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(71) Applicant *(for all designated States except US)*: **OPKO OPHTHALMICS, LLC** [US/US]; Suite 1180, 4400 Biscayne Boulevard, Miami, FL 33137 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **DEJNEKA, Nadine** [US/US]; Apt. B412, 200 North Wynnewood Avenue, Wynnewood, PA 19096 (US).

(74) Agent: **SCOLNICK, Daniel, M.**; Pepper Hamilton LLP, 50th Floor, 500 Grant Street, One Mellon Center, Pittsburgh, PA 15219-2502 (US).

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(54) Title: COMPOSITIONS AND METHODS FOR INHIBITION OF VEGF

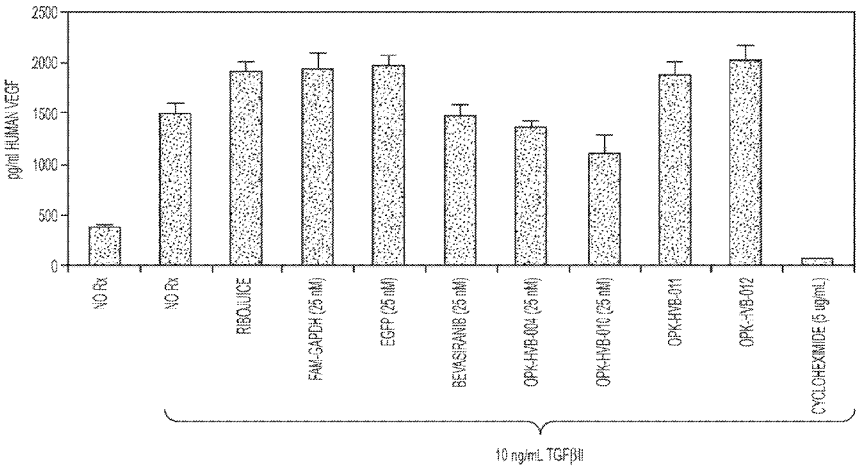

FIG. 14

(57) Abstract: Disclosed herein are siRNA compositions and methods useful for inhibiting expression of vascular endothelial growth factor (VEGF) isoforms. Diseases which involve angiogenesis stimulated by overexpression of VEGF, such as diabetic retinopathy, age related macular degeneration and many types of cancer, can be treated by administering small interfering RNAs as disclosed.

A. Title: Compositions and Methods for Inhibition of VEGF

B. Cross-Reference to Related Applications:

[0001] This application claims benefit of priority to U.S. Provisional Patent Application No. 61/266,645 filed on December 4, 2009 entitled "COMPOSITIONS AND METHODS FOR INHIBITION OF VEGF," the entire contents of which are hereby incorporated by reference.

C. Government Interests: Not applicable

D. Parties to a Joint Research Agreement: Not applicable

E. Incorporation by Reference of Material submitted on a Compact Disc: Not applicable

F. Background

1. **Field of Invention:** Not applicable
2. **Description of Related Art:** Not applicable

G. Brief summary of the invention

[0002] Angiogenesis, defined as the growth of new capillary blood vessels or "neovascularization," plays a fundamental role in growth and development. In mature humans, the ability to initiate angiogenesis is present in all tissues, but is held under strict control. A key regulator of angiogenesis is vascular endothelial growth factor ("VEGF"), also called vascular permeability factor ("VPF"). Angiogenesis is initiated when secreted VEGF binds to the Flt-1 and Flk-1/KDR receptors (also called VEGF receptor 1 and VEGF receptor 2), which are expressed on the surface of endothelial cells. Flt-1 and Flk-1/KDR are transmembrane protein tyrosine kinases, and binding of VEGF initiates a cell signal cascade resulting in the ultimate neovascularization in the surrounding tissue.

[0003] There are three main different VEGF alternative splice forms (i.e., isoforms) in humans ($VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$), while a number of other variants also exist ($VEGF_{206}$, $VEGF_{183}$, $VEGF_{148}$, $VEGF_{165b}$ and $VEGF_{145}$). Remarkably, not all of the isoforms are pro-angiogenic. It has been demonstrated that at least $VEGF_{165b}$ is capable of counteracting the effects of $VEGF_{165}$ induced angiogenesis. Without being bound by theory, it appears that $VEGF_{165b}$ is capable of preventing VEGF Receptor 2 signaling. As such, secretion of $VEGF_{165b}$ may be able to prevent or retard angiogenesis in pathological states.

[0004] Aberrant angiogenesis, or the pathogenic growth of new blood vessels, is implicated in a number of conditions. Among these conditions are diabetic retinopathy, psoriasis, exudative or "wet" age-related macular degeneration ("ARMD"), rheumatoid arthritis and other inflammatory diseases, and most cancers. The diseased tissues or tumors

associated with these conditions express abnormally high levels of VEGF, and show a high degree of vascularization or vascular permeability.

[0005] ARMD in particular is a clinically important angiogenic disease. This condition is characterized by choroidal neovascularization in one or both eyes in aging individuals, and is the major cause of blindness in industrialized countries.

[0006] RNA interference (hereinafter "RNAi") is a method of post-transcriptional gene regulation that is conserved throughout many eukaryotic organisms. RNAi is induced by short (*i.e.*, <30 nucleotide) double stranded RNA ("dsRNA") molecules which are present in the cell. These short dsRNA molecules, called "short interfering RNA" or "siRNA," cause the destruction of messenger RNAs ("mRNAs") which share sequence homology with the siRNA to within one nucleotide resolution. It is believed that the siRNA and the targeted mRNA bind to an "RNA-induced silencing complex" or "RISC", which cleaves the targeted mRNA. The siRNA is apparently recycled much like a multiple-turnover enzyme, with 1 siRNA molecule capable of inducing cleavage of approximately 1000 mRNA molecules. siRNA-mediated RNAi degradation of an mRNA is therefore more effective than currently available technologies for inhibiting expression of a target gene. However, such methods are not directly able to be translated into therapeutic agents for treatment of disease.

[0007] What is needed, therefore, are agents which selectively inhibit expression of pro-angiogenic VEGF in catalytic or sub-stoichiometric amounts in mammals, while inducing or maintaining the secretion of anti-angiogenic VEGF isoforms.

[0008] The present disclosure is directed to siRNAs which specifically target and cause RNAi-induced degradation of mRNA from VEGF and its isoforms. The siRNA compounds and compositions of the disclosure are used to inhibit angiogenesis, in particular for the treatment of cancerous tumors, age-related macular degeneration, and other angiogenic diseases.

[0009] Thus, the disclosure provides an isolated siRNA which targets human VEGF mRNA, or an alternative splice form, mutant or cognate thereof. For example, in one embodiment, the siRNA targets pro-angiogenic VEGF mRNA isoforms such as $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$, $VEGF_{183}$, $VEGF_{148}$, and/or $VEGF_{145}$. In certain embodiments, the siRNA comprises a sense RNA strand and an antisense RNA strand which form an RNA duplex. The sense RNA strand comprises a nucleotide sequence identical to a target sequence of about 19 to about 25 contiguous nucleotides in the target mRNA.

[0010] The disclosure also provides recombinant plasmids and viral vectors which express the siRNA disclosed herein, as well as pharmaceutical compositions comprising such an siRNA and a pharmaceutically acceptable carrier.

[0011] The disclosure further provides a method of inhibiting expression of human pro-angiogenic VEGF mRNA, or an alternative splice form, mutant or cognate thereof, while sparing anti-angiogenic VEGF mRNA, comprising administering to a subject an effective amount of siRNA such that the target mRNA is degraded.

[0012] The disclosure further provides a method of inhibiting angiogenesis in a subject, comprising administering to a subject an effective amount of an siRNA targeted to pro-angiogenic human VEGF mRNA or an alternative splice form, mutant or cognate thereof.

[0013] The disclosure further provides a method of treating an angiogenic disease, comprising administering to a subject in need of such treatment an effective amount of an siRNA targeted to human proangiogenic VEGF mRNA or an alternative splice form, mutant or cognate thereof, such that angiogenesis associated with the angiogenic disease is inhibited.

H. Description of Drawings

[0014] The file of this patent contains at least one photograph or drawing executed in color. Copies of this patent with color drawing(s) or photograph(s) will be provided by the Patent and Trademark Office upon request and payment of necessary fee.

[0015] For a fuller understanding of the nature and advantages of the present invention, reference should be had to the following detailed description taken in connection with the accompanying drawings, in which:

[0016] **Fig. 1A** and **1B** are a histograms of VEGF concentration (in pg/ml) in hypoxic 293 and HeLa cells treated with no siRNA ("-"); nonspecific siRNA ("nonspecific"); or siRNA targeting human VEGF mRNA ("VEGF"). VEGF concentration (in pg/ml) in non-hypoxic 293 and HeLa cells is also shown. Each bar represents the average of four experiments, and the error is the standard deviation of the mean.

[0017] **Fig. 2** is a histogram of murine VEGF concentration (in pg/ml) in hypoxic NIH 3T3 cells treated with no siRNA ("-"); nonspecific siRNA ("nonspecific"); or siRNA targeting human VEGF mRNA ("VEGF"). Each bar represents the average of six experiments and the error is the standard deviation of the mean.

[0018] Fig. 3 is a histogram of human VEGF concentration (pg/total protein) in retinas from mice injected with adenovirus expressing human VEGF ("AdVEGF") in the presence of either GFP siRNA (dark gray bar) or human VEGF siRNA (light grey bar). Each bar represents the average of 5 eyes and the error bars represent the standard error of the mean.

[0019] Fig. 4 is a histogram showing the mean area (in mm^2) of laser-induced CNV in control eyes given subretinal injections of GFP siRNA (N=9; "GFP siRNA"), and in eyes given subretinal injections of mouse VEGF siRNA (N=7; "Mouse VEGF siRNA"). The error bars represent the standard error of the mean.

[0020] Fig. 5 is a schematic representation of pAAVsiRNA, a cis-acting plasmid used to generate a recombinant AAV viral vector of the invention. "ITR": AAV inverted terminal repeats; "U6": U6 RNA promoters; "Sense": siRNA sense coding sequence; "Anti": siRNA antisense coding sequence; "PolyT": polythymidine termination signals.

[0021] Fig. 6 shows histograms of the mean area (in mm^2) of laser-induced CNV in treatment in mouse eyes injected **(A)** subretinally or **(B)** intravitreally with a mouse anti-VEGF siRNA ("mVEGF1.siRNA") or control siRNA ("GFP1.siRNA"). The error bars represent the standard error of the mean. **(C)** is a histogram of the mean area (in mm^2) of laser-induced CNV in mouse eyes injected intravitreally with: phosphate-buffered saline with no siRNA at 1 day post-laser induction ("PBS"; CNV area measured at 14 days post-laser induction); control siRNA at 14 days post-laser induction ("GFP1.siRNA"; CNV area measured at 21 days post-laser induction); or a mouse anti-VEGF siRNA at 14 days post-laser induction ("mVEGF1.siRNA"; CNV area measured at 21 days post-laser induction). The error bars represent the standard error of the mean.

[0022] Fig. 7 is a graph of the percent of VEGF ("%VEGF") protein in mouse eyes injected sub-retinally with human anti-VEGF siRNA ("Cand5") and control siRNA ("GFP1.siRNA") at 0 (n=2; pre-siRNA injection), 6 (n=3), 10 (n=3) and 14 (n=3) days post-injection. %VEGF = ([VEGF] in the Cand5 eye/[VEGF] in the GFP1.siRNA eye) *100.

[0023] Fig. 8 is a graph of hVEGF protein levels in 293 cells transfected with transfected with human VEGF siRNAs, non-specific siRNA (EGFP siRNA) or mock transfections without siRNA.

[0024] Fig. 9 is a graph of the dose response studies with Cand5 (bevasrianib), hVEGF#1, hVEGF#2, hVEGF#3, hVEGF#4, hVEGF#6 and hVEGF#7.

[0025] Fig. 10 is a schematic of the various isoforms of VEGF and their exon usage.

[0026] Fig. 11 is a diagram comparing the homology of $VEGF_{165}$ and $VEGF_{165b}$ at the exon 7/8 junction.

[0027] Fig. 12 depicts the amount of VEGF protein expressed for various siRNAs targeting the $VEGF_{165}$ exon 7/8 junction.

[0028] Fig. 13 depicts the percent knockdown of human VEGF protein for various siRNAs targeting the $VEGF_{165}$ exon 7/8 junction.

[0029] Fig. 14 depicts the amount of VEGF protein expressed for a secondary screen of siRNAs targeting the $VEGF_{165}$ exon 7/8 junction.

[0030] Fig. 15 depicts the percent knockdown of human VEGF protein for a secondary screen of siRNAs targeting the $VEGF_{165}$ exon 7/8 junction.

[0031] Fig. 16 depicts the percent knockdown of human VEGF protein for a secondary screen of siRNAs targeting the $VEGF_{165}$ exon 7/8 junction at varying concentrations.

[0032] Fig. 17 depicts the percent knockdown of human VEGF protein over seven days for a secondary screen of siRNAs targeting the $VEGF_{165}$ exon 7/8 junction.

[0033] Fig. 18 depicts the effect of siRNA targeting the $VEGF_{165}$ exon 7/8 junction on GAPDH mRNA expression using RT-PCR.

[0034] Fig. 19 depicts the effect of siRNA targeting the $VEGF_{165}$ exon 7/8 junction on $VEGF_{165}$ mRNA expression using RT-PCR.

[0035] Fig. 20 depicts the effect of siRNA targeting the VEGF exon 7/8 junction on $VEGF_{189}$ mRNA expression using RT-PCR.

[0036] Fig. 21 depicts the effect of siRNA targeting the VEGF exon 7/8 junction on $VEGF_{121}$ mRNA expression using RT-PCR.

[0037] Fig. 22 depicts the effect of siRNA targeting the $VEGF_{165}$ exon 7/8 junction on $VEGF_{165b}$ mRNA expression using RT-PCR. Double banding at about 600bp is artefactual.

[0038] Fig. 23 depicts the cytokine profile of ARPE19 cells following treatment with selected siRNAs.

[0039] Fig. 24 depicts the effect of siRNAs on total VEGF protein secretion by ARPE19 cells.

[0040] Fig. 25 depicts the effect of siRNAs on total VEGF protein secretion by ARPE19 cells.

[0041] Fig. 26 depicts the effect of siRNAs on total VEGF protein secretion by ARPE19 cells.

[0042] Fig. 27 depicts the effect of siRNAs on total VEGF protein secretion by ARPE19 cells.

[0043] Fig. 28 depicts the stability of bevasiranib under different temperature conditions over time.

[0044] Fig. 29 depicts the stability of bevasiranib under different temperature conditions over time.

[0045] Fig. 30 depicts the stability of OPK-HVB-004 under different temperature conditions over time.

[0046] Fig. 31 depicts the stability of OPK-HVB-009 under different temperature conditions over time.

