlined amino acids are the adjacent exons corresponding to the zebrafish exons) >gi|14149677|ref|NP_056051.1| Lec3 or latrophilin 3 [Homo sapiens]

MWPSQLLIFMMLLAPIIHAFSRAPIPMAVVRRELSCESYPIELRCPGTDVIMIESANYGRTDDKICDSDP
AQMENIRCYLPDAYKIMSQRCNNRTQCAVVAGPDVFPDPCPGTYKYLEVQYECVPYKVEQKVFLCPGLLK
GVYQSEHLFESDHQSGAWCKDPLQASDKIYYMPWTPYRTDTLTEYSSKDDFIAGRPTTTYKLPHRVDGTG
FVVYDGALFFNKERTRNIVKFDLRTRIKSGEAIIANANYHDTSPYRWGGKSDIDLAVDENGLWVIYATEQ
NNGKIVISQLNPYTLRIEGTWDTAYDKRSASNAFMICGILYVVKSVYEDDDNEATGNKIDYIYNTDQSKD
SLVDVPFPNSYQYIAAVDYNPRDNLLYVWNNYHVVKYSLDFGPLDSRSGQAHHGQVSYISPPIHLDSELE
RPSVKDISTTGPLGMGSTTTSTTLRTTTLSPGRSTTPSVSGRRNRSTSTPSPAVEVLDDMTTHLPSASSQ
IPALEESCEAVEAREIMWFKTRQGQIAKQPCPAGTIGVSTYLCLAPDGIWDP**QGPDLSNCSSPWVNHITQ**
KLKSGETAANIARELAEQTRNHLNAGDITYSVRAMDQLVGLLDVQLRNLTPGGKDSAARSLNKLQKRERS
CRAYVQAMVETVNNLLQPQALNAWRDLTTSDQLRAATMLLHTVEESAFVLADNLLKTDIVRENTDNIKLE
VARLSTEGNLEDLKFPENMGHGSTIQLSANTLKQNGRNGEIRVAFVLYNNLGPYLSTENASMKLGTEALS
TNHSVIVNSPVITAAINKEFSNKVYLADPVVFTVKHIKQSEENFNPNCSFWSYSKRTMTGYWSTQGCRLL
TTNKTHTTCSCNHLTNFAVLMAHVEVKHSDAVHDLLLDVITWVGILLSLVCLLICIFTFCFFRGLQSDRN
TIHKNLCISLFVAELLFLIGINRTDQPIACAVFAALLHFFFLAAFTWMFLEGVQLYIMLVEVFESEHSRR
KYFYLVGYGMPALIVAVSAAVDYRSYGTDKVCWLRLDTYFIWSFIGPATLIIMLNVIFLGIALYKMFHHT
AILKPESGCLDNINYEDNRPFIKSWVIGAIALLCLLGLTWAFGLMYINESTVIMAYLFTIFNSLQGMFIF
IFHCVLQKKVRKEYGKCLRTHCCSGKSTESSIGSGKTSGSRTPGRYSTGSQSRIRRMWNDTVRKQSESSF
ITGDINSSASLNREPYRETSMGVKLNIAYQIGASEQCQGYKCHGYSTTEW

[0167] The portion of the protein that spans the splice junction in the human Lec3 was compared to the same portion of the zebrafish Lec3 sequence and was found to have high homology (shown below where conservative substitutions are shown as a "+"):

```
Human Lec3 543th_aa QGPDLSNCSSPWVNHITQKLKSGETAANIARELAEQTRN 581 (SEQ ID NO:27)
                    +GPDLSNC+SPW NHITQ+++SGETAA +ARELAEQT+
Zebrafish Lec3      KGPDLSNCTSPWTNHITQRMRSGETAAIVARELAEQTKG____(SEQ ID NO:28)
```

[0168] An alignment of the cDNA (SEQ ID NO:29) and amino acid (SEQ ID NO:10) sequences of the zebrafish Lec3 shows the introns, exons and spliced portion:

```
CTTGGCTACTGGGACCCGAAGGGACCAGATCTGAGCAACTGCACCTCACCG
L  G  Y  W  D  P  K  G  P  D  L  S  N  C  T  S  P
TGGACCAATCATATCACTCAAAGAgtgagtaaaaacatgacaccatgggaa
W  T  N  H  I  T  Q  R
atatgctaatggagtatcagccaatttaaaaacattcagtggagagcgata
tgcctatagcaaatattcttaaagcataatttggcctccagctttttaac
```

aaagcacaaaagcttatcacaaagagcagcctgtagcgaaacagcttagag
gaaatttagtgtacaacaaaatgagccaaaattcaactgtcaagatcagcc
gcaatttgtatcactttgtggcaaaattacgtagggacgacagtgcagcca
aaagcgtcgtgcgacagcagcttaaaataaagtggcacgtgtgtatgtgtg
tgtgtgctgcgagagtgcatgtgtgtgtaaagtgcaatgatgactgaagct
taacagtaatgtaaaatggtttcctttgtaccacagagagcaaatcggaaa
ataggcagaatcacaaatctatgaagccatttgttcatccacttattcgac
catcaagggacagagcgaataaaatcaatatacacacacgtacatatctct
(continue but different frame translation)

***ctcgctctctctttctctttctgtag*ATGAGATCGGGCGAAACTGCTGCTA**

M R S G E T A A I

TAGTAGCCCGGGAATTGGCTGAACAGACAAAGGGAGAGTTGAGACCCGGAG

V A R E L A E Q T K G E L R P G D

ATGTCCCATCAACAGTCAAAGCCATGGCACAGCTGGTGGAACTACTGGACG

V P S T V K A M A Q L V E L L D V
TGCAGCTGCGAAACCTCACCCCTGGGGGAAAAGACACCGCCGCACGCAGTT
Q L R N L T P G G K D T A A R S L
TAAACAAGGTACACGAGCTGTTAGCAAACCACACGCACACTG
N K V H E L L A N H T H T

[0169] The splice inhibition morpholino was designed to inhibit splicing of the two exons. Below is shown the zebrafish Lec3 genomic DNA sequence showing one exon in bold and the adjoining exon with underlining. The sequence of the morpholino targeted sequence is shown in capital italics.

AAGGGACCAGATCTGAGCAACTGCACCTCACCGTGGACCAATCATATCACTCAAAGAGTGAGTAAAAACATGACACC
ATGGGAAATATGCTAATGGAGTATCAGCCAATTTAAAAACATTCAGTGGAGAGCGATATGCCTATAGCAAATATTCT
TAAAGCATAATTTGGCCTCCAGCTTTTTTAACAAAGCACAAAAGCTTATCACAAAGAGCAGCCTGTAGCGAAACAGC
TTAGAGGAAATTTAGTGTACAACAAAATGAGCCAAAATTCAACTGTCAAGATCAGCCGCAATTTGTATCACTTTGTG
GCAAAATTACGTAGGGACGACAGTGCAGCCAAAAGCGTCGTGCGACAGCAGCTTAAAATAAAGTGGCACGTGTGTAT
GTGTGTGTGTGCTGCGAGAGTGCATGTGTGTGTAAAGTGCAATGATGACTGAAGCTTAACAGTAATGTAAAATGGTT
TCCTTTGTACCACAGAGAGCAAATCGGAAAATAGGCAGAATCACAAATCTATGAAGCCATTTGTTCATCCACTTATT
CGACCATCAAGGGACAGAGCGAATAAAATCAATATACACACACGTACATATCTCTCTCGCTCT*CTCTTTCTCTTTCT*
*GTAGATGAGATCGGGCG*aaactgctgctatagtagcccgggaattggctgaacagacaa

[0170] Thus, the morpholino for the targeted area is the reverse-complement of the sequence shown in italics, or 5'-cgcccgatctcatctacagaaagag-3' (SEQ ID NO:24).

[0171] For the CXCR4 translation inhibition morpholino, a morpholino was designed to span the translation initiation site. The targeted sequence for the morpholino is shown below in boldface (the initiating methionine is shown in capital letters):