[0047] Fig. 32 depicts the stability of OPK-HVB-010 under different temperature conditions over time

[0048] Fig. 33 depicts the homology between human, rat and mouse VEGF sequences at the 3' terminal end.

[0049] Fig. 34 depicts the effect of siRNAs on rat VEGF secretion by C6 cells.

[0050] Fig. 35 depicts the effect of siRNAs on mouse VEGF secretion by NIH3T3 cells.

[0051] Fig. 36 depicts the effect of siRNAs on VEGF secretion by ARPE19 cells.

[0052] Fig. 37 depicts the effect of siRNAs on VEGF secretion by ARPE19 cells.

[0053] Fig. 38 depicts the effect of siRNAs on VEGF secretion by ARPE19 cells.

[0054] Fig. 39 depicts the effect of siRNAs on mouse VEGF secretion by NIH3T3 cells

[0055] Fig. 40 depicts the effect of siRNAs on VEGF mRNA message in ARPE19 cells

[0056] Fig. 41 depicts the effect of siRNAs on VEGF expression in C6 cells.

I. Detailed Description

[0057] Before the present compositions and methods are described, it is to be understood that this invention is not limited to the particular processes, compositions, or methodologies described, as these may vary. It is also to be understood that the terminology used in the description is for the purpose of describing the particular versions or embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the present invention, the preferred methods, devices, and materials are now described. All publications mentioned herein are incorporated by reference in their entirety. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

[0058] It must also be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to a "molecule" is a reference to one or more molecules and equivalents thereof known to those skilled in the art, and so forth. As used herein, the term "about" means plus or minus 10% of the numerical value of the number with which it is being used. Therefore, about 50% means in the range of 45%-55%.

[0059] As used herein, a "subject" includes a human being or non-human animal. In certain embodiments, the subject is a human being.

[0060] As used herein, an "effective amount" of the siRNA is an amount sufficient to cause RNAi-mediated degradation of the target mRNA in cell. The term clinically effective amount is an amount that when administered to a subject, will inhibit the progression of angiogenesis in a subject by RNA silencing.

[0061] As used herein, "isolated" means altered or removed from the natural state through human intervention. For example, an siRNA naturally present in a living animal is not "isolated," but a synthetic siRNA, or an siRNA partially or completely separated from the coexisting materials of its natural state is "isolated." An isolated siRNA can exist in

substantially purified form, or can exist in a non-native environment such as, for example, a cell into which the siRNA has been delivered.

[0062] As used herein, "target mRNA" means an mRNA comprising a complementary sense sequence to an siRNA antisense strand. Such a target mRNA need not be 100% homologous to the siRNA antisense strand, as long as the siRNA functions to silence or otherwise form a RISC complex with the target mRNA. Target mRNAs of particular use in the methods of the disclosure include, for example, pro-angiogenic VEGF mRNA isoforms such as $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$, $VEGF_{206}$, $VEGF_{183}$, $VEGF_{148}$, and $VEGF_{145}$ and combinations thereof. In certain other embodiments, the target mRNA does not comprise anti-angiogenic $VEGF_{165b}$ mRNA, but targets at least one other VEGF isoforms.

[0063] As used herein the term "partially non-complementary" is intended to mean an siRNA sequence which although, perhaps sharing some sequence homology to a non-target sequence still differs sufficiently such that RNA silencing does not occur for the non-target sequence. Partially non-complementary include sequences that are 90% homologous, 85%, homologous, 80% homologous, 75% homologous, 70% homologous, 65% homologous, 60%, homologous, 55% homologous, 50% homologous, 45% homologous, 40% homologous, 35%, homologous, 30% homologous, 25% homologous, 20% homologous, 15% homologous, 10%, homologous, 5% homologous, 2% homologous, and 1% homologous to a non-target sequence. A sequence that is entirely non-homologous to a non-target sequence is considered non-complementary to the sequence.

[0064] As used herein, a gene or mRNA which is "cognate" to human VEGF or mRNA from another mammalian species which is homologous to human VEGF. For example, the cognate VEGF mRNA from the mouse is given in SEQ ID NO: 1.

[0065] Unless otherwise indicated, all nucleic acid sequences herein are given in the 5' to 3' direction. Also, all deoxyribonucleotides in a nucleic acid sequence are represented by capital letters (*e.g.*, deoxythymidine is "T"), and ribonucleotides in a nucleic acid sequence are represented by lower case letters (*e.g.*, uridine is "u").

[0066] Compositions and methods comprising siRNA targeted to VEGF and its various isoforms can be used to inhibit angiogenesis, in particular for the treatment of angiogenic disease. The siRNA are believed to cause the RNAi-mediated degradation of these mRNAs, so that the protein product of the VEGF and its isoforms are not produced or is

produced in reduced amounts. Because VEGF binding to the Flt-1 or Flk-1/KDR receptors is required for initiating and maintaining angiogenesis, the siRNA-mediated degradation of VEGF and its isoforms as well as Flt-1 or Flk-1/KDR mRNA may also be used to inhibit the angiogenic process.

[0067] One aspect of the present disclosure therefore provides isolated siRNA comprising short double-stranded RNA from about 17 nucleotides to about 29 nucleotides in length, and in certain embodiments from about 19 to about 25 nucleotides in length, that are targeted to the target mRNA. The siRNA comprise a sense RNA strand and a complementary antisense RNA strand annealed together by standard Watson-Crick base-pairing interactions (hereinafter "base-paired"). As is described in more detail below, the sense strand comprises a nucleic acid sequence which is identical or closely homologous to a target sequence contained within the target mRNA.

[0068] The sense and antisense strands of the siRNA can comprise two complementary, single-stranded RNA molecules or can comprise a single molecule in which two complementary portions are base-paired and are covalently linked by a single-stranded "hairpin" area. Without wishing to be bound by any theory, it is believed that the hairpin area of the latter type of siRNA molecule is cleaved intracellularly by the "Dicer" protein (or its equivalent) to form an siRNA of two individual base-paired RNA molecules .

[0069] Splice variants of human VEGF are known, including pro-angiogenic VEGF mRNA isoforms such as $VEGF_{121}$ (SEQ ID NO: 2), $VEGF_{165}$ (SEQ ID NO: 3), and $VEGF_{189}$(SEQ ID NO: 4), $VEGF_{206}$(SEQ ID NO: 5; GenBank Accession No. CS245579), $VEGF_{183}$ (GenBank Accession No. AJ010438), $VEGF_{148}$ (GenBank Accession No. AF091352), and $VEGF_{145}$ (GenBank Accession No. CS245578), as well as anti-angiogenic $VEGF_{165b}$ mRNA (GenBank Accession No. AF430806). The mRNA transcribed from the human VEGF and its isoforms, as well as Flt-1 (SEQ ID NO: 6) or Flk-1/KDR (SEQ ID NO: 7) genes can be analyzed for further alternative splice forms using techniques well-known in the art. Such techniques include reverse transcription-polymerase chain reaction (RT-PCR), northern blotting and *in-situ* hybridization. Techniques for analyzing mRNA sequences are described, for example, in Busting SA (2000), *J. Mol. Endocrinol.* 25: 169-193, the entire disclosure of which is herein incorporated by reference. Representative techniques for identifying alternatively spliced mRNAs are also described below.

[0070] For example, databases that contain nucleotide sequences related to a given disease gene can be used to identify alternatively spliced mRNA. Such databases include GenBank, Embase, and the Cancer Genome Anatomy Project (CGAP) database. The CGAP database, for example, contains expressed sequence tags (ESTs) from various types of human cancers. An mRNA or gene sequence from the VEGF and its isoforms as well as Flt-1 or Flk-1/KDR genes can be used to query such a database to determine whether ESTs representing alternatively spliced mRNAs have been found for a these genes.

[0071] A technique called "RNAse protection" can also be used to identify alternatively spliced VEGF and its isoforms as well as Flt-1 or Flk-1/KDR mRNAs. RNAse protection involves translation of a gene sequence into synthetic RNA, which is hybridized to RNA derived from other cells; for example, cells from tissue at or near the site of neovascularization. The hybridized RNA is then incubated with enzymes that recognize RNA:RNA hybrid mismatches. Smaller than expected fragments indicate the presence of alternatively spliced mRNAs. The putative alternatively spliced mRNAs can be cloned and sequenced by methods well known to those skilled in the art.

[0072] RT-PCR can also be used to identify alternatively spliced VEGF and its isoforms as well as Flt-1 or Flk-1/KDR mRNAs. In RT-PCR, mRNA from tissue or cells is converted into cDNA by the enzyme reverse transcriptase, using methods well-known to those of ordinary skill in the art. The coding sequence of the cDNA is then amplified via PCR using a forward primer located in the 5' translated region, and a reverse primer located in the 3' translated region. In some embodiments, all the bases encoding the cDNA are amplified. The amplified products can be analyzed for alternative splice forms, for example by comparing the size of the amplified products with the size of the expected product from normally spliced mRNA, *e.g.*, by agarose gel electrophoresis. Any change in the size of the amplified product can indicate alternative splicing.

[0073] mRNA produced from mutant VEGF and its isoforms as well as Flt-1 or Flk-1/KDR genes can also be readily identified through the techniques described above for identifying alternative splice forms. As used herein, "mutant" VEGF and its isoforms as well as Flt-1 or Flk-1/KDR genes or mRNA include human VEGF and its isoforms as well as Flt-1 or Flk-1/KDR genes or mRNA which differ in sequence from the VEGF and its isoforms as well as Flt-1 or Flk-1/KDR sequences set forth herein. Thus, allelic forms of these genes, and the mRNA produced from them, are considered "mutants" for purposes of this invention.

[0074] It is understood that human VEGF and its isoforms, as well as Flt-1 or Flk-1/KDR mRNA may contain target sequences in common with their respective alternative splice forms, cognates or mutants. A single siRNA comprising such a common targeting sequence can therefore induce RNAi-mediated degradation of different RNA types which contain the common targeting sequence. For example, as shown in Figure 10, all VEGF isoforms share exons 1-5. However, in $VEGF_{121}$ (SEQ ID NO: 2) exons 6 and 7 (7a and 7b) are deleted. In $VEGF_{165}$ (SEQ ID NO: 3) exon 6 (6a and 6b) is deleted. In $VEGF_{189}$ (SEQ ID NO: 4) exon 6b is deleted. In $VEGF_{183}$ a portion of exon 6a is deleted as well as the complete exon 6b sequence. $VEGF_{148}$ has a deletion of exon 6 (6a and 6b) as well as exon 7b and a portion of exon 8. In $VEGF_{145}$ exon 6b and exon 7 (7a and 7b) are deleted. The only known anti-angiogenic isoform of VEGF, $VEGF_{165}^{b}$, lacks exon 6 (6a and 6b), but additionally comprises a pseudo-exon 9. The pseudo-exon 9 is a result of a reading frame shift caused by the deletion of a stop codon, thus allowing a portion of the 3'UTR to be translated as protein. See for example, Bates et al., Can. Res. 62:4123 (2002), herein incorporated by reference in its entirety. $VEGF_{206}$ (SEQ ID NO: 5) is the full length sequence VEGF with no deletions. Thus, in certain embodiments, the siRNA targets one or more isoforms, such as $VEGF_{121}$ (SEQ ID NO: 2), $VEGF_{165}$ (SEQ ID NO: 3), and $VEGF_{189}$(SEQ ID NO: 4), $VEGF_{206}$(SEQ ID NO: 5; GenBank Accession No. CS245579), $VEGF_{183}$ (GenBank Accession No. AJ010438), $VEGF_{148}$ (GenBank Accession No. AF091352), and/or $VEGF_{145}$ (GenBank Accession No. CS245578), but spares others, such as $VEGF_{165b}$, because the siRNA targets a shared exon among certain isoforms but not others.

[0075] In one embodiment, provided is an isolated siRNA comprising of a duplex of a first RNA strand and a second RNA strand, said first RNA strand comprising a nucleotide sequence identical to a target sequence of about 19 to about 25 contiguous nucleotides to a vascular endothelial growth factor (VEGF) isoform selected from the group consisting of human $VEGF_{121}$, $VEGF_{165}$ $VEGF_{189}$, $VEGF_{206}$, $VEGF_{183}$, $VEGF_{148}$, $VEGF_{145}$ and combinations thereof; further wherein said siRNA is at least partially non-complementary to $VEGF_{165b}$, with the proviso that the human VEGF mRNA is not SEQ ID NO. 42. Further embodiments include methods of using such siRNA to inhibit angiogenesis and pharmaceutical compositions comprising a therapeutically effective amount of such siRNA to inhibit angiogenesis.

[0076] The siRNA can comprise partially purified RNA, substantially pure RNA, synthetic RNA, or recombinantly produced RNA, as well as altered RNA that differs from

naturally-occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the siRNA or to one or more internal nucleotides of the siRNA, including modifications that make the siRNA resistant to nuclease digestion.

[0077] One or both strands of the siRNA can also comprise a 3' overhang. As used herein, a "3' overhang" refers to at least one unpaired nucleotide extending from the 3'-end of a duplexed RNA strand. In some embodiments, the siRNA does not comprise a overhang and has a blunt end. In some embodiments, both ends of the siRNA comprise a blunt end. In some embodiments, the siRNA comprises a 17mer that contiguous with a target mRNA and dTdT overhang. In some embodiments, the siRNA is a siRNA that can inhibit the secretion or production of VEGF from cells from different species. For example, in some embodiments, the siRNA can inhibit VEGF secretion or inhibition from a human cell, a rat cell, and/or a mouse cell. In some embodiments, the siRNA can inhibit the secretion or production of VEGF from a mouse cell and a human cell, but not from a rat cell. In some embodiments, the siRNA can inhibit the secretion or production of VEGF from a rat cell and a human cell, but not from a mouse cell. In some embodiments, the siRNA can inhibit the secretion or production of VEGF from a human cell, a mouse cell, and a rat cell. The selectivity of the siRNA can be based upon the homology between the different sequences. For example, Figure 33 shows the homology between the terminal codons encoding human, mouse and rat VEGF. These differences can be exploited to produce siRNAs that can selectively inhibit the production of VEGF from one or more species.

[0078] In some embodiments, siRNAs comprising less than 21 nucleotides, *e.g.* 17, 18, 19, or 20, can be used to avoid any potential non-specific *in vivo* responses. (See, Ambati, Nature, 452, 591-597 (3 April 2008)). For example, siRNAs comprising less than 21 nucleotides can be used to avoid activating a TLR3 response *in vivo*.

[0079] Thus in one embodiment, the siRNA comprises at least one 3' overhang of from 1 to about 6 nucleotides (which includes ribonucleotides or deoxynucleotides) in length, preferably from 1 to about 5 nucleotides in length, more preferably from 1 to about 4 nucleotides in length, and particularly preferably from about 2 to about 4 nucleotides in length.

[0080] In the embodiment in which both strands of the siRNA molecule comprise a 3' overhang, the length of the overhangs can be the same or different for each strand. In a most

preferred embodiment, the 3' overhang is present on both strands of the siRNA, and is 2 nucleotides in length. For example, each strand of the siRNA can comprise 3' overhangs of dithymidylic acid ("TT") or diuridylic acid ("uu").

[0081] In order to enhance the stability of the present siRNA, the 3' overhangs can be also stabilized against degradation. In one embodiment, the overhangs are stabilized by including purine nucleotides, such as adenosine or guanosine nucleotides. Alternatively, substitution of pyrimidine nucleotides by modified analogues, *e.g.*, substitution of uridine nucleotides in the 3' overhangs with 2'-deoxythymidine, is tolerated and does not affect the efficiency of RNAi degradation. In particular, the absence of a 2' hydroxyl in the 2'-deoxythymidine significantly enhances the nuclease resistance of the 3'overhang in tissue culture medium.

[0082] In certain embodiments, the siRNA comprises the sequence AA(N19)TT or NA(N21), where N is any nucleotide. These siRNA comprise approximately 30-70% GC, and preferably comprise approximately 50% G/C. The sequence of the sense siRNA strand corresponds to (N19)TT or N21 (*i.e.*, positions 3 to 23), respectively. In the latter case, the 3' end of the sense siRNA is converted to TT. The rationale for this sequence conversion is to generate a symmetric duplex with respect to the sequence composition of the sense and antisense strand 3' overhangs. The antisense RNA strand is then synthesized as the complement to positions 1 to 21 of the sense strand.

[0083] Because position 1 of the 23-nt sense strand in these embodiments is not recognized in a sequence-specific manner by the antisense strand, the 3'-most nucleotide residue of the antisense strand can be chosen deliberately. However, the penultimate nucleotide of the antisense strand (complementary to position 2 of the 23-nt sense strand in either embodiment) is generally complementary to the targeted sequence.

[0084] In another embodiment, the siRNA comprises the sequence NAR(N17)YNN, where R is a purine (*e.g.*, A or G) and Y is a pyrimidine (*e.g.*, C or U/T). The respective 21-nt sense and antisense RNA strands of this embodiment therefore generally begin with a purine nucleotide. Such siRNA can be expressed from pol III expression vectors without a change in targeting site, as expression of RNAs from pol III promoters is only believed to be efficient when the first transcribed nucleotide is a purine.

[0085] In a further embodiment, the siRNA comprises a sequence having no more than five (5) consecutive purines or pyrimidines. In a further embodiment, the siRNA

comprises a sequence having no more than five (5) consecutive nucleotides having the same nucleobase (i.e., A, C, G, or U/T).

[0086] The siRNA can be targeted to any stretch of approximately 19-25 contiguous nucleotides in any of the target mRNA sequences (the "target sequence"). Techniques for selecting target sequences for siRNA are given, for example, in Tuschl T et al., "The siRNA User Guide," revised Oct. 11, 2002, the entire disclosure of which is herein incorporated by reference. "The siRNA User Guide" is available on the world wide web at a website maintained by Dr. Thomas Tuschl, Department of Cellular Biochemistry, AG 105, Max-Planck-Institute for Biophysical Chemistry, 37077 Göttingen, Germany, and can be found by accessing the website of the Max Planck Institute and searching with the keyword "siRNA." Thus, the sense strand of the present siRNA comprises a nucleotide sequence identical to any contiguous stretch of about 19 to about 25 nucleotides in the target mRNA.

[0087] In some embodiments, the siRNA is 19 nucleotides and comprises 17 nucleotides that are identical to a target mRNA. In some embodiments, the siRNA is 19 nucleotides in length comprising at least one blunt end. In some embodiments, each end of the 19mer has a blunt end. In some embodiments, the 19mer comprises at least one dT overhang. In some embodiments, the 19mer comprises two dT overhangs.

[0088] Generally, a target sequence on the target mRNA can be selected from a given cDNA sequence corresponding to the target mRNA, preferably beginning 50 to 100 nt downstream (*i.e.*, in the 3' direction) from the start codon. The target sequence can, however, be located in the 5' or 3' untranslated regions, or in the region nearby the start codon (see, *e.g.*, the target sequences of SEQ ID NOS: 73 and 74 in Table 1 below, which are within 100 nt of the 5'-end of the $VEGF_{121}$ cDNA.

[0089] In a further embodiment of the present invention, the target mRNA sequence comprises no more than five (5) consecutive purines or pyrimidines. For example, a suitable target sequence in the $VEGF_{121}$ cDNA sequence is:

TCATCACGAAGTGGTGAAG (SEQ ID NO: 8)

[0090] Thus, an siRNA targeting this sequence, and which has 3' uu overhangs on each strand (overhangs shown in bold), is:

5'-ucaucacgaaguggugaag**uu**-3' (SEQ ID NO: 9)

3'-**uu**aguagugcuucaccacuuc-5' (SEQ ID NO: 10)

[0091] An siRNA targeting this same sequence, but having 3' TT overhangs on each strand (overhangs shown in bold) is:

5'-ucaucacgaaguggugaag**TT**-3' (SEQ ID NO: 11)

3'-**TT**aguagugcuucaccacuuc-5' (SEQ ID NO: 12)

[0092] Other $VEGF_{121}$ target sequences from which siRNA can be derived are given in Table 1. It is understood that all $VEGF_{121}$ target sequences listed in Table 1 are within that portion of the $VEGF_{121}$ alternative splice form which is common to all human VEGF alternative splice forms. Thus, the $VEGF_{121}$ target sequences in Table 1 can also target $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ mRNA. Target sequences which target a specific VEGF isoform can also be readily identified. For example, a target sequence which targets $VEGF_{165}$ mRNA but not $VEGF_{121}$ mRNA is AACGTACTTGCAGATGTGACA (SEQ ID NO: 13). Conversely, target sequences which target pro-angiogenic VEGF mRNA isoforms such as $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$, $VEGF_{206}$, $VEGF_{183}$, $VEGF_{148}$, and $VEGF_{145}$ and combinations thereof, but does not target anti-angiogenic $VEGF_{165b}$ mRNA include the sequences found in Table 2, with the proviso that the VEGF mRNA is not SEQ ID No. 42. In certain embodiments, said human VEGF mRNA is selected from the group consisting of SEQ ID NO: 86; SEQ ID NO: 87; SEQ ID NO: 88; SEQ ID NO: 89 ;SEQ ID NO: 90; SEQ ID NO: 91; SEQ ID NO: 92; SEQ ID NO: 93; SEQ ID NO: 94; SEQ ID NO: 95; SEQ ID NO: 96; SEQ ID NO: 97; and SEQ ID NO: 98. In certain embodiments, said human VEGF mRNA is selected from SEQ ID NO. 88 and SEQ ID NO. 94.

[0093] By selectively targeting the angiogenic isoforms of VEGF, while sparing the anti-angiogenic isoform, it is possible to enhance the anti-angiogenic effects of siRNA treatment. As shown in Figure 11, the region between exon 7 and 9 differ between the angiogenic and antiangiogenic sequences. According to the various embodiments, it is possible to selectively target this region where the siRNA is at least partially complementary to the angiogenic isoforms, but at least partially or fully non-complementary to the anti-angiogenic isoform. Consequently, in certain embodiments, the siRNA would not inhibit the expression of the anti-angiogenic isoform, $VEGF_{165b}$ with the proviso that the VEGF mRNA is not SEQ ID No. 42. In certain embodiments, said human VEGF mRNA is selected from the group consisting of SEQ ID NO: 86; SEQ ID NO: 87; SEQ ID NO: 88; SEQ ID NO: 89; SEQ ID NO: 90; SEQ ID NO: 91; SEQ ID NO: 92; SEQ ID NO: 93; SEQ ID NO: 94; SEQ ID NO: 95; SEQ ID NO: 96; SEQ ID NO: 97; SEQ ID NO: 98, SEQ ID NO 99, SEQ ID NO

100, SEQ ID NO 101, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 105, SEQ ID NO: 106, SEQ ID NO: 107, SEQ ID NO: 108, SEQ ID NO: 109, SEQ ID NO: 110, SEQ ID NO: 111, SEQ ID NO: 112, SEQ ID NO: 113, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, and SEQ ID NO: 118. In certain embodiments, said human VEGF mRNA is selected from SEQ ID NO. 88 and SEQ ID NO. 94.

[0094] Exemplary target sequences for human Flt-1 for human Flk-1/KDR are given in PCT/US2003/0022444 filed July 18, 2003, herein incorporated by reference in its entirety.

Table 1 - VEGF Target Sequences

target sequence	SEQ ID NO:		target sequence	SEQ ID NO:
cognate VEGF mRNA sequence	1		GATAGAGCAAGACAAGAAA	26
Splice variant $VEGF_{121}$ sequence	2		GACAAGAAAATCCCTGTGG	27
Splice variant $VEGF_{165}$ sequence	3		GAAAATCCCTGTGGGCCTT	28
Splice variant $VEGF_{189}$ sequence	4		AATCCCTGTGGGCCTTGCT	29
Splice variant $VEGF_{206}$ sequence	5		TCCCTGTGGGCCTTGCTCA	30
TCATCACGAAGTGGTGAAG	8		GCATTTGTTTGTACAAGAT	31
UCAUCACGAAGUGGUGAAGUU	9		GATCCGCAGACGTGTAAAT	32
UUAGUAGUGCUUCACCACUUC	10		ATGTTCCTGCAAAAACACA	33
UCAUCACGAAGUGGUGAAGTT	11		TGTTCCTGCAAAAACACAG	34
TTAGUAGUGCUUCACCACUUC	12		AAACACAGACTCGCGTTGC	35
AACGTACTTGCAGATGTGACA	13		AACACAGACTCGCGTTGCA	36
GTTCATGGATGTCTATCAG	14		ACACAGACTCGCGTTGCAA	37
TCGAGACCCTGGTGGACAT	15		CACAGACTCGCGTTGCAAG	38
TGACGAGGGCCTGGAGTGT	16		GGCGAGGCAGCTTGAGTTA	39
TGACGAGGGCCTGGAGTGT	17		ACGAACGTACTTGCAGATG	40
CATCACCATGCAGATTATG	18		CGAACGTACTTGCAGATGT	41
ACCTCACCAAGGCCAGCAC	19		CGTACTTGCAGATGTGACA	42
GGCCAGCACATAGGAGAGA	20		GTGGTCCCAGGCTGCACCC	43
CAAATGTGAATGCAGACCA	21		GGAGGAGGGCAGAATCATC	44
ATGTGAATGCAGACCAAAG	22		GTGGTGAAGTTCATGGATG	45
TGCAGACCAAAGAAAGATA	23		AATCATCACGAAGTGGTGAAG	46
AGAAAGATAGAGCAAGACA	24		AAGTTCATGGATGTCTATCAG	47

GAAAGATAGAGCAAGACAA	25		AATCGAGACCCTGGTGGACAT	48
AATGACGAGGGCCTGGAGTGT	49		AATGTTCCTGCAAAAACACAGAC	65
AACATCACCATGCAGATTATG	50		AAAAACACAGACTCGCGTTGCAA	66
AAACCTCACCAAGGCCAGCAC	51		AAAACACAGACTCGCGTTGCAAG	67
AAGGCCAGCACATAGGAGAGA	52		AAACACAGACTCGCGTTGCAAGG	68
AACAAATGTGAATGCAGACCA	53		AACACAGACTCGCGTTGCAAGGC	69
AAATGTGAATGCAGACCAAAG	54		AAGGCGAGGCAGCTTGAGTTAAA	70
AATGCAGACCAAAGAAAGATA	55		AAACGAACGTACTTGCAGATGTG	71
AAAGAAAGATAGAGCAAGACA	56		AACGAACGTACTTGCAGATGTGA	72
AAGAAAGATAGAGCAAGACAA	57		AAGTGGTCCCAGGCTGCACCCAT	73
AAGATAGAGCAAGACAAGAAAAT	58		AAGGAGGAGGGCAGAATCATCAC	74
AAGACAAGAAAATCCCTGTGGGC	59		AAGTGGTGAAGTTCATGGATGTC	75
AAGAAAATCCCTGTGGGCCTTGC	60		AAAATCCCTGTGGGCCTTGCTCA	76
AATCCCTGTGGGCCTTGCTCAGA	61		ACCUCACCAAGGCCAGCACTT	77
AAGCATTTGTTTGTACAAGATCC	62		GUGCUGGCCUUGGUGAGGUTT	78
AAGATCCGCAGACGTGTAAATGT	63		GGCTACGTCCAGCGCACC	79
AAATGTTCCTGCAAAAACACAGA	64		AAACCUCACCAAAGCCAGCAC	80
ACCUCACCAAGGCCAGCAC	119		GUGCUGGCCUUGGUGAGGU	120

Table 2 - VEGF Target Sequences selectively excluding $VEGF_{165b}$

siRNA Name	Target sequence (5'-3')
OPK-HVB-001	AACGTACTTGCAGATGTGA (SEQ ID NO: 86)
OPK-HVB-002	ACGTACTTGCAGATGTGAC (SEQ ID NO: 87)
OPK-HVB-003	CGTACTTGCAGATGTGACA (SEQ ID NO: 42)
OPK-HVB-004	GTACTTGCAGATGTGACAA (SEQ ID NO: 88)
OPK-HVB-005	TACTTGCAGATGTGACAAG (SEQ ID NO: 89)
OPK-HVB-006	ACTTGCAGATGTGACAAGC (SEQ ID NO: 90)
OPK-HVB-007	CTTGCAGATGTGACAAGCC (SEQ ID NO: 91)
OPK-HVB-008	TTGCAGATGTGACAAGCCG (SEQ ID NO: 92)
OPK-HVB-009	TGCAGATGTGACAAGCCGA (SEQ ID NO: 93)
OPK-HVB-010	GCAGATGTGACAAGCCGAG (SEQ ID NO: 94)
OPK-HVB-011	CAGATGTGACAAGCCGAGG (SEQ ID NO: 95)
OPK-HVB-012	AGATGTGACAAGCCGAGGC (SEQ ID NO: 96)
OPK-HVB-013	GATGTGACAAGCCGAGGCG (SEQ ID NO: 97)
OPK-HVB-014	ATGTGACAAGCCGAGGCGG (SEQ ID NO: 98)
OPK-HVB-004be	GTACTTGCAGATGTGACAA (SEQ ID NO: 99)
OPK-HVB-009be	TGCAGATGTGACAAGCCGA (SEQ ID NO: 100)

OPK-HVB-010be	GCAGATGTGACAAGCCGAG (SEQ ID NO: 101)
OPK-HVB-012be	AGATGTGACAAGCCGAGGC (SEQ ID NO: 102)
OPK-HVB-001a	AACGTACTTGCAGATGT (SEQ ID NO: 103)
OPK-HVB-002a	ACGTACTTGCAGATGTG (SEQ ID NO: 104)
OPK-HVB-003a	CGTACTTGCAGATGTGA (SEQ ID NO: 105)
OPK-HVB-004a	GTACTTGCAGATGTGAC (SEQ ID NO: 106)
OPK-HVB-005a	TACTTGCAGATGTGACA (SEQ ID NO: 107)
OPK-HVB-006a	ACTTGCAGATGTGACAA (SEQ ID NO: 108)
OPK-HVB-007a	CTTGCAGATGTGACAAG (SEQ ID NO: 109)
OPK-HVB-008a	TTGCAGATGTGACAAGC (SEQ ID NO: 110)
OPK-HVB-009a	TGCAGATGTGACAAGCC (SEQ ID NO: 111)
OPK-HVB-010a	GCAGATGTGACAAGCCG (SEQ ID NO: 112)
OPK-HVB-011a	CAGATGTGACAAGCCGA (SEQ ID NO: 113)
OPK-HVB-012a	AGATGTGACAAGCCGAG (SEQ ID NO: 114)
OPK-HVB-013a	GATGTGACAAGCCGAGG (SEQ ID NO: 115)
OPK-HVB-014a	ATGTGACAAGCCGAGGC (SEQ ID NO: 116)
OPK-HVB-015a	TGTGACAAGCCGAGGCG (SEQ ID NO: 117)
OPK-HVB-016a	GTGACAAGCCGAGGCGG (SEQ ID NO: 118)

The sequences with the names "OPK-HVB-XXXbe" refer to sequences that are 19mer blunt end counterparts of the similar 21mers. The sequences with the names "OPVHVB-XXXa" refer to 19 mers where there is a 17 bp nucleotide sequence with a dTdT overhang. Other sequences not specifically exemplified herein but targeting VEGF while sparing VEGF165b can also be made with similar properties.