Zebrafish CXCR4a (SEQ ID NO:32) polynucleotide (NM_131882) and translation (SEQ ID NO:33) (1..56th 5'UTR; 57-1139th open reading frame, start codon in capital letters, boldface = morpholino targeted sequence):

```
  1 aagaaaaata ctgaagactt gctggagact gaaggagctg gagaagtctt ttagag  56
 57 ATGgcttattacggacacatcgtctttgaagatgatttatcagct
    M  A  Y  Y  G  H  I  V  F  E  D  D  L  S  A
102 gataacagctctgaattcggctcgggggacatcggagccaacttt
```

```
     D  N  S  S  E  F  G  S  G  D  I  G  A  N  F
 147 gaggtcccgtgtgatgtggaggtcagtcacgacttccagaggatc
     E  V  P  C  D  V  E  V  S  H  D  F  Q  R  I
 192 tttctcccaacggtgtacgggatcatatttgctttgggtcttatc
     F  L  P  T  V  Y  G  I  I  F  A  L  G  L  I
 237 gggaacggactggttgttctggtaatgggttgccagaagaaatcc
     G  N  G  L  V  V  L  V  M  G  C  Q  K  K  S
 282 agaaccatgacagacaagtaccgtctgcacctctcagtggcggac
     R  T  M  T  D  K  Y  R  L  H  L  S  V  A  D
 327 ctcctgtttgtgctcactctgccattctgggctgtggacgtggcc
     L  L  F  V  L  T  L  P  F  W  A  V  D  V  A
 372 aaagactggtactttggagggttcatgtgcgtggccgtgcatatg
     K  D  W  Y  F  G  G  F  M  C  V  A  V  H  M
 417 atttacacggtaaacttgtacagcagcgtcctcatcctcgccttc
     I  Y  T  V  N  L  Y  S  S  V  L  I  L  A  F
 462 atcagtttggaccggtacctcgccgtagtgcgcgccacgaacagc
     I  S  L  D  R  Y  L  A  V  V  R  A  T  N  S
 507 cagggtccgaggaaacttctggccaaccgcatcatttacgtgggc
     Q  G  P  R  K  L  L  A  N  R  I  I  Y  V  G
 552 gtgtggctccccgccgcgctcctcactgtgcccgacttggtgttc
     V  W  L  P  A  A  L  L  T  V  P  D  L  V  F
 597 gccaaagcggagagcagcgccatccgcaccttctgcgagcgcata
     A  K  A  E  S  S  A  I  R  T  F  C  E  R  I
 642 tacccgcaggactcgtttgtcacctgggtggtcgctttccgcttc
     Y  P  Q  D  S  F  V  T  W  V  V  A  F  R  F
 687 cagcacatcctcgtgggcttcgtgcttcccgggctcgtgatcctc
     Q  H  I  L  V  G  F  V  L  P  G  L  V  I  L
 732 atctgctactgcatcatcatctccaagctgtcgcgcggctccaaa
     I  C  Y  C  I  I  I  S  K  L  S  R  G  S  K
 777 ggcacgcagaagcgcaaggcgctgaagaccaccgtggttctgatc
     G  T  Q  K  R  K  A  L  K  T  T  V  V  L  I
 822 gtctgctttttcgtctgctggttgccgtattgcggagggatcctg
     V  C  F  F  V  C  W  L  P  Y  C  G  G  I  L
 867 ctggacacgctgatgatgctggaggtgattccccacagctgcgag
     L  D  T  L  M  M  L  E  V  I  P  H  S  C  E
 912 ctggagcaggggctgcagaagtggatcttcgtgacggaagcgctc
     L  E  Q  G  L  Q  K  W  I  F  V  T  E  A  L
 957 gcgtactttcactgctgtctcaacccgatcctgtacgcctttctg
     A  Y  F  H  C  C  L  N  P  I  L  Y  A  F  L
1002 ggggtgaagttcaagaagtccgctcgcagcgctctgtctcccagc
     G  V  K  F  K  K  S  A  R  S  A  L  S  P  S
1047 cgggggtccagtcttaaaattctgtccaagaagaggacaggaatg
     R  G  S  S  L  K  I  L  S  K  K  R  T  G  M
1092 tcatccgtgtccacagaatctgaatcttccagctttcactctagt
     S  S  V  S  T  E  S  E  S  S  S  F  H  S  S
1137 taa 1139
     *  .
```

[0172] Thus, the morpholino for the targeted area is the reverse-complement of the sequence shown in boldface, or 5'-taagccatctctaaaagacttctcc-3' (SEQ ID NO:25).

[0173] For the EDNRA translation inhibition morpholino, a morpholino was designed to span the translation initiation site. The targeted sequence for the morpholino is shown below in boldface (the initiating methionine is shown in capital letters):

Zebrafish EDNRA polynucleotide (SEQ ID NO:35) (acc. # CK141648) and translation product (SEQ ID NO:12): (1..242th 5'UTR; 243-677th open reading frame, start codon in capital letters, boldface = morpholino targeted sequence)

```
  1 ctttggtgcg tcgtggcgtc cacatgggat tctgctagac tttgatagtt ggttaattac
 61 tcatatgata ccacacagat gtctgggggg ctctagccga atattcgtct ctcactaact
121 cactgactga ctgactgacg ggatcctggg gaaggcagac atttaaggag cgctgtgcta
181 aataacccta atcaggctct tatcacctca tctggaaatc aaagagcggc cagtgttctg
241 ca
243 ATGgccattacgacgctacaattgtttctgctaatggccgttctg
    M  A  I  T  T  L  Q  L  F  L  L  M  A  V  L

288 gccactggtggattatgtctgataaatggcacagaggaagcccag
    A  T  G  G  L  C  L  I  N  G  T  E  E  A  Q

333 gatgctttatatccaaactctactacctccaaaaccaacgtacac
    D  A  L  Y  P  N  S  T  T  S  K  T  N  V  H

378 aagggtttccagccccccacaaaaaaagacgcttcggttttcaat
    K  G  F  Q  P  P  T  K  K  D  A  S  V  F  N

423 atgaagcatccaccctgtagggatcccacttccatcaagctttat
    M  K  H  P  P  C  R  D  P  T  S  I  K  L  Y

468 tttaagtatatcaacacaatcatttcctgcatcgtgtttgtggtg
    F  K  Y  I  N  T  I  I  S  C  I  V  F  V  V

513 ggaatagtgggtaatgccactttattgaagattatttaccagaat
    G  I  V  G  N  A  T  L  L  K  I  I  Y  Q  N

558 aagtgtatgaggaacggacccaatgccctcatcgccagtctggct
    K  C  M  R  N  G  P  N  A  L  I  A  S  L  A

603 cttggagacctcatttacatcactatagacatccctataaatgtc
    L  G  D  L  I  Y  I  T  I  D  I  P  I  N  V

648 tacaagctgctggtcacaagtggccattga
    Y  K  L  L  V  T  S  G  H  *
```

[0174] Thus, the morpholino for the targeted area is the reverse-complement of the sequence shown in boldface, or 5'_gccattgcagaacactggccgctct-3' (SEQ ID NO:26).

Microinjection of mRNA and morpholino antisense oligo:

[0175] Capped sense RNA was synthesized using SP6 RNA polymerase and the mMESSAGE mMACHINE system (Ambion). For microinjection of mRNA or morpholino antisense oligos, zebrafish embryos were injected with 0.25 to 0.5 nl into the yolk at the early one-cell stage, subsequently incubated in 0.3x Danieau's medium at 28.5°C. Embryos were maintained in the above condition until they had reached bud stage or 24 hour-post-fertilization and then collected for total RNA preparation or fixed in 4% paraformaldehyde for whole-mount *in situ* hybridization.

***In situ* hybridization:**

[0176] The *flk in situ* construct was kindly provided by Dr. Brant Weinstein (Lawson, N.D. *et al.* (2003) *Genes Dev.* 17(11):1346-1351). The *in situ* hybridization procedure was modified from Leung, T. *et al.* (2003) *Development* 130(16):3639-3649. In brief, embryos were hybridized at 68°C overnight, then washed by 66%Hyb/33% 2X SSC for 30 minutes, then washed by 33% Hyb/66% 2X SSC for 30 minutes at 68°C, then by 2X SSC for 15 minutes at 68°C, followed by 0.2X SSC for 1 hour at 68°C. The hybridized probes were detected by NBT/BCIP staining (Roche). After color staining, the embryos were washed in 100% ethanol for 1 hour.

Identification of the Zebrafish *lec3, cxcr4 and ednra* genes

[0177] As a first step, we sought to identify the *lec3, cxcr4* and *ednra* specifically expressed in the developing vasculature of the zebrafish embryos. Using the human and/or mouse protein sequences to probe the zebrafish sequence database, we identified the zebrafish *lec3, cxcr4* and *ednr* genes (SEQ ID NOS:9, 7, and 11, respectively). The expression pattern of zebrafish *lec3, cxcr4* and *ednra* genes was very dynamic during embryonic development (**Figs. 9-11**). *lec3* was expressed in dorsal aorta of 1dpf zebrafish embryos where the intersomitic vessels will be formed by angiogenic sprouting in 24- and 28-hour-postfertilization (**Fig. 12**).

[0178] Similarly, *cxcr4a* was expressed in developing blood vessels at 1dpf. *cxcr4a* is expressed in the dorsal aorta, intersomitic vessels and cranial vessels in 28-hour-postfertilization zebrafish embryos (**Fig. 13**).

[0179] Expression of *ednra* was found in axial vasculature and developing vessels. Like *lec3*, *ednra* is expressed in the dorsal aorta where the intersomitic vessels will be formed by angiogenic sprouting in 24- and 28-hour-postfertilization zebrafish embryos (**Fig. 14**).

Targeted knockdown of zebrafish *lec3, cxcr4* and *ednra* inhibits angiogenesis

[0180] In order to uncover a possible role of *lec3, cxcr4* and/or *ednra* in angiogenesis, we used the morpholino antisense knockdown approach in the zebrafish embryos (Nasevicius A. and SC Ekker (2000) *Nat. Genet.* 26:216-220). When injected into zebrafish embryos, the morpholinos induced angiogenic defect, as revealed by molecular markers of the vascular endothelial cells (see below).

[0181] These inter-somitic vessels are particularly interesting because they develop by angiogenic sprouting from the dorsal aorta and cardinal vein, a process that closely resembles tumor angiogenesis (Bergers G, and LE Benjamin (2003) *Nature Rev. Cancer* 3:401-410).

Molecular analysis of *lec3, cxcr4* and *ednra* knockdown phenotypes

[0182] To examine the nature of the angiogenic defect in *lec3, cxcr4* and *ednra* knockdown embryos, we used the endothelial specific marker *flk1*, which encodes the VEGFR-2 receptor (*Flk-1*/KDR), to visualize the developing vasculature (Liao W. *et al.* (1997) *Development* 124:381-389). As shown by *in situ* hybridization of 1 dpf control embryos, the *flk1* transcript was abundantly expressed in the cranial vessels, axial vasculature (including the dorsal aorta and cardinal vein), and more importantly, in the inter-somitic vessels sprouting from the dorsal aorta (**Fig. 8**). *In situ* hybridization analysis of *lec3, cxcr4* and *ednra* knockdown embryos at the same stage revealed expression of the *flk1* transcript along the dorsal aorta and cardinal vein, indicating that endothelial cell differentiation along the axial vasculature was not defective. However, *lec3, cxcr4* and *ednra* knockdown embryos showed greatly reduced staining of the intersomitic vessels sprouting from the axial vasculature (**Fig. 12-14**), suggesting that the defect was specific to the angiogenic process.