[0095] Other blunt end nucleic acid molecules can also be used, but that do not necessarily spare VEGF165b. For example, an siRNA comprising a sense strand SEQ ID NO: 119 and an antisense strand comprising SEQ ID NO: 120 can be used. An siRNA comprising SEQ ID NO: 119 and SEQ ID NO: 120, wherein each siRNA comprises blunt ends can also be referred to as bevasiranib-be. For example, in some embodiments, the siRNA is a 19mer with a blunt ends comprising SEQ ID NO: 119 and SEQ ID NO: 120 (See Figure 36).

[0096] The siRNA can be obtained using a number of techniques known to those of skill in the art. For example, the siRNA can be chemically synthesized or recombinantly produced using methods known in the art, such as the Drosophila *in vitro* system described in U.S. published application 2002/0086356 of Tuschl et al., the entire disclosure of which is herein incorporated by reference.

[0097] In certain embodiments, the siRNA are chemically synthesized using appropriately protected ribonucleoside phosphoramidites and a conventional DNA/RNA synthesizer. The siRNA can be synthesized as two separate, complementary RNA molecules, or as a single RNA molecule with two complementary regions. Commercial suppliers of synthetic RNA molecules or synthesis reagents include Proligo (Hamburg, Germany), Dharmacon Research (Lafayette, CO, USA), Pierce Chemical (part of Perbio Science, Rockford, IL, USA), Glen Research (Sterling, VA, USA), ChemGenes (Ashland, MA, USA) and Cruachem (Glasgow, UK).

[0098] Alternatively, siRNA can also be expressed from recombinant circular or linear DNA plasmids using any suitable promoter. Suitable promoters for expressing siRNA from a plasmid include, for example, the U6 or H1 RNA pol III promoter sequences and the cytomegalovirus promoter. Selection of other suitable promoters is within the skill in the art. The recombinant plasmids of the invention can also comprise inducible or regulatable promoters for expression of the siRNA in a particular tissue or in a particular intracellular environment.

[0099] The siRNA expressed from recombinant plasmids can either be isolated from cultured cell expression systems by standard techniques, or can be expressed intracellularly at or near the area of neovascularization *in vivo*. The use of recombinant plasmids to deliver siRNA to cells *in vivo* is discussed in more detail below.

[00100] siRNA can be expressed from a recombinant plasmid either as two separate, complementary RNA molecules, or as a single RNA molecule with two complementary regions.

[00101] Selection of plasmids suitable for expressing siRNA, methods for inserting nucleic acid sequences for expressing the siRNA into the plasmid, and methods of delivering the recombinant plasmid to the cells of interest are within the skill in the art. See, for example Tuschl, T. (2002), *Nat. Biotechnol*, 20**:** 446-448; Brummelkamp TR et al. (2002), *Science* 296**:** 550-553; Miyagishi M et al. (2002), *Nat. Biotechnol.* 20**:** 497-500; Paddison PJ et al. (2002), *Genes Dev.* 16**:** 948-958; Lee NS *et al.* (2002), *Nat. Biotechnol.* 20**:** 500-505; and Paul CP et al. (2002), *Nat. Biotechnol.* 20**:** 505-508, the entire disclosures of which are herein incorporated by reference.

[00102] A plasmid comprising nucleic acid sequences for expressing an siRNA is described in Example 7 below. That plasmid, called pAAVsiRNA, comprises a sense RNA

strand coding sequence in operable connection with a polyT termination sequence under the control of a human U6 RNA promoter, and an antisense RNA strand coding sequence in operable connection with a polyT termination sequence under the control of a human U6 RNA promoter. The plasmid pAAVsiRNA is ultimately intended for use in producing an recombinant adeno-associated viral vector comprising the same nucleic acid sequences for expressing an siRNA.

[00103] As used herein, "in operable connection with a polyT termination sequence" means that the nucleic acid sequences encoding the sense or antisense strands are immediately adjacent to the polyT termination signal in the 5' direction. During transcription of the sense or antisense sequences from the plasmid, the polyT termination signals act to terminate transcription.

[00104] As used herein, "under the control" of a promoter means that the nucleic acid sequences encoding the sense or antisense strands are located 3' of the promoter, so that the promoter can initiate transcription of the sense or antisense coding sequences.

[00105] The siRNA can also be expressed from recombinant viral vectors intracellularly at or near the area of neovascularization *in vivo*. The recombinant viral vectors of the invention comprise sequences encoding the siRNA and any suitable promoter for expressing the siRNA sequences. Suitable promoters include, for example, the U6 or H1 RNA pol III promoter sequences and the cytomegalovirus promoter. Selection of other suitable promoters is within the skill in the art. The recombinant viral vectors of the invention can also comprise inducible or regulatable promoters for expression of the siRNA in a particular tissue or in a particular intracellular environment. The use of recombinant viral vectors to deliver siRNA to cells *in vivo* is discussed in more detail below.

[00106] siRNA can be expressed from a recombinant viral vector either as two separate, complementary RNA molecules, or as a single RNA molecule with two complementary regions.

[00107] Any viral vector capable of accepting the coding sequences for the siRNA molecule(s) to be expressed can be used, for example vectors derived from adenovirus (AV); adeno-associated virus (AAV); retroviruses (*e.g,* lentiviruses (LV), Rhabdoviruses, murine leukemia virus); herpes virus, and the like. The tropism of the viral vectors can also be modified by pseudotyping the vectors with envelope proteins or other surface antigens from

other viruses. For example, an AAV vector of the invention can be pseudotyped with surface proteins from vesicular stomatitis virus (VSV), rabies, Ebola, Mokola, and the like.

[00108] Selection of recombinant viral vectors suitable for use in the invention, methods for inserting nucleic acid sequences for expressing the siRNA into the vector, and methods of delivering the viral vector to the cells of interest are within the skill in the art. See, for example, Dornburg R (1995), *Gene Therap.* 2: 301-310; Eglitis MA (1988), *Biotechniques* 6: 608-614; Miller AD (1990), *Hum Gene Therap.* 1: 5-14; and Anderson WF (1998), *Nature* 392: 25-30, the entire disclosures of which are herein incorporated by reference.

[00109] Preferred viral vectors are those derived from AV and AAV. In a particularly preferred embodiment, the siRNA is expressed as two separate, complementary single-stranded RNA molecules from a recombinant AAV vector comprising, for example, either the U6 or H1 RNA promoters, or the cytomegalovirus (CMV) promoter.

[00110] A suitable AV vector for expressing the siRNA, a method for constructing the recombinant AV vector, and a method for delivering the vector into target cells, are described in Xia H et al. (2002), *Nat. Biotech.* 20: 1006-1010.

[00111] Suitable AAV vectors for expressing the siRNA, methods for constructing the recombinant AAV vector, and methods for delivering the vectors into target cells are described in Samulski R et al. (1987), *J. Virol.* 61: 3096-3101; Fisher KJ et al. (1996), *J. Virol.*, 70: 520-532; Samulski R et al. (1989), *J. Virol.* 63: 3822-3826; U.S. Pat. No. 5,252,479; U.S. Pat. No. 5,139,941; International Patent Application No. WO 94/13788; and International Patent Application No. WO 93/24641, the entire disclosures of which are herein incorporated by reference. An exemplary method for generating a recombinant AAV vector of the invention is described in Example 7 below.

[00112] The ability of an siRNA containing a given target sequence to cause RNAi-mediated degradation of the target mRNA can be evaluated using standard techniques for measuring the levels of RNA or protein in cells. For example, siRNA can be delivered to cultured cells, and the levels of target mRNA can be measured by Northern blot or dot blotting techniques, or by quantitative RT-PCR. Alternatively, the levels of VEGF and its isoforms as well as Flt-1 or Flk-1/KDR receptor protein in the cultured cells can be measured by ELISA or Western blot. A suitable cell culture system for measuring the effect of the present siRNA on target mRNA or protein levels is described in Example 1 below.

[00113] RNAi-mediated degradation of target mRNA by an siRNA containing a given target sequence can also be evaluated with animal models of neovascularization, such as the ROP or CNV mouse models. For example, areas of neovascularization in an ROP or CNV mouse can be measured before and after administration of an siRNA and, in some embodiments, compared to an untreated animal. A reduction in the areas of neovascularization in these models upon administration of the siRNA indicates, in some embodiments, the down-regulation of the target mRNA (see Example 6 below).

[00114] As discussed above, the siRNA is capable of targeting and causing the RNAi-mediated degradation of VEGF and its isoforms as well as Flt-1 or Flk-1/KDR mRNA, or alternative splice forms, mutants or cognates thereof, preferably VEGF, and more preferably human VEGF. Degradation of the target mRNA by the present siRNA reduces the production of a functional gene product from the VEGF and its isoforms as well as Flt-1 or Flk-1/KDR genes. Thus, another embodiment of the present invention provides a method of inhibiting expression of VEGF and its isoforms, such as $VEGF_{121}$ (SEQ ID NO: 2), $VEGF_{165}$ (SEQ ID NO: 3), and $VEGF_{189}$(SEQ ID NO: 4), $VEGF_{206}$(SEQ ID NO: 5; GenBank Accession No. CS245579), $VEGF_{183}$ (GenBank Accession No. AJ010438), $VEGF_{148}$ (GenBank Accession No. AF091352), and/or $VEGF_{145}$ (GenBank Accession No. CS245578), as well as Flt-1 or Flk-1/KDR in a subject, comprising administering an effective amount of an siRNA to the subject, such that the target mRNA is degraded. As the products of the VEGF and its isoforms as well as Flt-1 and Flk-1/KDR genes are required for initiating and maintaining angiogenesis, another embodiment of the present invention provides a method of inhibiting angiogenesis in a subject by the RNAi-mediated degradation of the target mRNA by the present siRNA.

[00115] RNAi-mediated degradation of the target mRNA can be detected by measuring levels of the target mRNA or protein in the cells of a subject, using standard techniques for isolating and quantifying mRNA or protein as described above.

[00116] Inhibition of angiogenesis can be evaluated by directly measuring the progress of pathogenic or nonpathogenic angiogenesis in a subject; for example, by observing the size of a neovascularized area before and after treatment with the siRNA. An inhibition of angiogenesis is indicated if the size of the neovascularized area stays the same or is reduced. Techniques for observing and measuring the size of neovascularized areas in a subject are within the skill in the art; for example, areas of choroid neovascularization can be observed, for example, by fluorescein angiography.

[00117] Inhibition of angiogenesis can also be inferred through observing a change or reversal in a pathogenic condition associated with the angiogenesis. For example, in ARMD, a slowing, halting or reversal of vision loss indicates an inhibition of angiogenesis in the choroid. For tumors, a slowing, halting or reversal of tumor growth, or a slowing or halting of tumor metastasis, indicates an inhibition of angiogenesis at or near the tumor site. Inhibition of non-pathogenic angiogenesis can also be inferred from, for example, fat loss or a reduction in cholesterol levels upon administration of the siRNA.

[00118] It is understood that the siRNA can degrade the target mRNA (and thus inhibit angiogenesis) in substoichiometric amounts. Without wishing to be bound by any theory, it is believed that the siRNA causes degradation of the target mRNA in a catalytic manner. Thus, compared to standard anti-angiogenic therapies, significantly less siRNA needs to be delivered at or near the site of neovascularization to have a therapeutic effect.

[00119] One skilled in the art can readily determine an effective amount of the siRNA to be administered to a given subject, by taking into account factors such as the size and weight of the subject; the extent of the neovascularization or disease penetration; the age, health and sex of the subject; the route of administration; and whether the administration is regional or systemic. Generally, an effective amount of the siRNA comprises an intercellular concentration at or near the neovascularization site of from about 1 nanomolar (nM) to about 100 nM, preferably from about 2 nM to about 50 nM, more preferably from about 2.5 nM to about 10 nM. It is contemplated that greater or lesser amounts of siRNA can be administered.

[00120] The present methods can be used to inhibit angiogenesis which is non-pathogenic; *i.e.*, angiogenesis which results from normal processes in the subject. Examples of non-pathogenic angiogenesis include endometrial neovascularization, and processes involved in the production of fatty tissues or cholesterol. Thus, the invention provides a method for inhibiting non-pathogenic angiogenesis, *e.g.*, for controlling weight or promoting fat loss, for reducing cholesterol levels, or as an abortifacient.

[00121] The present methods can also inhibit angiogenesis which is associated with an angiogenic disease; *i.e.*, a disease in which pathogenicity is associated with inappropriate or uncontrolled angiogenesis. For example, most cancerous solid tumors generate an adequate blood supply for themselves by inducing angiogenesis in and around the tumor site. This tumor-induced angiogenesis is often required for tumor growth, and also allows metastatic cells to enter the bloodstream.

[00122] Other angiogenic diseases include diabetic retinopathy, age-related macular degeneration (ARMD), psoriasis, rheumatoid arthritis and other inflammatory diseases. These diseases are characterized by the destruction of normal tissue by newly formed blood vessels in the area of neovascularization. For example, in ARMD, the choroid is invaded and destroyed by capillaries. The angiogenesis-driven destruction of the choroid in ARMD eventually leads to partial or full blindness.

[00123] Preferably, an siRNA is used to inhibit the growth or metastasis of solid tumors associated with cancers; for example breast cancer, lung cancer, head and neck cancer, brain cancer, abdominal cancer, colon cancer, colorectal cancer, esophagus cancer, gastrointestinal cancer, glioma, liver cancer, tongue cancer, neuroblastoma, osteosarcoma, ovarian cancer, pancreatic cancer, prostate cancer, retinoblastoma, Wilm's tumor, multiple myeloma; skin cancer (*e.g.*, melanoma), lymphomas and blood cancer.

[00124] More preferably, an siRNA is used to inhibit choroidal neovascularization in age-related macular degeneration.

[00125] For treating angiogenic diseases, the siRNA can administered to a subject in combination with a pharmaceutical agent which is different from the present siRNA. Alternatively, the siRNA can be administered to a subject in combination with another therapeutic method designed to treat the angiogenic disease. For example, the siRNA can be administered in combination with therapeutic methods currently employed for treating cancer or preventing tumor metastasis (*e.g.*, radiation therapy, chemotherapy, and surgery). For treating tumors, the siRNA is preferably administered to a subject in combination with radiation therapy, or in combination with chemotherapeutic agents such as cisplatin, carboplatin, cyclophosphamide, 5-fluorouracil, adriamycin, daunorubicin or tamoxifen.

[00126] In the present methods, the present siRNA can be administered to the subject either as naked siRNA, in conjunction with a delivery reagent, or as a recombinant plasmid or viral vector which expresses the siRNA.

[00127] Suitable delivery reagents for administration in conjunction with the present siRNA include, but not limited to, the Mirus Transit TKO lipophilic reagent; lipofectin; lipofectamine; cellfectin; or polycations (*e.g.*, polylysine), or liposomes. In some embodiments the delivery reagent is RiboJuice™ (Novagen), a siRNA transfection reagent, which comprises amine and lipid based reagents. A preferred delivery reagent is a liposome. In some embodiments, the siRNA is delivered free of a liposomal delivery agent.

[00128] Liposomes can aid in the delivery of the siRNA to a particular tissue, such as retinal or tumor tissue, and can also increase the blood half-life of the siRNA. Liposomes suitable for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral or negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of factors such as the desired liposome size and half-life of the liposomes in the blood stream. A variety of methods are known for preparing liposomes, for example as described in Szoka et al. (1980), *Ann. Rev. Biophys. Bioeng.* 9: 467; and U.S. Pat. Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369, the entire disclosures of which are herein incorporated by reference.

[00129] Preferably, the liposomes encapsulating the present siRNA comprises a ligand molecule that can target the liposome to a particular cell or tissue at or near the site of angiogenesis. Ligands which bind to receptors prevalent in tumor or vascular endothelial cells, such as monoclonal antibodies that bind to tumor antigens or endothelial cell surface antigens, are preferred.

[00130] Particularly preferably, the liposomes encapsulating the present siRNA are modified so as to avoid clearance by the mononuclear macrophage and reticuloendothelial systems, for example by having opsonization-inhibition moieties bound to the surface of the structure. In one embodiment, a liposome of the invention can comprise both opsonization-inhibition moieties and a ligand.

[00131] Opsonization-inhibiting moieties for use in preparing the liposomes of the invention are typically large hydrophilic polymers that are bound to the liposome membrane. As used herein, an opsonization inhibiting moiety is "bound" to a liposome membrane when it is chemically or physically attached to the membrane, *e.g.*, by the intercalation of a lipid-soluble anchor into the membrane itself, or by binding directly to active groups of membrane lipids. These opsonization-inhibiting hydrophilic polymers form a protective surface layer which significantly decreases the uptake of the liposomes by the macrophage-monocyte system ("MMS") and reticuloendothelial system ("RES"); *e.g.*, as described in U.S. Pat. No. 4,920,016, the entire disclosure of which is herein incorporated by reference. Liposomes modified with opsonization-inhibition moieties thus remain in the circulation much longer than unmodified liposomes. For this reason, such liposomes are sometimes called "stealth" liposomes.

[00132] Stealth liposomes are known to accumulate in tissues fed by porous or "leaky" microvasculature. Thus, target tissue characterized by such microvasculature defects, for example solid tumors, will efficiently accumulate these liposomes; *see* Gabizon, et al. (1988), *P.N.A.S., USA*, 18: 6949-53. In addition, the reduced uptake by the RES lowers the toxicity of stealth liposomes by preventing significant accumulation in the liver and spleen. Thus, liposomes of the invention that are modified with opsonization-inhibition moieties can deliver the present siRNA to tumor cells.

[00133] Opsonization inhibiting moieties suitable for modifying liposomes are preferably water-soluble polymers with a number-average molecular weight from about 500 to about 40,000 daltons, and more preferably from about 2,000 to about 20,000 daltons. Such polymers include polyethylene glycol (PEG) or polypropylene glycol (PPG) derivatives; *e.g.*, methoxy PEG or PPG, and PEG or PPG stearate; synthetic polymers such as polyacrylamide or poly N-vinyl pyrrolidone; linear, branched, or dendrimeric polyamidoamines; polyacrylic acids; polyalcohols, e.g., polyvinylalcohol and polyxylitol to which carboxylic or amino groups are chemically linked, as well as gangliosides, such as ganglioside GM_1. Copolymers of PEG, methoxy PEG, or methoxy PPG, or derivatives thereof, are also suitable. In addition, the opsonization inhibiting polymer can be a block copolymer of PEG and either a polyamino acid, polysaccharide, polyamidoamine, polyethyleneamine, or polynucleotide. The opsonization inhibiting polymers can also be natural polysaccharides containing amino acids or carboxylic acids, e.g., galacturonic acid, glucuronic acid, mannuronic acid, hyaluronic acid, pectic acid, neuraminic acid, alginic acid, carrageenan; aminated polysaccharides or oligosaccharides (linear or branched); or carboxylated polysaccharides or oligosaccharides, e.g., reacted with derivatives of carbonic acids with resultant linking of carboxylic groups.

[00134] Preferably, the opsonization-inhibiting moiety is a PEG, PPG, or derivatives thereof. Liposomes modified with PEG or PEG-derivatives are sometimes called "PEGylated liposomes."

[00135] The opsonization inhibiting moiety can be bound to the liposome membrane by any one of numerous well-known techniques. For example, an N-hydroxysuccinimide ester of PEG can be bound to a phosphatidyl-ethanolamine lipid-soluble anchor, and then bound to a membrane. Similarly, a dextran polymer can be derivatized with a stearylamine lipid-soluble anchor via reductive amination using $Na(CN)BH_3$ and a solvent mixture such as tetrahydrofuran and water in a 30:12 ratio at 60 °C.

[00136] Recombinant plasmids which express siRNA are discussed above. Such recombinant plasmids can also be administered directly or in conjunction with a suitable delivery reagent, including the Mirus Transit LT1 lipophilic reagent; lipofectin; lipofectamine; cellfectin; polycations (*e.g.*, polylysine) or liposomes. Recombinant viral vectors which express siRNA are also discussed above, and methods for delivering such vectors to an area of neovascularization in a patient are within the skill in the art.

[00137] The siRNA can be administered to the subject by any means suitable for delivering the siRNA to the cells of the tissue at or near the area of neovascularization. For example, the siRNA can be administered by gene gun, electroporation, or by other suitable parenteral or enteral administration routes.

[00138] Suitable enteral administration routes include oral, rectal, or intranasal delivery.

[00139] Suitable parenteral administration routes include intravascular administration (e.g. intravenous bolus injection, intravenous infusion, intra-arterial bolus injection, intra-arterial infusion and catheter instillation into the vasculature); peri- and intra-tissue administration (*e.g.*, peri-tumoral and intra-tumoral injection, intra-retinal injection or subretinal injection); subcutaneous injection or deposition including subcutaneous infusion (such as by osmotic pumps); direct (*e.g.*, topical) application to the area at or near the site of neovascularization, for example by a catheter or other placement device (*e.g.*, a corneal pellet or a suppository, eye-dropper, or an implant comprising a porous, non-porous, or gelatinous material); and inhalation. Suitable placement devices include the ocular implants described in U.S. Pat. Nos. 5,902,598 and 6,375,972, and the biodegradable ocular implants described in U.S. Pat. No 6,331,313, the entire disclosures of which are herein incorporated by reference. Such ocular implants are available from Control Delivery Systems, Inc. (Watertown, MA) and Oculex Pharmaceuticals, Inc. (Sunnyvale, CA).

[00140] In a preferred embodiment, injections or infusions of the siRNA are given at or near the site of neovascularization. More preferably, the siRNA is administered topically to the eye, *e.g.* in liquid or gel form to the lower eye lid or conjunctival cul-de-sac, as is within the skill in the art (see, *e.g.*, Acheampong AA et al, 2002, *Drug Metabol. and Disposition* 30: 421-429, the entire disclosure of which is herein incorporated by reference).

[00141] Typically, the siRNA is administered topically to the eye in amounts of from about 5 microliters to about 75 microliters, for example from about 7 microliters to about 50

microliters, preferably from about 10 microliters to about 30 microliters. It is understood that topical instillation in the eye of siRNA in volumes greater than 75 microliters can result in loss of siRNA from the eye through spillage and drainage. Thus, it is preferable to administer a high concentration of siRNA (*e.g.*, 100-1000 nM) in as small a volume as possible.

[00142] A particularly preferred parenteral administration route is intraocular administration. It is understood that intraocular administration of the present siRNA can be accomplished by injection or direct (*e.g.*, topical) administration to the eye, as long as the administration route allows the siRNA to enter the eye. In addition to the topical routes of administration to the eye described above, suitable intraocular routes of administration include intravitreal, intraretinal, subretinal, subtenon, peri- and retro-orbital, trans-corneal and trans-scleral administration. Such intraocular administration routes are within the skill in the art; see, *e.g.*, and Acheampong AA et al, 2002, *supra*; and Bennett et al. (1996), *Hum. Gene Ther.* 7: 1763-1769 and Ambati J et al., 2002, *Progress in Retinal and Eye Res.* 21: 145-151, the entire disclosures of which are herein incorporated by reference. In another preferred embodiment, the siRNA is administered by intravitreal injection.

[00143] The siRNA can be administered in a single dose or in multiple doses. Where the administration of the siRNA is by infusion, the infusion can be a single sustained dose or can be delivered by multiple infusions. Injection of the agent directly into the tissue is at or near the site of neovascularization preferred. Multiple injections of the agent into the tissue at or near the site of neovascularization are particularly preferred.

[00144] One skilled in the art can also readily determine an appropriate dosage regimen for administering the siRNA to a given subject. For example, the siRNA can be administered to the subject once, such as by a single injection or deposition at or near the neovascularization site. Alternatively, the siRNA can be administered to a subject multiple times daily or weekly. For example, the siRNA can be administered to a subject once weekly for a period of from about three to about twenty-eight weeks, and alternatively from about seven to about ten weeks. In a certain dosage regimen, the siRNA is injected at or near the site of neovascularization (*e.g.*, intravitreally) once a week for seven weeks. It is understood that periodic administrations of the siRNA for an indefinite length of time may be necessary for subjects suffering from a chronic neovascularization disease, such as wet ARMD or diabetic retinopathy.

[00145] Where a dosage regimen comprises multiple administrations, it is understood that the effective amount of siRNA administered to the subject can comprise the total amount of siRNA administered over the entire dosage regimen.

[00146] The siRNA are preferably formulated as pharmaceutical compositions prior to administering to a subject, according to techniques known in the art. Pharmaceutical compositions of the present invention are characterized as being at least sterile and pyrogen-free. As used herein, "pharmaceutical formulations" include formulations for human and veterinary use. Methods for preparing pharmaceutical compositions of the invention are within the skill in the art, for example as described in *Remington's Pharmaceutical Science*, 17th ed., Mack Publishing Company, Easton, Pa. (1985), the entire disclosure of which is herein incorporated by reference.

[00147] In one embodiment, the pharmaceutical formulations comprise an siRNA (*e.g.*, 0.1 to 90% by weight), or a physiologically acceptable salt thereof, mixed with a physiologically acceptable carrier medium. Preferred physiologically acceptable carrier media are water, buffered water, saline solutions (*e.g.*, normal saline or balanced saline solutions such as Hank's or Earle's balanced salt solutions), 0.4% saline, 0.3% glycine, hyaluronic acid and the like.

[00148] Pharmaceutical compositions can also comprise conventional pharmaceutical excipients and/or additives. Suitable pharmaceutical excipients include stabilizers, antioxidants, osmolality adjusting agents, buffers, and pH adjusting agents. Suitable additives include physiologically biocompatible buffers (*e.g.*, tromethamine hydrochloride), additions of chelants (such as, for example, DTPA or DTPA-bisamide) or calcium chelate complexes (as for example calcium DTPA, CaNaDTPA-bisamide), or, optionally, additions of calcium or sodium salts (for example, calcium chloride, calcium ascorbate, calcium gluconate or calcium lactate). Pharmaceutical compositions of the invention can be packaged for use in liquid form, or can be lyophilized.

[00149] For topical administration to the eye, conventional intraocular delivery reagents can be used. For example, pharmaceutical compositions of the invention for topical intraocular delivery can comprise saline solutions as described above, corneal penetration enhancers, insoluble particles, petrolatum or other gel-based ointments, polymers which undergo a viscosity increase upon instillation in the eye, or mucoadhesive polymers. Preferably, the intraocular delivery reagent increases corneal penetration, or prolongs

preocular retention of the siRNA through viscosity effects or by establishing physicochemical interactions with the mucin layer covering the corneal epithelium.

[00150] Suitable insoluble particles for topical intraocular delivery include the calcium phosphate particles described in U.S. Pat. No. 6,355,271 of Bell et al., the entire disclosure of which is herein incorporated by reference. Suitable polymers which undergo a viscosity increase upon instillation in the eye include polyethylenepolyoxypropylene block copolymers such as poloxamer 407 (*e.g.*, at a concentration of 25%), cellulose acetophthalate (*e.g.*, at a concentration of 30%), or a low-acetyl gellan gum such as Gelrite® (available from CP Kelco, Wilmington, DE). Suitable mucoadhesive polymers include hydrocolloids with multiple hydrophilic functional groups such as carboxyl, hydroxyl, amide and/or sulfate groups; for example, hydroxypropylcellulose, polyacrylic acid, high-molecular weight polyethylene glycols (*e.g.*, >200,000 number average molecular weight), dextrans, hyaluronic acid, polygalacturonic acid, and xylocan. Suitable corneal penetration enhancers include cyclodextrins, benzalkonium chloride, polyoxyethylene glycol lauryl ether (*e.g.*, Brij® 35), polyoxyethylene glycol stearyl ether (*e.g.*, Brij® 78), polyoxyethylene glycol oleyl ether (*e.g.*, Brij® 98), ethylene diamine tetraacetic acid (EDTA), digitonin, sodium taurocholate, saponins and polyoxyethylated castor oil such as Cremaphor EL.