***cxcr4, lec3*, and *ednra* genetically interacts with the *vegf* pathway**

[0183] Vascular endothelial growth factor (VEGF) is a major mediator for embryonic and tumor angiogenesis (Tammela *et al.*, 2005; Ferrara *et al.*, 2005). The *lec3, cxcr4* and *ednra* knockdown phenotypes shared striking similarity with that of *vegf* knockdown embryos (Nasevicius A. *et al.* (2000) *Yeast* 17:294-301; Childs S. *et al.* (2002) *Development* 129:973-982), suggesting they may function in a common or converging pathway. Using the zebrafish model, we discovered that GPCR interacts with vegf signaling and both GPCR and VEGF may function in a common or converging pathway in angiogenesis. Here we provide an example to demonstrate a novel synergistic interaction between cxcr4a- and vegf-dependent pathways to regulate the angiogenic process in a whole animal model (**Fig. 15**). At a sub-effective dosage of either morpholino alone, there was no effect on angiogenesis (**Fig. 15 E-F**, vegf knockdown; **Fig. 15 C-D**, cxcr4a knockdown). However, the simultaneous inhibition of both vegf and cxcr4a at the same dose significantly eliminated the process of angiogenic sprouting in the zebrafish model (**Fig. 15 G-H**). Our findings provide a new opportunity for co-targeting this GPCR- and VEGF-dependent pathways to synergistically block pathological angiogenesis, which may lead to a safer and more efficacious therapeutic regimen to fight cancer.

Example 2

Anti-angiogenesis therapy for retinopathy

[0184] Recently, the mouse model of oxygen-induced retinopathy has been used to recapitulate the human disease, retinopathy of prematurity (ROP), a retinal, neo-vascularizing disease affecting premature infants. Use of this mouse model to test novel anti-angiogenic compounds developed in our laboratory offers several important advantages compared to other species: (1) the mouse model of oxygen-induced retinopathy is a well-validated and highly-reproducible method leading to abnormal development of blood vessels in the retina; (2) the retina is an excellent organ for studying this process since the growth of abnormal blood vessels can be monitored by fundoscopy and the entire retinal vasculature can be viewed in flat-mounted retinal preparations; and, (3) the restricted delivery to the retina allows the efficacy of these novel anti-angiogenic compounds to be assessed, and at the same time, limits any potential toxicity of these compounds to the retina.

Mouse model of oxygen-induced retinopathy

[0185] This model serves as a paradigm for the human condition retinopathy of prematurity. On postnatal day P7, up to two, nursing dams and their litters are placed in an animal cage in a sealed container ventilated by a mixture of oxygen and compressed air to a final oxygen concentration of 75% ± 2% (ProOx 110 System, Biospherix, Ltd). This exposure to hyperoxia blocks vascular development in the retina. On postnatal day P12, the animals are returned to room air. This return to room air induces new vascular development (neovascularization) in the retina resulting from the relative state of hypoxia. To monitor oxygen-induced changes in the retina, animals are sacrificed by lethal injection of sodium pentobarbital (120 mg/kg) on P7, prior to oxygen exposure; on P12, immediately following oxygen exposure; and on P17, the time of maximal retinal neovascularization. The eyes from one litter are processed for histological analysis to confirm abnormal blood vessel development in the retina, while the eyes from another litter are processed for RNA and protein analyses to identify gene expression changes in the retina. This experiment is repeated three times to assess the reproducibility of results.

Uptake of fluorescent phosphorodiamidate morpholino oligomer (PMO)

[0186] A comparison is made of the uptake of fluorescently labeled PMO alone, or PMO conjugated with peptide (PMO-pep) into retinal cells. On postnatal day P12, neonatal mice receive an intraviteral injection of PMO alone in one eye, or PMO-pep in the contralateral eye. Three hours after injection, animals are euthanized by lethal injection of sodium pentobarbital (120 mg/kg). Cryosections of the eyes are prepared to visualize the location of the injected

substance by fluorescence microscopy. This experiment is repeated three times to assess the reproducibility of the results. The results of this study will show whether peptide conjugation confers a delivery advantage compared to PMO alone.

Effectiveness of PMO and PMO conjugates

[0187] A study is performed to assess the effectiveness of PMO- and PMOpep-dependent inhibition of *lec3*, *ednra*, or *cxcr4* expression to suppress retinal neovascularization in the mouse model of oxygen-induced retinopathy. Retinopathy will be produced as described above by a five-day exposure of neonatal mice to 75% oxygen. On postnatal day P12, which is the day when the oxygen exposure ends, these mice with bilateral retinopathy will receive intravitreal injections of PMO or PMO-pep targeting *lec3*, *ednra*, or *cxcr4* in one eye. Because intravitreal injection itself can inhibit neovascularization to some extent, these mice will also injections of 6-base mismatch, control PMO or PMO-pep in the contralateral eye. At postnatal day P17, which corresponds to period of maximal neovascularization, these mice will be euthanized and their eyes processed for histological analysis. Areas of neovascularization, ischemia, and normal vasculature will be quantified by an observer blinded to the treatment. The efficacy of treatment will be measured as the difference between the area of neovascularization in the treated eye versus the control eye. The finding that targeted knockdown of *lec3*, *ednra*, or *cxcr4* can inhibit pathologic neovascularization without blocking physiologic angiogenesis will identify these genes as a potential therapeutic target for a variety of vascular- related retinopathies.

Combination therapy with *vegf*

[0188] A study is performed to compare the relative effectiveness of PMO-dependent inhibition of gng2, *lec3*, *ednra*, or *cxcr4* in combination with *vegf* or *gng2*, *lec3*, *ednra*, or *cxcr4* /*vegf* expression to suppress retinal neovascularization. These experiments will be performed as described above. The finding that targeted knockdown of *gng2*, *lec3*, *ednra*, or *cxcr4* is as efficacious or more than corresponding knockdown of *vegf* will identify a second potential target for drug development. This will be important because not all retinopathies show similar dependency on the *vegf* pathway. Therefore, the identification of additional targets for drug development will expand the treatment options to a wider range of patients and retinal disorders. Likewise, the finding that simultaneous knockdown of , *lec3*, *ednra*, or *cxcr4* in combination with *vegf* provides greater efficacy and lower side effects than either treatment alone will allow optimization of the treatment options.

What is Claimed:

1. A method for promoting angiogenesis in an animal comprising administering to an animal an isolated expression vector comprising a polynucleotide selected from:

(a) SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 and SEQ ID NO:11, and

(b) a sequence encoding a polypeptide having the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10 and SEQ ID NO:12.

2. An antibody that specifically binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:10 with high affinity.

3. An antibody that specifically binds to an extracellular portion of a polypeptide comprising the amino acid sequence of SEQ ID NO:4 with high affinity.

4. The antibody of claim 2, wherein said antibody is monoclonal.

5. The antibody of claim 2, wherein said antibody is polyclonal.

6. The antibody of claim 3, wherein said antibody is monoclonal.

7. The antibody of claim 3, wherein said antibody is polyclonal.

8. A method of producing a zebrafish LEC3, CXCR4 and/or EDNRA comprising culturing the host cell comprising an expression vector, wherein said expression vector comprises the sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11 operably linked to an expression control sequence, under culture conditions suitable for the expression of zebrafish LEC3, CXCR4 and/or EDNRA, and isolating said LEC3, CXCR4 and/or EDNRA.

9. A therapeutic composition comprising, an isolated polynucleotide comprising a nucleic acid sequence encoding LEC3, CXCR4 and/or EDNRA and a pharmaceutically acceptable carrier.

10. The therapeutic composition of claim 9 wherein said nucleic acid sequence encodes the polypeptide of SEQ ID NO:4 or SEQ ID NO:10.

11. A therapeutic composition comprising the antibody of claim 3 and a pharmaceutically acceptable carrier.

12. A therapeutic composition comprising a polypeptide comprising at least one amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, and SEQ ID NO:12 and a pharmaceutically acceptable carrier.

13. A method of treating an angiogenesis-related disease comprising administering to a patient in need of such treatment a polynucleotide of that inhibits the expression of LEC3 in an amount sufficient to inhibit angiogenesis.

14. The method of claim 13, wherein the angiogenesis-related disease is selected from the group consisting of angiogenesis-dependent cancers; benign tumors; rheumatoid arthritis; psoriasis; ocular angiogenesis diseases; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; wound granulation; intestinal adhesions, atherosclerosis, scleroderma, hypertrophic scars, cat scratch disease and *Helicobacter pylori* ulcers.

15. The method of claim 13, wherein the angiogenesis-related disease is angiogenesis-dependent cancer.

16. The method of claim 13 wherein said angiogenesis-related disease is an angiogenesis-dependent tumor comprising, and wherein said polynucleotide is administered in an amount sufficient to cause tumor regression.

17. A method of promoting angiogenesis in an animal in need thereof, comprising administering to an animal an effective amount of a polynucleotide encoding a LEC3, CXCR4 and/or EDNRA polypeptide.

18. The method of claim 17 wherein said LEC3, CXCR4 and/or EDNRA polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, and SEQ ID NO:12.