[00151] For solid compositions, conventional nontoxic solid carriers can be used; for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like.

[00152] For example, a solid pharmaceutical composition for oral administration can comprise any of the carriers and excipients listed above and 10-95%, preferably 25%-75%, of one or more siRNA. A pharmaceutical composition for aerosol (inhalational) administration can comprise 0.01-20% by weight, preferably 1%-10% by weight, of one or more siRNA encapsulated in a liposome as described above, and propellant. A carrier can also be included as desired; *e.g.*, lecithin for intranasal delivery.

[00153] The invention will now be illustrated with the following non-limiting examples.

[00154] **Example 1 - siRNA Transfection and Hypoxia Induction *In Vitro***

[00155] *siRNA Design* - A 19 nt sequence located 329 nt from the 5' end of human VEGF mRNA was chosen as a target sequence: AAACCTCACCAAGGCCAGCAC (SEQ ID NO: 51). To ensure that it was not contained in the mRNA from any other genes, this

target sequence was entered into the BLAST search engine provided by NCBI. The use of the BLAST algorithm is described in Altschul et al. (1990), *J. Mol. Biol.* 215: 403-410 and Altschul et al. (1997), *Nucleic Acids Res.* 25: 3389-3402, the disclosures of which are herein incorporated by reference in their entirety. As no other mRNA was found which contained the target sequence, an siRNA duplex was synthesized to target this sequence (Dharmacon Research, Inc., Lafayette, CO).

[00156] The siRNA duplex had the following sense and antisense strands.

sense:

5'-accucaccaaggccagcacTT-3' (SEQ ID NO: 77).

antisense:

5'-gugcuggccuuggugagguTT-3' (SEQ ID NO: 78).

[00157] Together, the siRNA sense and antisense strands formed a 19 nt double-stranded siRNA with TT 3' overhangs (shown in bold) on each strand. This siRNA was termed "Candidate 5" or "Cand5." Other siRNA which target human VEGF mRNA were designed and tested as described for Cand5 (bevasiranib).

[00158] An siRNA targeting the following sequence in green fluorescent protein (GFP) mRNA was used as a nonspecific control: GGCTACGTCCAGCGCACC (SEQ ID NO: 79). The siRNA was purchased from Dharmacon (Lafayette, CO).

[00159] *siRNA Transfection and Hypoxia Induction* In Vitro - Human cell lines (293; Hela and ARPE19) were separately seeded into 24-well plates in 250 microliters of complete DMEM medium one day prior to transfection, so that the cells were ~50% confluent at the time of transfection. Cells were transfected with 2.5 nM Cand5 siRNA, and with either no siRNA or 2.5 nM non-specific siRNA (targeting GFP) as controls. Transfections were performed in all cell lines with the "Transit TKO Transfection" reagent, as recommended by the manufacturer (Mirus).

[00160] Twenty four hours after transfection, hypoxia was induced in the cells by the addition of deferoxamine mesylate to a final concentration of 130 micromolar in each well. Twenty four hours post-transfection, the cell culture medium was removed from all wells, and a human VEGF ELISA (R&D systems, Minneapolis, MN) was performed on the culture medium as described in the Quantikine human VEGF ELISA protocol available from the manufacturer, the entire disclosure of which is herein incorporated by reference.

[00161] As can be seen in Fig. 1, RNAi degradation induced by Cand5 siRNA significantly reduces the concentration of VEGF produced by the hypoxic 293 and HeLa cells. There was essentially no difference in the amount of VEGF produced by hypoxic cells treated with either no siRNA or the non-specific siRNA control. Similar results were also seen with human ARPE19 cells treated under the same conditions. Thus, RNA interference with VEGF-targeted siRNA disrupts the pathogenic up-regulation of VEGF in human cultured cells *in vitro*.

[00162] The experiment outlined above was repeated on mouse NIH 3T3 cells using a mouse-specific VEGF siRNA (see Example 6 below), and VEGF production was quantified with a mouse VEGF ELISA (R&D systems, Minneapolis, MN) as described in the Quantikine mouse VEGF ELISA protocol available from the manufacturer, the entire disclosure of which is herein incorporated by reference. Results similar to those reported in Fig. 1 for the human cell lines were obtained.

[00163] Example 2 - Effect of Increasing siRNA Concentration on VEGF Production in Human Cultured Cells

[00164] The experiment outlined in Example 1 was repeated with human 293, HeLa and ARPE19 cells using a range of siRNA concentrations from 10 nM to 50 nM. The ability of the Cand5 siRNA to down-regulate VEGF production increased moderately up to approximately 13 nM siRNA, but a plateau effect was seen above this concentration. These results highlight the catalytic nature of siRNA-mediated RNAi degradation of mRNA, as the plateau effect appears to reflect VEGF production from the few cells not transfected with the siRNA. For the majority of cells which had been transfected with the siRNA, the increased VEGF mRNA production induced by the hypoxia is outstripped by the siRNA-induced degradation of the target mRNA at siRNA concentrations greater than about 13 nM.

[00165] Example 3 - Specificity of siRNA Targeting

[00166] NIH 3T3 mouse fibroblasts were grown in 24-well plates under standard conditions, so that the cells were ~50% confluent one day prior to transfection. The human VEGF siRNA Cand5 was transfected into a NIH 3T3 mouse fibroblasts as in Example 1. Hypoxia was then induced in the transfected cells, and murine VEGF concentrations were measured by ELISA as in Example 1.

[00167] The sequence targeted by the human VEGF siRNA Cand5 differs from the murine VEGF mRNA by one nucleotide. As can be seen in Fig. 2, the human VEGF siRNA

has no affect on the ability of the mouse cells to up-regulate mouse VEGF after hypoxia. These results show that siRNA induced RNAi degradation is sequence-specific to within a one nucleotide resolution.

[00168] **Example 4 - *In Vivo* delivery of siRNA to Murine Retinal Pigment Epithelial Cells**

[00169] VEGF is upregulated in the retinal pigment epithelial (RPE) cells of human patients with age-related macular degeneration (ARMD). To show that functional siRNA can be delivered to RPE cells *in vivo*, GFP was expressed in mouse retinas with a recombinant adenovirus, and GFP expression was silenced with siRNA. The experiment was conducted as follows.

[00170] One eye from each of five adult C57/Black6 mice (Jackson Labs, Bar Harbor, ME) was injected subretinally as described in Bennett et al. (1996), *supra.*, with a mixture containing ~$1x10^8$ particles of adenovirus containing eGFP driven by the CMV promoter and 20 picomoles of siRNA targeting eGFP conjugated with transit TKO reagent (Mirus).

[00171] As positive control, the contralateral eyes were injected with a mixture containing ~$1x10^8$ particles of adenovirus containing eGFP driven by the CMV promoter and 20 picomoles of siRNA targeting human VEGF conjugated with transit TKO reagent (Mirus). Expression of GFP was detected by fundus ophthalmoscopy 48 hours and 60 hours after injection. Animals were sacrificed at either 48 hours or 60 hours post-injection. The eyes were enucleated and fixed in 4% paraformaldehyde, and were prepared either as flat mounts or were processed into 10 micron cryosections for fluorescent microscopy.

[00172] No GFP fluorescence was detectable by ophthalmoscopy in the eyes which received the siRNA targeted to GFP mRNA in 4 out of 5 mice, whereas GFP fluorescence was detectable in the contralateral eye which received the non-specific control siRNA. A representative flat mount analyzed by fluorescence microscopy showed a lack of GFP fluorescence in the eye which received GFP siRNA, as compared to an eye that received the non-specific control siRNA. Cryosections of another retina showed that the recombinant adenovirus efficiently targets the RPE cells, and when the adenovirus is accompanied by siRNA targeted to GFP mRNA, expression of the GFP transgene is halted.

[00173] While there is some GFP fluorescence detectable by fluorescence microscopy in eyes that received siRNA targeted to GFP mRNA, the fluorescence is greatly

suppressed as compared to controls that received non-specific siRNA. These data demonstrate that functional siRNA can be delivered *in vivo* to RPE cells.

[00174] Example 5 - *In Vivo* Expression and siRNA-Induced RNAi Degradation of Human VEGF in Murine Retinas

[00175] In order to demonstrate that siRNA targeted to VEGF functioned *in vivo*, an exogenous human VEGF expression cassette was delivered to mouse RPE cells via an adenovirus by subretinal injection, as in Example 4. One eye received Cand5 siRNA, and the contralateral eye received siRNA targeted to GFP mRNA. The animals were sacrificed 60 hours post-injection, and the injected eyes were removed and snap frozen in liquid N_2 following enucleation. The eyes were then homogenized in lysis buffer, and total protein was measured using a standard Bradford protein assay (Roche, Germany). The samples were normalized for total protein prior to assaying for human VEGF by ELISA as described in Example 1.

[00176] The expression of VEGF was somewhat variable from animal to animal. The variability of VEGF levels correlated well to those observed in the GFP experiments of Example 4, and can be attributed to some error from injection to injection, and the differential ability of adenovirus to delivery the target gene in each animal. However, there was a significant attenuation of VEGF expression in each eye that received VEGF siRNA, as compared to the eyes receiving the non-specific control siRNA (Figure 4). These data indicate that the Cand5 siRNA was potent and effective in silencing human VEGF expression in murine RPE cells *in vivo*.

[00177] Example 6 - Inhibition of Choroidal Neovascularization in the Mouse CNV Model

There is evidence that choroidal neovascularization in ARMD is due to the upregulation of VEGF in the RPE cells. This human pathologic condition can be modeled in the mouse by using a laser to burn a spot on the retina ("laser photo-coagulation" or "laser induction"). During the healing process, VEGF is believed to be up-regulated in the RPE cells of the burned region, leading to re-vascularization of the choroid. This model is called the mouse choroidal neovascularization ("CNV") model.

[00178] For rescue of the mouse CNV model, a mouse siRNA was designed that incorporated a one nucleotide change from the human "Cand5" siRNA from Example 1. The mouse siRNA specifically targeted mouse VEGF mRNA at the sequence

AAACCUCACCAAAGCCAGCAC (SEQ ID NO: 80). Other siRNA that target mouse VEGF were also designed and tested. The GFP siRNA used as a nonspecific control in Example 1 was also used as a non-specific control here.

[00179] Twenty four hours after laser induction, one eye from each of eleven adult C57/Black6 mice (Jackson Labs, Bar Harbor, ME) was injected subretinally with a mixture containing ~$1x10^8$ particles of adenovirus containing LacZ driven by the CMV promoter and 20 picomoles of siRNA targeting mouse VEGF conjugated with transit TKO reagent (Mirus), as in Example 4. As a control, contralateral eyes received a mixture containing ~$1x10^8$ particles of adenovirus containing LacZ driven by the CMV promoter and 20 picomoles of siRNA targeting GFP conjugated with transit TKO reagent (Mirus).

[00180] Fourteen days after the laser treatment, the mice were perfused with fluorescein and the area of neovascularization was measured around the burn spots. Areas of the burn spots in the contra-lateral eye were used as a control. The site of neovascularization around the burn spots in animals that received siRNA targeting mouse VEGF was, on average, 1/4 the area of the control areas. These data support the use of VEGF-directed siRNA (also called "anti-VEGF siRNA") for therapy of ARMD.

[00181] **Example 7 - Generation of an Adeno-Associated Viral Vector for Expression of siRNA**

A "cis-acting" plasmid for generating a recombinant AAV vector for delivering an siRNA was generated by PCR based subcloning, essentially as described in Samulski R et al. (1987), *supra*. The cis-acting plasmid was called "pAAVsiRNA."

[00182] The *rep* and *cap* genes of psub201 were replaced with the following sequences in this order: a 19 nt sense RNA strand coding sequence in operable connection with a polyT termination sequence under the control of a human U6 RNA promoter, and a 19 nt antisense RNA strand coding sequence in operable connection with a polyT termination sequence under the control of a human U6 RNA promoter. A schematic representation of pAAVsiRNA is given if Fig. 5.

[00183] A recombinant AAV siRNA vector was obtained by transfecting pAAVsiRNA into human 293 cells previously infected with E1-deleted adenovirus, as described in Fisher KJ et al. (1996), *supra*. The AAV rep and cap functions were provided by a trans-acting plasmid pAAV/Ad as described in Samulski R et al. (1989), *supra*.

Production lots of the recombinant AAV siRNA vector were titered according to the number of genome copies/ml, as described in Fisher KJ et al. (1996), *supra*.

[00184] Example 8 – VEGF-Directed siRNA Inhibits Experimental Choroidal Neovascularization

The ability of murine VEGF-directed siRNA to inhibit experimental laser-induced choroidal neovascularization (CNV) in mice was tested as follows.

[00185] The retinas of adult female C57BL/6 mice were laser photocoagulated using an 810 nm diode laser (75 um, 140 mw, 0.10 seconds) (OcuLight Six; IRIS Medical, Mountain View, CA). Three laser spots were applied to both eyes of each mouse. Thirty-six hours following laser photocoagulation, an siRNA targeted to mouse VEGF ("mVEGF1.siRNA") was delivered subretinally or intravitreally to one eye of each mouse. For subretinal injection, the siRNA was conjugated with Transit TKO transfection reagent (Mirus) and mixed with recombinant adenovirus (rAdenovirus). For intravitreal injection, the siRNA was delivered in the absence of transfection reagent and rAdenovirus. As a control, the contralateral eyes of each mouse received subretinal or intravitreal injections of identical formulations with an siRNA targeted to GFP ("GFP1.siRNA"), which has no homology to mouse VEGF.

[00186] Fourteen days following laser treatment, all animals were perfused with high molecular weight FITC-dextran, choroidal flat mounts were prepared as described above, and the flat mounts were photographed and analyzed microscopically in a masked fashion. The area of CNV in each flat mount was measured with Openlab software (Improvision, Boston, MA). The mean areas of CNV in eyes treated with mVEGF1.siRNA were significantly smaller than those areas from GFP1.siRNA-treated eyes for both subretinal (Fig. 6A; $P<0.003$) and intravitreal (Fig. 6B; $P<0.04$) delivery.

[00187] In a second experiment, the retinas of adult female C57BL/6 mice were laser photocoagulated as described above, and the animals were divided into control and test groups. One day following laser photocoagulation, phosphate buffered saline was delivered intravitreally to the animals of the control group, which were perfused with dextran-fluorescein 14 days after laser treatment. Choroidal flat mounts were then prepared and the areas of CNV in each flat mount were measured as above.

[00188] Fourteen days following laser photocoagulation, mVEGF1.siRNA was delivered by intravitreal injection into one eye of each mouse in the test group. Contralateral

eyes were injected with GFP1.siRNA as a control. The test group animals were perfused with high molecular weight dextran-fluorescein 21 days after laser treatment. Choroidal flat mounts were then prepared and the areas of CNV in each flat mount were measured, as above.