19. The method of claim 17 wherein said LEC3 polypeptide comprises the amino acid sequence of SEQ ID NO:4 or SEQ ID NO:10.

20. A method of treating an angiogenesis-related disease comprising administering to a patient in need of such treatment a first polynucleotide that inhibits the expression of *lec3, cxcr4,* and/or *ednra* and a second polynucleotide that inhibits the expression of *vegf,* wherein said first polynucleotide and said second polynucleotide are provided in an sufficient to inhibit angiogenesis.

21. The method of claim 20, wherein said first polynucleotide comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11 and combinations thereof.

22. The method of claim 20, wherein said second polynucleotide comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:59, SEQ ID NO:61, SEQ ID NO:69, SEQ ID NO:71, SEQ ID NO:65, SEQ ID NO:63, SEQ ID NO:73, SEQ ID NO:75, SEQ ID NO:67 and combinations thereof.

23. The method of claim 20, wherein the angiogenesis-related disease is angiogenesis-dependent cancer.

24. A method of treating a patient with an angiogenesis-dependent tumor comprising administering to a patient in need of such treatment a first compound that inhibits the expression or function of *lec3, cxcr4* and/or *ednra* and a second compound that inhibits the expression or function of *vegf* wherein said first compound and said second compound are provided in amount sufficient to cause tumor regression.

25. A method of promoting angiogenesis in an animal in need thereof comprising administering to an animal an effective amount of a first polynucleotide encoding a LEC3,

CXCR4 and/or EDNRA polypeptide and an effective amount of a second polynucleotide encoding a VEGF polypeptide.

26. The method of claim 25 wherein said LEC3, CXCR4 and/or EDNRA polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:6, SEQ ID NO:12 and SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22.

27. The method of claim 25 wherein said VEGF polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:60, SEQ ID NO:62, SEQ ID NO:70, SEQ ID NO:72, SEQ ID NO:66, SEQ ID NO:64, SEQ ID NO: 74, SEQ ID NO:76, and SEQ ID NO:68.

28. The method of claim 25 wherein said LEC3 polypeptide comprises the amino acid sequence of SEQ ID NO:4 or SEQ ID NO:10 and said VEGF polypeptide comprises the amino acid sequence of SEQ ID NO:60, SEQ ID NO:62, SEQ ID NO:70, SEQ ID NO:72, SEQ ID NO:66, SEQ ID NO:64, SEQ ID NO: 74, SEQ ID NO:76, and SEQ ID NO:68.

29. The method of claim 24 wherein said second compound is an antibody that specifically binds VEGF.

30. The method of claim 24 wherein said first compound is an antibody that specifically binds LEC3.

31. A method of treating an angiogenesis-related disease comprising administering to a patient in need of such treatment a first compound that inhibits the function of LEC3, CXCR4 and/or EDNRA and a second compound that inhibits the function of VEGF, wherein said first compound and said second compound are provided in an sufficient to inhibit angiogenesis.

32. The method of claim 31 wherein said first compound is an antibody that specifically binds LEC3.

33. The method of claim 31 wherein said second compound is an antibody that specifically binds VEGF.

34. The method of claim 32 wherein said antibody binds to at least one extracellular portion of the LEC3 protein.

35. A method for identifying a compound that inhibits LEC3, CXCR4 and/or EDNRA activity comprising contacting a test compound with a LEC3, CXCR4 and/or EDNRA polypeptide and determining whether said test compound inhibits the activity of LEC3, CXCR4 and/or EDNRA, wherein a test compound that inhibits the activity of LEC3, CXCR4 and/or EDNRA is identified as an antagonist of LEC3, CXCR4 and/or EDNRA.

36. The method of claim 35 wherein said biological activity of LEC3, CXCR4 and/or EDNRA is measured by binding of said test compound to LEC3, CXCR4 and/or EDNRA.

37. The method of claim 35 wherein said biological activity of LEC3, CXCR4 and/or EDNRA is measured by inhibition of angiogenesis in a model system.

38. The method of claim 37 wherein said model system is a zebrafish development system.

39. The method of claim 37 wherein said model system is a transgenic animal system.

40. The method of claim 37 wherein said model system is an *in vitro* cell system.

41. A method of inhibiting angiogenesis comprising administering to a cell an effective amount of a cell permeable peptide that inhibits the biological function of LEC3, CXCR4 and/or EDNRA.

42. The method of claim 41 further comprising the administration of a compound that inhibits the expression or biological function of VEGF.

43. A method of promoting angiogenesis comprising administering to a subject an effective amount of a compound selected from the group consisting of:

(a) an isolated expression vector comprising a polynucleotide having a nucleotide sequence of SEQ ID NO:41, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:47, SEQ ID

(b) an isolated expression vector comprising a polynucleotide having a sequence encoding a polypeptide having the amino acid sequence of SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO:52, SEQ ID NO:54, SEQ ID NO:56, and SEQ ID NO:58;

(c) a polypeptide comprising the amino acid sequence of SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO:52, SEQ ID NO:54, SEQ ID NO:56, and SEQ ID NO:58.

44. A method of treating an angiogenesis-related disease comprising administering to a patient in need of such treatment an antibody that binds to SDF-1 or ET-1 in an amount sufficient to inhibit angiogenesis.

45. The method of claim 44, wherein the angiogenesis-related disease is selected from the group consisting of angiogenesis-dependent cancers; benign tumors; rheumatoid arthritis; psoriasis; ocular angiogenesis diseases; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; wound granulation; intestinal adhesions, atherosclerosis, scleroderma, hypertrophic scars, cat scratch disease and *Helicobacter pylori* ulcers.

46. The method of claim 44, wherein the angiogenesis-related disease is angiogenesis-dependent cancer.

47. The method of claim 44 wherein said angiogenesis-related disease is an angiogenesis-dependent tumor comprising, and wherein said antibody is administered in an amount sufficient to cause tumor regression.

48. The method of claim 44 wherein said SDF-1 comprises an amino acid sequence of SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:48, or SEQ ID NO:50.

49. The method of claim 44 wherein said ET-1 comprises an amino acid sequence of, SEQ ID NO:52, SEQ ID NO:54, SEQ ID NO:56, or SEQ ID NO:58.

50. A method for preventing or treating retinopathy comprising administering to the eye

a CXCR4, LEC3 and/or EDNRA polypeptide that inhibits the expression or function of a protein selected from the group consisting of LEC3, EDNRA and CXCR4 in an amount sufficient to inhibit angiogenesis.

51. The method of claim 50 wherein said polynucleotide is an antisense oligonucleotide or a phosphorodiamidate morpholino oligomer.

52. The method of claim 51 wherein said polynucleotide is conjugated to a peptide to promote cellular uptake of said polynucleotide.

53. The method of claim 50 wherein said retinopathy is age-related macular degeneration, diabetic retinopathy, or retinopathy of prematurity.

54. The method of claim 50 wherein said antibody is an antibody that specifically binds a CXCR4, LEC3 and/or EDNRA polypeptide.

Membrane Structure of Human CXCR4

```
MSIPLPLLQIYTSDNYTEEMGSGDYDSMKEPCFREENANFNKIFLPTIYSIIFLTGIVGNGLVILVMGYQKK
OOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOMMMMMMMMMMMMMMMMMMMMMiii

LRSMTDKYRLHLSVADLLFVITLPFWAVDAVANWYFGNFLCKAVHVIYTVNLYSSVLILAFISLDRYLAIVH
iiiiiiiiiMMMMMMMMMMMMMMMMMMoooooooooooooooooMMMMMMMMMMMMMMMMMMMMMiii

ATNSQRPRKLLAEKVVYVGVWIPALLLTIPDFIFANVSEADDRYICDRFYPNDLWVVVFQFQHIMVGLILPG
iiiiiiiiiiiiiiiMMMMMMMMMMMMMMMMMMoooooooooooooooooooooooooMMMMMMMMMMM

IVILSCYCIIISKLSHSKGHQKRKALKTTVILILAFFACWLPYYIGISIDSFILLEIIKQGCEFENTVHKWI
MMMMMMMMMMiiiiiiiiiiiiiiiiiiMMMMMMMMMMMMMMMMMMMMMoooooooooooooooooM

SITEALAFFHCCLNPILYAFLGAKFKTSAQHALTSVSRGSSLKILSKGKRGGHSSVSTESESSSFHS
MMMMMMMMMMMMMMMMMMMMMiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiii
```

Figure 1

CXCR4

```
Danio:   270 QKKSRTMTDKYRLHLSVADLLFVLTLPFWAVDVAKDWYFGGFMCVAVHMIYTVNLYSSVL 449
             QKK R+MTDKYRLHLSVADLLFV+TLPFWAVD +  WYFG F+C AVH+IYTVNLYSSVL
Human:   284 QKKLRSMTDKYRLHLSVADLLFVITLPFWAVDAVANWYFGNFLCKAVHVIYTVNLYSSVL 463

Danio:   450 ILAFISLDRYLAVVRATNSQGPRKLLANRIIYVGVWLPAALLTVPDLVFAKAESSAIRTF 629
             ILAFISLDRYLA+V ATNSQ PRKLLA +++YVGVW+PA LLT+PD +FA +     R
Human:   464 ILAFISLDRYLAIVHATNSQRPRKLLAEKVVYVGVWIPALLLTIPDFIFANVSEADDRYI 643

Danio:   630 CERIYPQDSFV                                                  662
             C+R YP D +V
Human:   644 CDRFYPNDLWV                                                  676
```

Figure 2

Membrane Structure of LEC3

MWPSQLLIFMMLLAPIIHAFSRAPIPMAVVRRELSCESYPIELRCPGTDVIMIESANYGRTDDKICDSDPAQ
OOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOO

MENIRCYLPDAYKIMSQRCNNRTQCAVVAGPDVFPDPCPGTYKYLEVQYECVPYKVEQKVFLCPGLLKGVYQ
OOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOO

SEHLFESDHQSGAWCKDPLQASDKIYYMPWTPYRTDTLTEYSSKDDFIAGRPTTTYKLPHRVDGTGFVVYDG
OOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOO

ALFFNKERTRNIVKFDLRTRIKSGEAIIANANYHDTSPYRWGGKSDIDLAVDENGLWVIYATEQNNGKIVIS
OOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOO

QLNPYTLRIEGTWDTAYDKRSASNAFMICGILYVVKSVYEDDDNEATGNKIDYIYNTDQSKDSLVDVPFPNS
OOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOO

YQYIAAVDYNPRDNLLYVWNNYHVVKYSLDFGPLDSRSGQAHHGQVSYISPPIHLDSELERPSVKDISTTGP
OOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOO

LGMGSTTTSTTLRTTTLSPGRSTTPSVSGRRNRSTSTPSPAVEVLDDMTTHLPSASSQIPALEESCEAVEAR
OOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOO

EIMWFKTRQGQIAKQPCPAGTIGVSTYLCLAPDGIWDPQGPDLSNCSSPWVNHITQKLKSGETAANIARELA
OOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOO

EQTRNHLNAGDITYSVRAMDQLVGLLDVQLRNLTPGGKDSAARSLNKLQKRERSCRAYVQAMVETVNNLLQP
OOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOO

Figure 3A

Membrane Structure of LEC3 (continued)

```
QALNAWRDLTTSDQLRAATMLLHTVEESAFVLADNLLKTDIVRENTDNIKLEVARLSTEGNLEDLKFPENMG
OOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOO

HGSTIQLSANTLKQNGRNGEIRVAFVLYNNLGPYLSTENASMKLGTEALSTNHSVIVNSPVITAAINKEFSN
OOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOO

KVYLADPVVFTVKHIKQSEENFNPNCSFWSYSKRTMTGYWSTQGCRLLTTNKTHTTCSCNHLTNFAVLMAHV
OOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOO

EVKHSDAVHDLLLDVITWVGILLSLVCLLICIFTFCFFRGLQSDRNTIHKNLCISLFVAELLFLIGINRTDQ
OOOOOOOOOOOOOOMMMMMMMMMMMMMMMMMMMMMiiiiiiiiiiiiMMMMMMMMMMMMMMMMMMoooo

PIACAVFAALLHFFFLAAFTWMFLEGVQLYIMLVEVFESEHSRRKYFYLVGYGMPALIVAVSAAVDYRSYGT
ooooMMMMMMMMMMMMMMMMMMMMMiiiiiiiiiiiiiiiiiMMMMMMMMMMMMMMMMMMoooooooo

DKVCWLRLDTYFIWSFIGPATLIIMLNVIFLGIALYKMFHHTAILKPESGCLDNINYEDNRPFIKSWVIGAI
ooooooooooMMMMMMMMMMMMMMMMMMMMMiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiMMMMMM

ALLCLLGLTWAFGLMYINESTVIMAYLFTIFNSLQGMFIFIFHCVLQKKVRKEYGKCLRTHCCSGKSTESSI
MMMMMMMMMMMMMMMMooMMMMMMMMMMMMMMMMMMMMMiiiiiiiiiiiiiiiiiiiiiiiiiii

GSGKTSGSRTPGRYSTGSQSRIRRMWNDTVRKQSESSFITGDINSSASLNREPYRETSMGVKLNIAYQIGAS
iiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiii

EQCQGYKCHGYSTTE
iiiiiiiiiiiiiii
```

Figure 3B

LEC3

```
Danio:   709 NLEDLKFPENMGHGSTIQLSANTLKQNGRNGEIRVAFVLYNNLGPYLSTENASMKLGTEA 768
             NL DL FP++ G++I LSANTLKQ GRNGEIR+A VLY NLG YLSTENAS++LG+EA
Human:     4 NLADLSFPQSGLTGNSIHLSANTLKQQGRNGEIRMALVLYKNLGQYLSTENASVRLGSEA 183

Danio:   769 LSTNHSVIVNSPVITAAINKEFSNKVYLADPVVFTVKHIKQSEENFNPNCSFWSYSKRTM 828
             N+S+IVNSPVITAAINK+ SNKVYL++PVVFTVKHI QSEENFNPNCSFWSYSKRTM
Human:   184 AYPNYSLIVNSPVITAAINKD-SNKVYLSEPVVFTVKHI-QSEENFNPNCSFWSYSKRTM 357

Danio:   829 TGYWSTQGCRLLTTNKTHTTCSCNHLTNFAVLMAHVEVKHSDAVHDLLLDVITWVGXXXX 888
             TG+WSTQ CRLL TN+THT+CSC HLTNFAVLMAHVEVK +D VH+LLLDVITWVG
Human:   358 TGFWSTQDCRLLGTNRTHTSCSCTHLTNFAVLMAHVEVKKADQVHELLLDVITWVGILLS 537

Danio:   889 XXXXXXXXXXXXXXRGLQSDRNT 911
                          RGLQSDRNT
Human:   538 LVCLLICIFTLNFFRGLQSDRNT 606
```

Figure 4

Zebrafish EDNRA

```
243 atggccattacgacgctacaattgtttctgctaatggccgttctg
     M  A  I  T  T  L  Q  L  F  L  L  M  A  V  L
288 gccactggtggattatgtctgataaatggcacagaggaagcccag
     A  T  G  G  L  C  L  I  N  G  T  E  E  A  Q
333 gatgctttatatccaaactctactacctccaaaaccaacgtacac
     D  A  L  Y  P  N  S  T  T  S  K  T  N  V  H
378 aagggtttccagccccccacaaaaaaagacgcttcggttttcaat
     K  G  F  Q  P  P  T  K  K  D  A  S  V  F  N
423 atgaagcatccaccctgtagggatcccacttccatcaagctttat
     M  K  H  P  P  C  R  D  P  T  S  I  K  L  Y
468 tttaagtatatcaacacaatcatttcctgcatcgtgtttgtggtg
     F  K  Y  I  N  T  I  I  S  C  I  V  F  V  V
513 ggaatagtgggtaatgccactttattgaagattatttaccagaat
     G  I  V  G  N  A  T  L  L  K  I  I  Y  Q  N
558 aagtgtatgaggaacggacccaatgccctcatcgccagtctggct
     K  C  M  R  N  G  P  N  A  L  I  A  S  L  A
603 cttggagacctcatttacatcactatagacatccctataaatgtc
     L  G  D  L  I  Y  I  T  I  D  I  P  I  N  V
648 tacaagctgctggtcacaagtggccattga 677
     Y  K  L  L  V  T  S  G  H  *
```

Figure 5

Membrane Structure of Human EDNRA

METLCLRASFWLALVGCVISDNPERYSTNLSNHVDDFTTFRGTELSFLVTTHQPTNLVLPSNGSMHNYCPQQ
OOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOO

TKITSAFKYINTVISCTIFIVGMVGNATLLRIIYQNKCMRNGPNALIASLALGDLIYVVIDLPINVFKLLAG
OOOOOOOOOMMMMMMMMMMMMMMMMMMMMMiiiiiiiiiiiiMMMMMMMMMMMMMMMMMMMMMMMMooooo

RWPFDHNDFGVFLCKLFPFLQKSSVGITVLNLCALSVDRYRAVASWSRVQGIGIPLVTAIEIVSIWILSFIL
ooooooooooMMMMMMMMMMMMMMMMMMMMMiiiiiiiiiiiiiiiiiiiMMMMMMMMMMMMMMMMMMMMM

AIPEAIGFVMVPFEYRGEQHKTCMLNATSKFMEFYQDVKDWWLFGFYFCMPLVCTAIFYTLMTCEMLNRRNG
MMoooooooooooooooooooooooooooooooooooooooMMMMMMMMMMMMMMMMMMMMMiiiiiiiii

SLRIALSEHLKQRREVAKTVFCLVVIFALCWFPLHLSRILKKTVYNEMDKNRCELLSFLLLMDYIGINLATM
iiiiiiiiiiiiiiiiiMMMMMMMMMMMMMMMMMMMMMoooooooooooooooooooMMMMMMMMMMMMMM

NSCINPIALYFVSKKFKNCFQSCLCCCCYQSKSLMTSVPMNGTSIQWKNHDQNNHNTDRSSHKDSM
MMMMMMMMMiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiii

Figure 6

EDNRA

```
Human:    66  HNYCPQQTKITSAFKYINTVISCTIFIVGMVGNATLLRIIYQNKCMRNGPNALIASLALG 125
              H  C   T I   FKYINT+ISC +F+VG+VGNATLL+IIYQNKCMRNGPNALIASLALG
Danio:    429 HPPCRDPTSIKLYFKYINTIISCIVFVVGIVGNATLLKIIYQNKCMRNGPNALIASLALG 608

Human:    126 DLIYVVIDLPINVFKLL 142
              DLIY+ ID+PINV+KLL
Danio:    609 DLIYITIDIPINVYKLL 659
```