[00189] In this latter experiment, the anti-VEGF siRNA was administered during CNV growth, as opposed to before CNV growth, and thus is more representative of the condition of human patients presenting with wet AMD. As can be seen from Fig. 6, the mean areas of CNV in mVEGF1.siRNA-treated eyes were significantly smaller than those areas measured in GFP1.siRNA-treated eyes (Fig. 6C; $P<0.05$). The mean areas of CNV in mVEGF1.siRNA-treated eyes at day 21 and control ("PBS") eyes at day 14 were not significantly different (Fig. 6C; $P=0.469$).

[00190] The results of these experiments indicate that age-related macular degeneration can be treated with anti-VEGF siRNA.

[00191] **Example 9 – In Vivo RNA Interference of Human VEGF Induced by anti-VEGF siRNA in Murine RPE Cells**

[00192] The ability of Cand5 siRNA to induce RNAi of VEGF *in vivo* over time was evaluated as follows.

[00193] AAV.CMV.VEGF, which expresses human VEGF from an adeno-associated viral vector, was generously provided by Dr. A. Auricchio. AAV.CMV.VEGF was injected subretinally and bilaterally in eyes of five C57Bl/6 mice. Twenty-eight days after injection of AAV.CMV.VEGF, Cand5 siRNA was delivered by intravitreal injection into one eye and control GFP1.siRNA was delivered by intravitreal injection in the contralateral eye of each animal.

[00194] At day 0 (pre-siRNA injection), and at 6, 10 and 14 days after siRNA injection, the mice were sacrificed and the eyes were snap frozen in liquid nitrogen following enucleation. The eyes were then homogenized in lysis buffer (Roche, Basel, Switzerland), and total protein was measured using a Bradford assay, as in Example 5 above. Two mice were used for the 0 day time point (n=2), and three mice each were used for the 6, 10 and 14 day time points (n=3). The samples were normalized for total protein prior to assaying for human VEGF by ELISA, according to the manufacturer's recommendations (R&D systems, Minneapolis, Minnesota). Percent of VEGF (%VEGF) for each mouse was calculated as the

concentration of VEGF ("[VEGF]") in the eye injected with Cand5 divided by the [VEGF] in the eye injected with GFP1.siRNA, multiplied by 100.

[00195] As can be seen from Figure 7, a single injection of Cand5 induced an RNAi-mediated decrease in VEGF levels of approximately 70% by day 6 post-siRNA injection, with a reduction in VEGF production of approximately 35% continuing through at least day 14 post-siRNA injection. These results indicate that siRNA directed against human VEGF is capable of inducing RNAi of human VEGF *in vivo* for a sustained period of time.

[00196] Example 10 – *In Vivo* RNA Interference of VEGF in Monkeys with Anti-VEGF siRNA

[00197] The objectives of this study were to determine the safety and efficacy of Cand5 when administered by single intravitreal injection to male cynomolgus monkeys following induction of CNV. Cand5 was administered in the vehicle control article to naive male cynomolgus monkeys in the following dose levels: 0 mg/eye (control), 0.07 mg/eye, 0.18 mg/eye, 0.35 mg/eye and, and 0.70 mg/eye.

[00198] CNV was induced by laser treatment to the maculae of both eyes of each animal, and the doses of Cand5 were given shortly following laser treatment. The animals were evaluated for changes in clinical signs, body weight and ocular condition (extensive ophthalmic examinations, electroretinography and tonometry). Fluorescein angiography was performed and blood samples were collected. At the end of the study (Day 44), all animals were euthanized and a complete gross necropsy was performed. Selected tissues were collected and preserved for histopathologic evaluation.

[00199] No adverse systemic or local (ocular) effects of Cand5 were detected when monkeys were administered a single intravitreal injection into both eyes at doses up to 0.70 mg/eye following laser lesioning of the macula and during subsequent development of CNV.

[00200] Example 11 – *In Vitro* RNA Interference of VEGF with Anti-VEGF siRNA in Human Embryonic Kidney 293 Cells

[00201] Human embryonic kidney 293 cells (obtained from ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle Medium (DMEM; obtained from Cellgro, Herndon, VA) with 10% fetal bovine serum (FBS; from JRH Biosciences, Lenexa, KS) and an antibiotic-antimycotic reagent, used for the prevention of cell culture growth contaminants (from Gibco, Carlsbad, CA).

[00202] siRNAs were synthesized by Integrated DNA Technologies (Coralville, IA). The siRNA target sequences are shown in Table 2. An additional siRNA was used in this study that targets the gene of enhanced green fluorescent protein (EGFP) as a negative control.

Table 2.

Name	GC Content	Nucleotide Start Site	Target Sequence 5'-3'
hVEGF#1	58%	92	aaggaggagggcagaatcatc (SEQ ID NO: 81)
hVEGF#2	42%	124	aagttcatggatgtctatcag (SEQ ID NO: 47)
hVEGF#3	58%	162	aatcgagaccctggtggacat (SEQ ID NO: 48)
hVEGF#4	42%	301	aacatcaccatgcagattatg (SEQ ID NO: 50)
hVEGF#5	58%	338	aaggccagcacataggagaga (SEQ ID NO: 52)
hVEGF#6	42%	380	aatgtgaatgcagaccaaaga (SEQ ID NO: 82)
hVEGF#7	37%	396	aaagaaagatagagcaagaca (SEQ ID NO: 56)
hVEGF#8	32%	450	aaagcatttgtttgtacaaga (SEQ ID NO: 83)
hVEGF#9	42%	467	aagatccgcagacgtgtaaat (SEQ ID NO: 84)
hVEGF#10	53%	498	aaacacacactcgcgttgcaa (SEQ ID NO: 85)
Cand5	63%	328	aaacctcaccaaggccagcac (SEQ ID NO: 51)

[00203] *siRNA Transfection and Hypoxia Induction In Vitro.* Human 293 cells were cultured in 24 well plates at 37°C with 5% CO_2 overnight. The next day, transfections were performed when cells were about 50%-70% confluent. Cells were transfected with siRNAs directed against human VEGF. siRNAs were mixed in a CaPi reagent and added to 20 µl of 250 mM $CaCl_2$ solution. The siRNA/$CaCl_2$ mixture was added drop-wise to 20 µl of 2X Hanks Balanced Salt Solution (HBS), while mixing by vortex. The siRNA/$CaCl_2$/HBS complex was added directly to the medium in each well (300 µL/well). After a 4-hour incubation at 37°C, the medium was removed, and the cells were further incubated with 10% DMSO-containing serum-free medium (300 µL/well at room temperature for 1-2 minutes). This medium was then removed, and the cells were fed again with growth medium (500 µL/well). Negative controls included transfection reagent lacking siRNA and nonspecific siRNA (EGFP1 siRNA). For screening experiments siRNAs were used at a concentration of 25nM. For dose response experiments, siRNAs were used at concentrations of 1 nM, 5nM and 25nM. Hypoxia was induced with desferrioxamine at a final concentration of 130 uM 4 hours after transfection was performed. Desferrioxamine mimics a hypoxic state, as it is proposed to disrupt normal oxygen-sensing pathways in mammalian cells by inhibiting heme-Fe2+ interactions.

[00204] *VEGF Protein Quantification.* Approximately 48 hours post transfection, the supernatant was removed from all wells and a human VEGF ELISA (R & D systems, Minneapolis, MN) was performed on the 293 cells as described in the Quantikine human VEGF ELISA protocol. VEGF-specific antibody was added to each well causing color development in proportion to the amount of VEGF bound to the plate. ELISA results were read on an AD340 plate reader at 450 nm (Beckman Coulter).

[00205] *Results. Human VEGF siRNAs Suppresses Hypoxia-Induced Up-regulation of Human VEGF Protein in 293 Cells.* Human VEGF was upregulated by the desferrioxamine-mediated induction of hypoxia. Readings of OD 450nm reflected the human VEGF protein levels in cell samples. The hypoxia-induced increase of hVEGF protein levels were significantly reduced in cells transfected with all of the human VEGF siRNAs (Figure 8). No effect on hVEGF levels were observed with transfections with nonspecific siRNA (EGFP siRNA) or mock transfections without siRNA. Dose response studies were performed on Cand5, hVEGF#1, hVEGF#2, hVEGF#3, hVEGF#4, hVEGF#6 and hVEGF#7 (Figure 9).

[00206] **Example 12 – *In Vitro* RNA Interference of VEGF isoforms**

[00207] $VEGF_{165b}$ has been identified as an endogenous anti-angiogenic VEGF isoform. siRNA were designed to selectively inhibit certain VEGF isoforms, such as $VEGF_{165}$, but spare $VEGF_{165b}$.

[00208] *Methods:* ARPE19 cells were seeded in 24 well plates (50,000 cells per well). Eighteen to twenty-four hours post-seeding, cells were 50-75% confluent and used for transfection. Fourteen human VEGF-A specific siRNAs were designed and tested. Cells were transfected with the siRNAs (25 nM) using RiboJuice™ siRNA Transfection Reagent (Novagen) following the manufacturer's protocol. Specifically, for a single well of cells, 40.5 µL serum free OPTI-MEM® was pipetted into an eppendorf tube then 2 µL of RiboJuice™ was added to the OPTI-MEM. The solution was mixed by gentle vortexing and centrifuged briefly to collect contents at bottom of the tube and incubated at room temperature for 5 minutes. siRNA (7.5 µL of a 1 µM stock) was added to the RiboJuice™ /medium mix and gently mixed and briefly centrifuged to collect contents at the bottom of the tube. The mixture was incubated at room temperature for 15 minutes. During the incubation, media was removed from cells and replaced with 250 µL of fresh complete ARPE19 growth media (DMEM/F12; 10% FBS, 1% penicillin/streptomycin). After the 15 minute incubation the siRNA/RiboJuice™/medium mixture (50µL) was added dropwise to the cells. The final

concentration of siRNA in the 300µL volume was 25 nM. Cells were maintained at 37°C, 5% CO_2 for 24 hours. In additional experiments, reactions were scaled up to transfect cells in triplicate wells with each siRNA. 24 hours post-transfection, the transfection mixture was removed and the cells were treated with 500µLs of serum free DMEM/F12, DMEM/F12 containing 10 ng/mL human recombinant TGFβII or DMEM/F12 containing 10ng/mL TGFβII and 5 µg/mL cycloheximide. The cells were returned to 37°C and 5% CO_2 for an additional 24 hours. Afterwards, the media was removed from the cells and analyzed for protein expression by ELISA (Quantikine human VEGF ELISA kit (R&D Systems)). Media was removed from cells and collected in eppendorf tubes and placed on ice and immediately analyzed for VEGF protein via ELISA, or stored at -80°C and analyzed for VEGF protein at a later time point.

[00209] Based on these results, a select number of siRNA candidates were put through an additional transfection screen. Cells were collected, RNA extracted, and semi-quantitative RT-PCR was performed to determine the siRNAs' inhibitory effect on $VEGF_{165}$, $VEGF_{165b}$, $VEGF_{121}$ and $VEGF_{189}$. GAPDH housekeeping gene expression was used as a control. Specifically, after removing the media from the wells, 200 µLs of lysis/binding solution from the RNAqueous Kit (Ambion) was added to each well. RNA was quantified via spectrophotometry (OD 260 nM). The lysed cells were collected and RNA was extracted following the manufacturer's protocol. RNA was reverse transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. cDNA was analyzed for GAPDH, $VEGF_{165}$, $VEGF_{165b}$, $VEGF_{121}$ and $VEGF_{189}$ using PCR. Primers used for PCR are shown in Table 3.

TABLE 3.

Primer Name	Description	Sequence 5'-3'
P121	Reverse primer VEGF121	GGCTTGTCACATTTTTCTTG
P165	Reverse primer VEGF165	CCCACAGGGATTTTCTTGTC
P189	Reverse primer VEGF189	CTTTCCCTTTCCTCGAACTG
hVEGF-E	Forward primer used for VEGF121, VEGF165 & VEGF 189	GCTACTGCCATCCAATCGAG
P165bR	Reverse primer for VEGF165b	GTCTTTCCTGGTGAGAGATC
hVEGF-A	Forward primer for VEGF165b	CTGTCTTGGGTGCATTGGAG

GAPDH-B	Reverse primer GAPDH	GAGGCAGGGATGATGTTCTG
GAPDH-A	Forward primer GAPDH	CATGGCAAATTCCATGGCAC

[00210] For PCR analysis, 3 µL cDNA was combined with 1 µL of each appropriate forward (10 µM) and reverse primer (10 µM) primer and 45 µL of Platinum PCR Supermix (Invitrogen) such that the final concentration of each primer was 200 nM. The cDNA was amplified in a thermocycler with the following PCR conditions:

[00211] Step 1: 94°C for 2 minutes

[00212] Step 2: 94°C for 15 seconds

[00213] Step 3: 55°C for 30 seconds

[00214] Step 4: 72°C for 30seconds

[00215] Step 5: Repeat steps 2-4 30 times for GAPDH, $VEGF_{165}$, $VEGF_{121}$ and $VEGF_{189}$ or 35 times for $VEGF_{165b}$

[00216] Step 6: 72°C for 10 minutes

[00217] Step 7: 4°C

[00218] PCR product was then visualized on a 2% agarose gel prepared in 1X TAE buffer.

[00219] *Results*: Treatment of ARPE19 cells with TGFβII induced VEGF production in ARPE19 cells and ELISA results demonstrated several siRNA candidates inhibited the production of TGFβII-induced VEGF in ARPE19 cells. RT-PCR confirmed that 2 candidates inhibited production of $VEGF_{165}$, $VEGF_{121}$ and $VEGF_{189}$, but spared $VEGF_{165b}$. As shown in Figure 12 (pg/mL hVEGF) and 13 (% knockdown hVEGF), VEGF siRNA candidates (Table 2) were screened for the ability to inhibit VEGF protein production by ARPE19 cells as tested by ELISA. Cells were treated with 10 ng/mL TGFβII to upregulate VEGF production. ELISA measured total VEGF protein and was not selective for any particular splice variant. Several candidates (OPK-HVB-004, OPK-HVB-010, and OPK-HVB-011) demonstrate an inhibitory effect and warranted further study. As shown in Figure 14 (pg/mL hVEGF) and 15 (% knockdown hVEGF), a secondary screen of VEGF production using the same methods as in Figure 12 and 13 demonstrated that OPK-HVB-004 and OPK-HVB-010 inhibited VEGF protein production and warranted further investigation.

[00220] Figures 16, 24 and 27 demonstrate a dose response efficacy of human VEGF knockdown with several candidates (OPK-HVB-004, OPK-HVB-010, and OPK-HVB-012) at varying concentrations.

[00221] Figure 17 demonstrates downregulation of human VEGF over one week (7 days) of several candidates (OPK-HVB-004, OPK-HVB-010, and OPK-HVB-012).