Figure 7

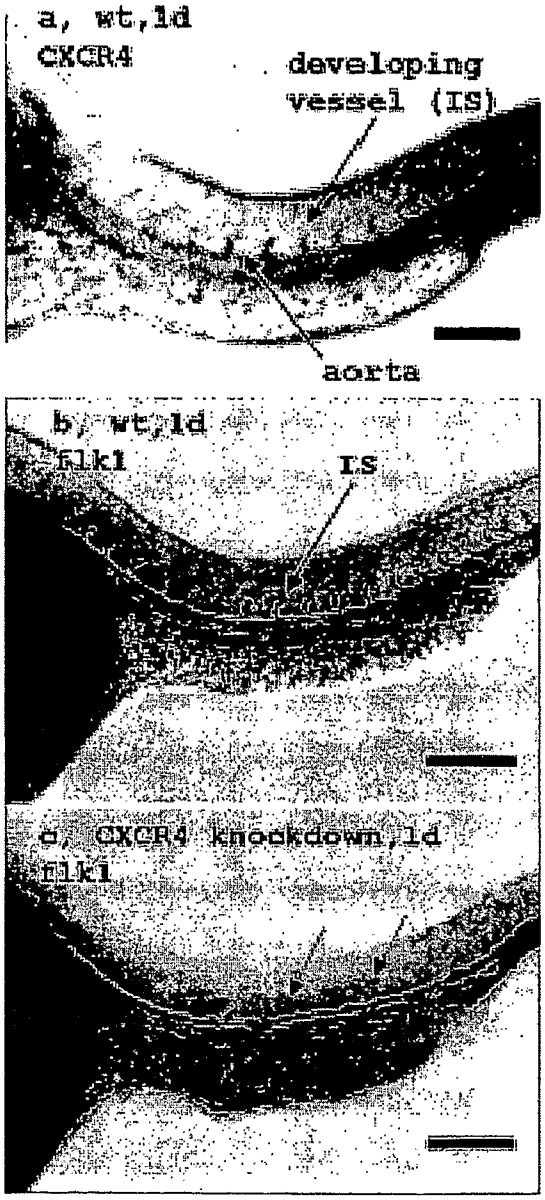
a, wt,1d
CXCR4
developing
vessel (IS)
aorta
b, wt,1d
flk1
IS
c, CXCR4 knockdown,1d
flk1

Figure 8

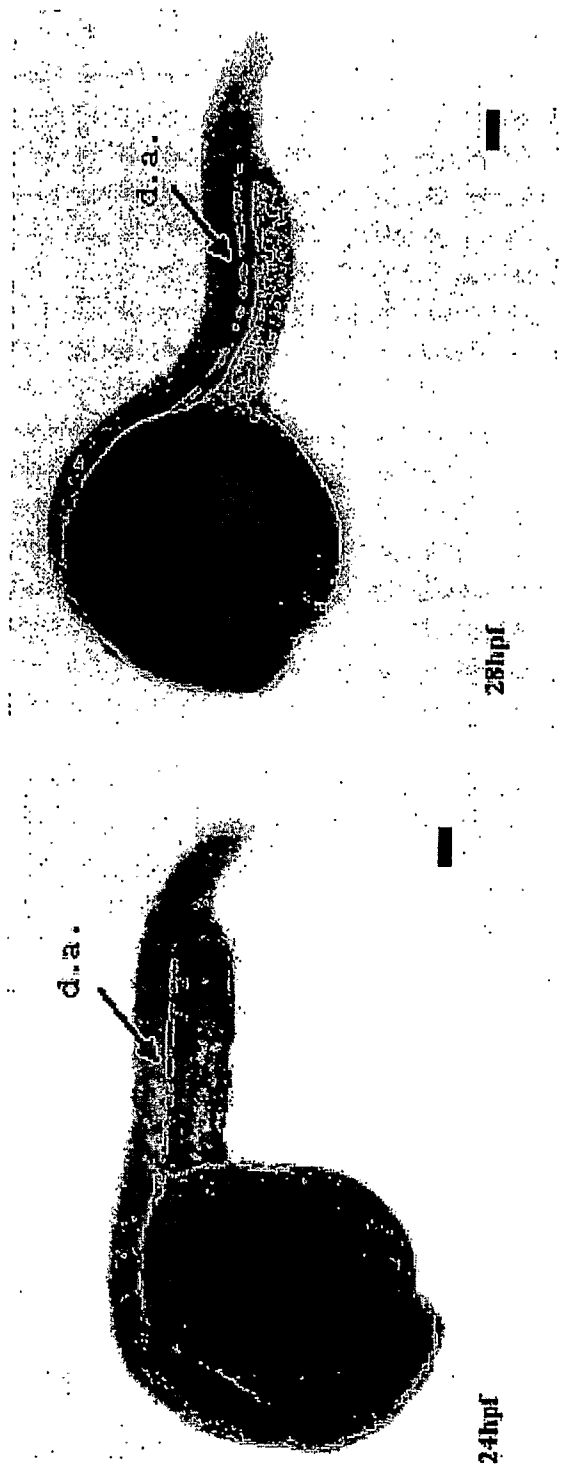

Figure 9

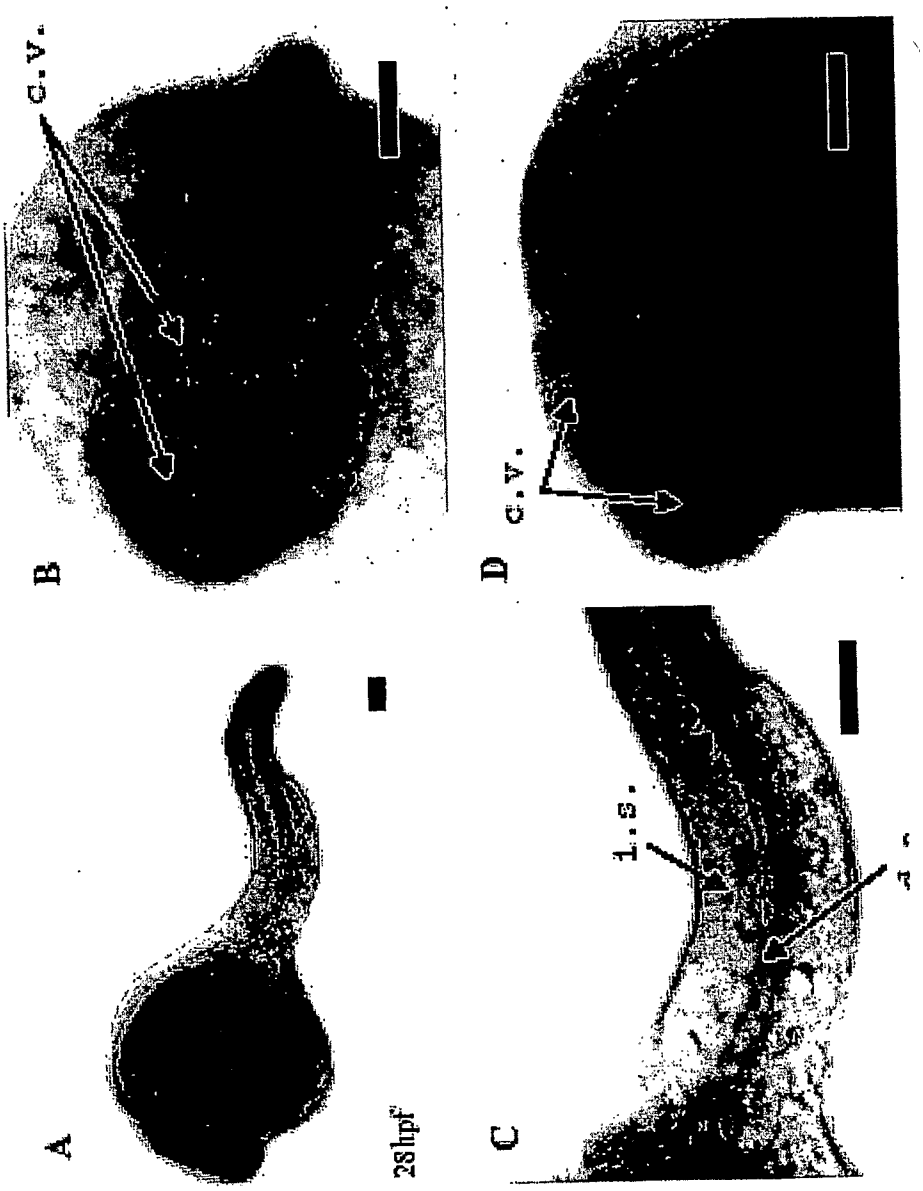

Figure 10

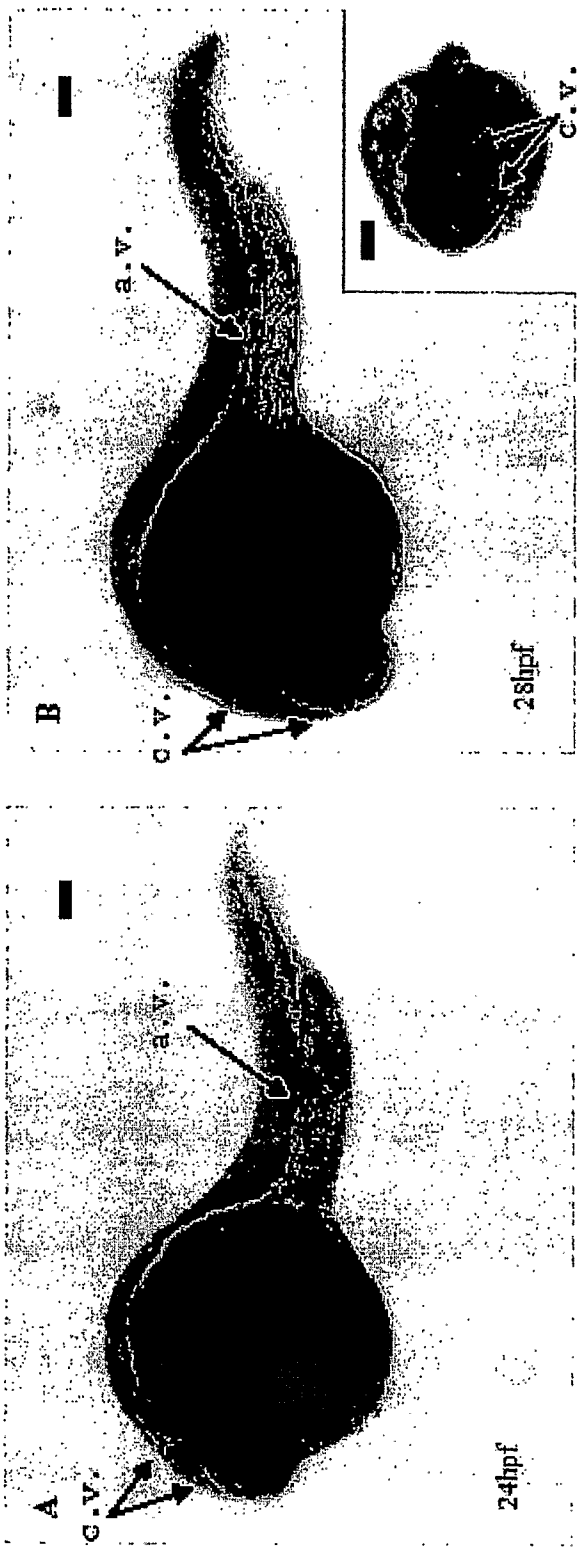

Figure 11

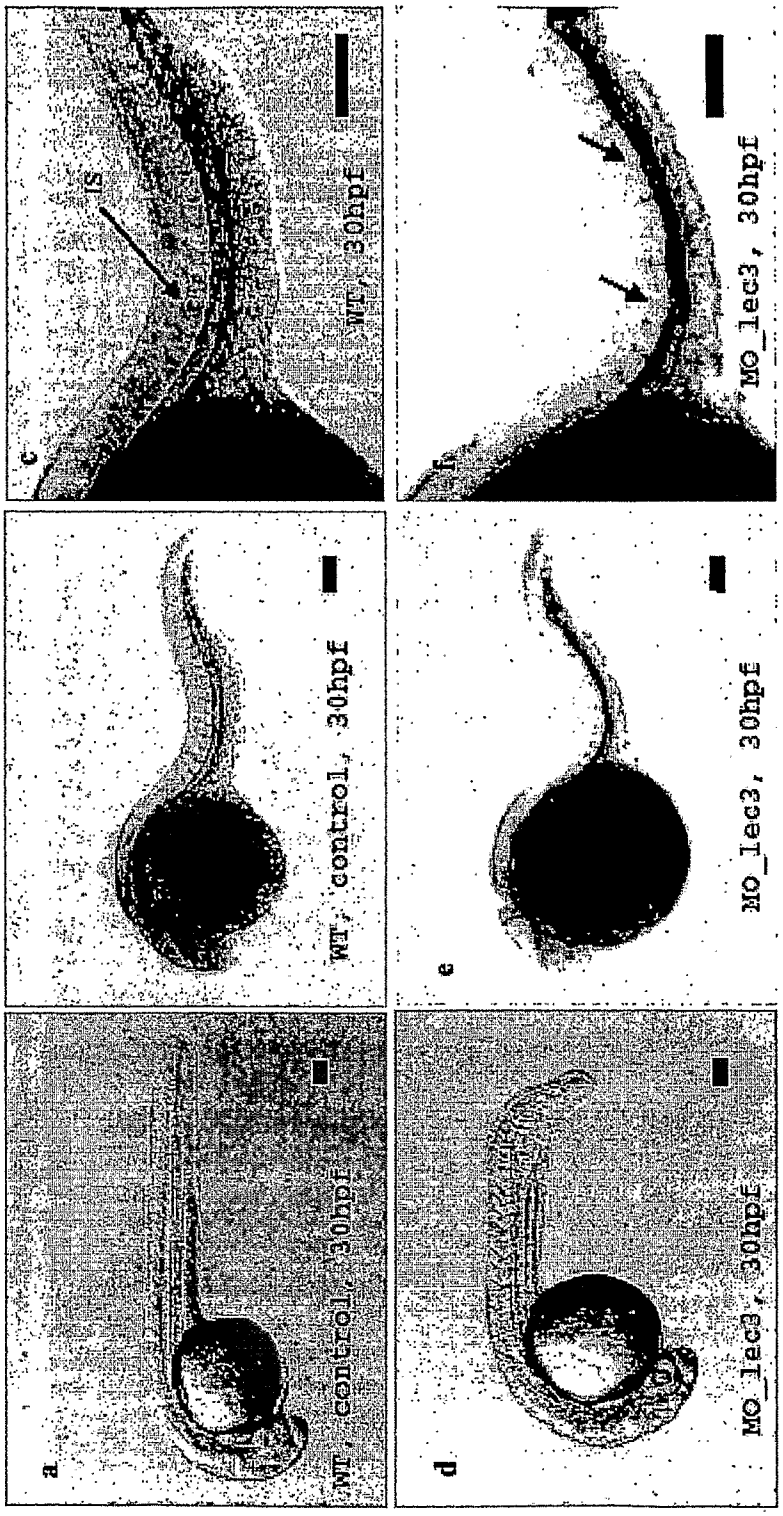
a
WT, control, 30hpf
b
WT, control, 30hpf
c
IS
WT, 30hpf
d
MO_lec3, 30hpf
e
MO_lec3, 30hpf
f
MO_lec3, 30hpf

Figure 12

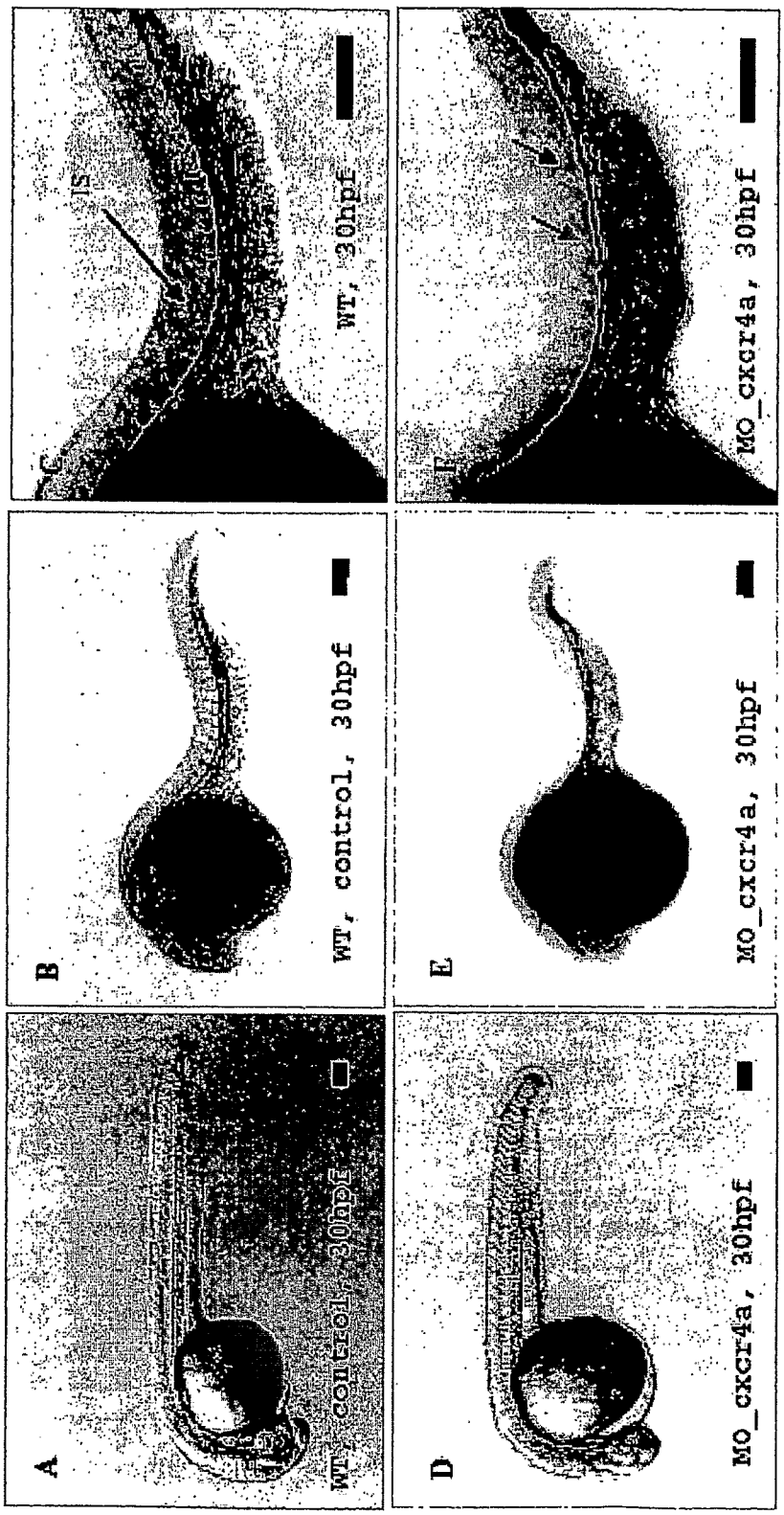
A
WT, control, 30hpf
B
WT, control, 30hpf
C
IS
WT, 30hpf
D
MO_cxcr4a, 30hpf
E
MO_cxcr4a, 30hpf
F
MO_cxcr4a, 30hpf

Figure 13

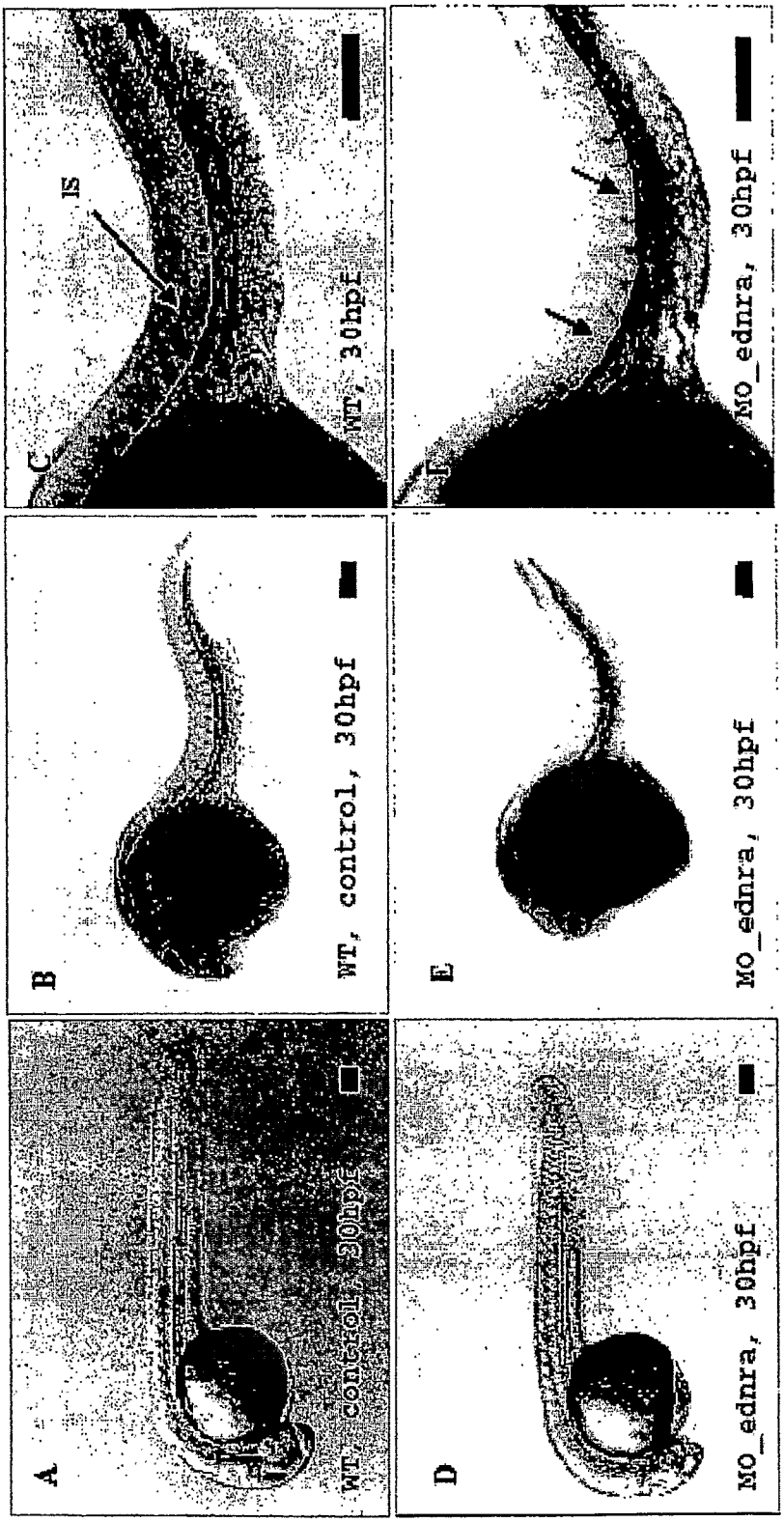
A
WT, control, 30hpf
B
WT, control, 30hpf
C
IS
WT, 30hpf
D
MO_ednra, 30hpf
E
MO_ednra, 30hpf
F
MO_ednra, 30hpf

Figure 14

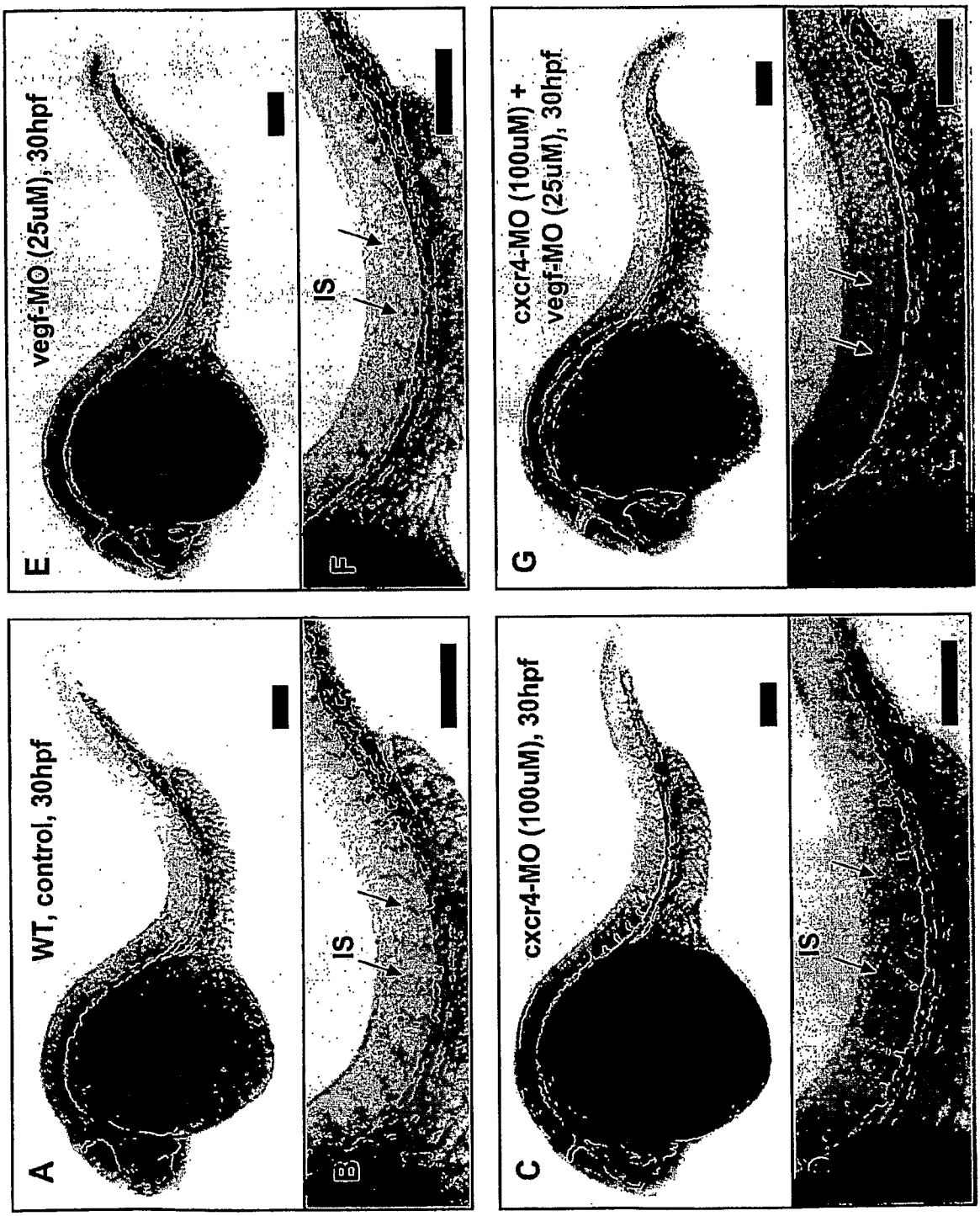
A
WT, control, 30hpf
B
IS
C
cxcr4-MO (100uM), 30hpf
IS
E
vegf-MO (25uM), 30hpf
F
IS
G
cxcr4-MO (100uM) +
vegf-MO (25uM), 30hpf

Figure 15

CXCR4	T	T	V	I	L	I	L	A	F	A	C	W	L	P	Y	Y	I	G	I	S	I
LEC3		D	K	R	S	A	S	N	A	F	M	I	C	G	I	L	Y	V	V	K	S	V	Y
EDNRA		T	V	F	C	L	V	V	I	F	A	L	C	W	F	P	L	H	L	S	R	I	L
α_{1B}-AR		T	L	G	I	V	V	G	M	F	I	L	C	W	L	P	F	F	I	A	L	P	L
α_{2C2}-AR		V	L	A	V	V	I	G	V	F	V	L	C	W	F	P	F	F	S	Y	S	L
β_2-AR		T	L	G	I	I	M	G	T	F	T	L	C	W	L	P	F	F	I	V	N	I	V
H_1		Q	L	G	F	I	M	A	A	F	I	L	C	W	I	P	Y	F	I	F	F	M	V
D_2		M	L	A	I	V	L	G	V	F	I	I	C	W	L	P	F	F	I	T	H	I	L
M_3		T	L	S	A	I	L	L	A	F	I	I	T	W	T	P	Y	N	I	M	V	L	V
AT_1		I	I	M	A	I	V	L	F	F	F	F	S	W	I	P	H	Q	I	F	T	F	L
Rhodopsin		M	V	I	I	M	V	I	A	F	L	I	C	W	L	P	Y	A	G	V	A	F	Y

Figure 16