[00222] As a control, GAPDH RT-PCR was performed on variously treated cells as shown in Figure 18. Although the actual amount of RNA present was not quantified, the procedures are semi-quantitative when compared to the reference control lane 3. Specifically, downregulation of RNA production is demonstrated when a band appears fainter. In this experiment, samples in Lanes 2-11 were treated with 10 ng/mL TGFβII to upregulate the production of VEGF. The FAM-GAPDH siRNA downregulated GAPDH message (lane 4), while the other treatments have no effect on GAPDH mRNA, thus confirming that there is no variability in total RNA production in the treated cells.

[00223] $VEGF_{165}$ isoform RT-PCR was also performed on the treated cells as shown in Figure 19. Samples in Lanes 2-11 were treated with 10 ng/mL TGFβII to upregulate the production of VEGF. 25 nM bevasiranib (lane 6), which is known to downregulate all VEGF isoforms, 25 nM OPK-HVB-004 (lane 7) and 25 nM OPK-HVB-010 (lane 8), downregulated the production of $VEGF_{165}$ mRNA following induction with TGFβII (lane 2), as demonstrated by the bands being lighter than control in lane 3.

[00224] $VEGF_{189}$ isoform RT-PCR was also performed as shown in Figure 20. Samples in Lanes 2-11 were treated with 10 ng/mL TGFβII to upregulate the production of VEGF.

[00225] 25 nM bevasiranib (lane 6), 25 nM OPK-HVB-004 (lane 7) and 25 nM OPK-HVB-010 (lane 8) downregulated the production of $VEGF_{189}$ mRNA following induction with TGFβII (lane 2) as demonstrated by the bands being lighter than control in lane 3.

[00226] $VEGF_{121}$ isoform RT-PCR was then performed as shown in Figure 21. Samples in Lanes 2-11 were treated with 10 ng/mL TGFβII to upregulate the production of VEGF.

[00227] $VEGF_{121}$ mRNA was downregulated in lane 6 (25 nM bevasiranib) as demonstrated by the bands being lighter than control in lane 3..

[00228] Finally, $VEGF_{165b}$ isoform RTPCR was performed as shown in Figure 22.

[00229] Samples in Lanes 2-11 were treated with 10 ng/mL TGFβII to upregulate the production of VEGF. As an initial matter, the double banding > 600bp was determined to be artifactual. However, $VEGF_{165b}$ mRNA is downregulated by bevasiranib (lane 6) as shown by the bands being fainter that the control of lane 3. In contrast, bands for OPK-HVB-004 (lane 7) and OPK-HVB-010 (lane 8) were not fainter that control in lane 3. Thus, these siRNA constructs preserved $VEGF_{165b}$ expression while also being able to inhibit various other VEGF isoforms. Thus, siRNAs sparing $VEGFA_{165b}$ can be synthesized and may be more efficacious then siRNAs that knockdown all VEGF-A isoforms. $VEGF_{165b}$ sparing siRNAs may be potent therapeutic candidates for the treatment of ocular neovascularization.

[00230] **Example 13 – Cytokine Profile Following Treatment with siRNAs**

[00231] The cytokine secretion profile of ARPE19 cells following treatment with polyinosinic-polycytidylic acid sodium salt [Poly (I:C)], a dsRNA analogue was determined. Further tests to determined whether or not siRNAs behaved like Poly (I:C) and caused the cells to produce the same cytokines were conducted.

[00232] *Methods.* ARPE19 cells were seeded in 24 well plates (50,000 cells per well). Twenty-four hours later, media was removed and cells were treated with Poly (I:C); 0-1000 mg/mL (Sigma, St. Louis, MO) or poly deoxyinosinic-deoxycytidylic acid sodium salt [Poly (dI:dC); 50mU/mL-800 mU/mL] (Sigma), prepared in serum free DMEM/F12(1:1) (Invitrogen, Carlsbad, CA). Forty-eight hours post-treatment, media was collected from cells and analyzed for IFN-α, IFN-β, IFN-γ, IL-8, IL-6, TNFα, ICAM, IL-12 and MCP-1 via ELISA (QUANTIKINE® Immunoassays for IFN-γ, IL-8, IL-6, TNFa, ICAM, IL-12 and MCP-1, R&D Systems, Minneapolis, MN); VeriKine™ ELISA kits for IFN-α and IFN-β, PBL Biomedical Laboratories, Piscataway, NJ) according to the manufacturers' protocols.

[00233] ARPE19 cells were transfected with bevasiranib, OPK-HVB-004, OPK-HVB-009, OPK-HVB-010 and OPK-HVB-012 (Dhamacon/Thermo Scientific, Chicago, IL). Cells were seeded in 24 well plates (40,000 cells per well). 24 hours later, cells were transfected with 25nM siRNA using RiboJuice™ Transfection Reagent (Novagen/EMD, San Diego, CA) according to the manufacturer's protocol. 24 hours post -ransfection, cells were treated with 10 ng/mL human recombinant TGFβII (R&D Systems). 48 hours post-transfection (ie. 24 hours post-TGFbII treatment), media was collected and cytokine levels were analyzed, as described above. Additionally, media was analyzed for hVEGF via ELISA (R&D Systems). Results are shown in Figure 23.

[00234] *Conclusions.* Based upon the foregoing it is suggested that (ii) ARPE19 cells produce several inflammatory cytokines in response to Poly (I:C), a dsRNA analogue, but do not produce three key mediators, IFN-α, IFN-β or IFN-γ; (ii) ARPE19 cells can be used to study the inflammatory potential and specific effects of dsRNAs such as siRNAs; and (iii) OPK-HVB-009 an OPK-HVB-010 did not cause ARPE19 cells to secrete any of the cytokines tested, suggesting they may have a low inflammatory potential.

[00235] Example 14: Dose Response Curves Shows Specificity of siRNAs.

[00236] A dose response curve was generated using various siRNAs, 21-mers, as shown in Figure 26 and 27. A dose response was seen with certain siRNAS indicating a specific response to the siRNAs used. A dose response curve was also generated for OPK-HVB-009 as shown in Figures 25 and 26. The cells were treated and transfected as described in Examples 12 and 13. Cells were seeded in 24 well plates (40,000 cells/well). Additionally, different concentrations were used, and therefore, the volumes of OPTI-MEM, RiboJuice™, and siRNA were adjusted accordingly when preparing the 50 µl transfection mix.

[00237] Example 15: Stability of siRNAs

[00238] ARPE19 cells were transfected with siRNAs that had been stored under various conditions as shown in Figures 28, 29, 30 31, and 32. The cells were transfected as described in Examples 12 and 13. It was found that the siRNA molecules were stable under various conditions as shown in Figures 28, 29, 30, 31, and 32. For example, 7.5 µM siRNA was aliquoted into 3 tubes and each tube was stored at a different temperature (37°C, room temperature, 4°C) for up to 8 weeks. Aliquots of each tube were collected at predetermined time points (24 hrs, 48 hrs and then weekly). Upon collection aliquots were stored at -80°C. Each aliquot was subsequently tested for efficacy in ARPE19 cells to see if the siRNAs maintained their stability under the different environmental conditions. siRNAs were transfected into ARPE19 cells using the methods described in Example 12 where 40,000 cells were seeded per well.

[00239] Example 16: Cross-species down regulation of VEGF.

[00240] C6 cells were seeded in 24 well plates (P12, 40,000 cells per well). Eighteen to twenty-four hours post-seeding, cells were 50-70% confluent and used for transfection. Cells were transfected with OPK-HVB-004, OPK-HVB-009, OPK-HVB-010 and OPK-HVB-012 using the RiboJuice™ siRNA Transfection Reagent (Novagen) following the

manufacturer's protocol. Briefly, for a single well serum-free OPTI-MEM (40.5 μL-47 μL) was pipetted into an eppendorf tube and then 2 μL of RiboJuice™ were added to the OPTI-MEM (Gibco). The solution was mixed by gentle vortexing and centrifuged briefly to collect the contents at the bottom of the tube and incubated at room temperature for 5 min. siRNA (0.3 μL-7.5 μL of a 100nM or 1 μM stock) was added to the RiboJuice™/medium mix and gently mixed and briefly centrifuged to collect contents at the bottom of the tube. The mixture was incubated at room temperature for 15 minutes. During the incubation, media was removed from cells and replaced with 250μL of fresh C6 growth media (F-12 Kaighn's, 2.5% fetal calf serum; 15% horse serum, 1% penicillin/streptomycin). After the 15 min incubation, the siRNA/RiboJuice™/medium mixture (50 μL) was added dropwise to the cells. The plate was gently rocked to ensure the complexes were evenly dispersed throughout the well. The final concentration of siRNA in the 300μL volume was 250pM, 500pM, 1nM, 5nM or 25nM. Cells were maintained at 37°C, 5% CO2 for 24 hours. All volumes were scaled up such that each siRNA was tested at each concentration in triplicate. 24 hours post-transfection, the transfection mixture was removed and cells were treated with 500 μLs of fresh C6 growth media or with fresh C6 growth media supplemented with 10 ng/mL human recombinant TGFβII. The cells were returned to 37°C, 5% CO_2 for an additional 24 hours. Afterwards the media was removed from the cells and analyzed for protein expression by ELISA (Quantikine rat VEGF ELISA kit, R&D Systems).

[00241] NIH3T3 cells were seeded in 24 well plates (P2-P6, 40,000 cells per well). Eighteen to twenty-four hours post-seeding, cells were 50-70% confluent and used for transfection. Cells were transfected with siRNAs using Lipofectamine™ Reagent 2000 (Invitrogen) following the manufacturer's protocol. Briefly for a single well, siRNA (1 μM or 7.5 μM) was diluted in 50 μL OPTI-MEM in an eppendorf tube and gently mixed and vortexed. In a second eppendorf tube 1μL of Lipofectamine 2000 was combined with 49 μL of OPTI-MEM. The mixture was gently mixed and vortexed and incubated for 5 minutes at room temperature. After the 5 minutes, the diluted siRNA (50μL volume) was added to the diluted Lipofectamine 2000 (50 μL). The contents were mixed gently and incubated at room temperature for 20 minutes. During the 20 minute incubation, media was removed from the cells and replaced with 500 μLs of fresh NIH3T3 growth media (DMEM, 10% fetal calf serum). After the 20 minutes the siRNA-Lipofectamine 2000 complex (100 μL) was added dropwise to the cells. The plate was gently rocked to ensure the complexes were evenly

dispersed throughout the well. The cells were then incubated at 37°C, 5% CO_2 for 24 hours. The final concentration of siRNA in the 500µL volume was 1nM, 5nM or 25nM. 24 hours post-transfection, the transfection mixture was removed and cells were treated with 500 µLs of fresh DMEM or with fresh DMEM supplemented with 10 ng/mL human recombinant TGFβII. The cells were returned to 37°C, 5% CO_2 for an additional 24 hours. Afterwards the media was removed from the cells and analyzed for protein expression by ELISA (Quantikine mouse VEGF ELISA kit, R&D Systems).

[00242] Results of the experiments are shown in Figures 34, 35 and 39. OPK-HVB-004 and OPK-HVB-009 were able to inhibit VEGF secretion by C6 cells as shown in Figure 34. Similar experiments were done in mouse cells (NIH3T3) and OPK-HVB-004, OPK-HVB-009, and OPK-HVB-010 were able to inhibit secretion of mouse VEGF as shown in Figures 35 and 39.

[00243] Example 17: Comparison of different siRNAs

[00244] 21mer siRNAs comprising an overhang were compared to a 19mer blunt-end counterpart. ARPE19 cells were transfected with the different siRNAs as described in Examples 12, 13, and 14 and VEGF production was measured. The Blunt end counterpart was found to knockdown VEGF production in ARPE19 cells equally effective as the 21mer as shown in Figure 36. For example, a blunt end version of bevasiranib comprising SEQ ID NO: 119 and SEQ ID NO: 120 was equally effective at knocking down VEGF production as shown in Figure 36.

[00245] Example 18: Screen of 19mers comprising 17bp and an overhang can inhibit VEGF production.

[00246] siRNAS comprising a 17mer and a dTdT overhang were transfected in ARPE19 cells as described in Examples 12, 13, and 14. Several siRNAs were found to inhibit VEGF production as shown in Figure 37.

[00247] Example 19: Dose Response of siRNAs

[00248] 19mers comprising a blunt end or an overhang 19mer (17bp +dTdT over) were transfected into ARPE19 cells at various doses as shown in Figure 38. A dose response curve was generated by measuring VEGF secretion as described in Examples 12, 13 and 16. The dose response seen indicates that the response to the siRNAs is specific to the siRNA and

not generated by a non-specific siRNA response. The results can be seen in Figure 38. Blunt end siRNAs tested in NIH3T3 cells showed a specific dose response. (See Figure 39).

[00249] Example 20: Effect of siRNAs on VEGF mRNA expression

[00250] ARPE19 cells were transfected with siRNAs (final concentration siRNA = 25nM). Cells were treated with 10ng/mL TGFβII to upregulate production of hVEGF. RNA was isolated from cells and reverse transcription PCR was performed to amplify GAPDH (Figure 40A; 472bp fragment), $VEGF_{165}$ (Figure 40B; 284bp fragment) and $VEGF_{165b}$ (Figure 40C; 199bp fragment). The Cy3-GAPDH siRNA (Figure 40A, Lane 4) silenced GAPDH message whereas the other treatments had no effect. OPK-HVB-004, OPK-HVB-009 and OPK-HVB-010 (Figure 40B, Lanes 6, 7 and 8) and OPK-HVB-004be, OPK-HVB-009be and OPK-HVB-010be (Figure 40B, Lanes 10, 11 and 12) silenced $VEGF_{165}$ message. Bevasiranib (Figure 40C, Lane 4) silenced $VEGF_{165b}$ whereas OPK-HVB-004, OPK-HVB-009, OPK-HVB-004be and OPK-HVB-009be (Figure 13C, Lanes 5, 6, 8 and 9) preserved levels of $VEGF_{165b.}$ (See Figure 40).

[00251] Example 21: Efficacy of siRNAs in rat C6 cells

[00252] Rat C6 cells were transfected with siRNAs (final concentration siRNA = 25nM). Cells were treated with 10ng/mL TGFβII to upregulate production of rat VEGF. Levels of total secreted VEGF were measured in media via ELISA. Percent knockdown reflects the level of VEGF produced by the cells relative to cells treated with RiboJuice™. OPK-HVB-004 and OPK-HVB-004be were the most effective in reducing levels of rat VEGF.

J. CLAIMS

1. An isolated siRNA comprising a sense RNA strand and an antisense RNA strand, wherein the sense and the antisense RNA strands form an RNA duplex, and wherein the sense RNA strand comprises a nucleotide sequence identical to a target sequence of about 19 to about 25 contiguous nucleotides in human VEGF mRNA, wherein the sense RNA strand comprises a nucleotide sequence that consists of SEQ ID NO: 119, and the antisense strand comprises a nucleotide sequence that consists of SEQ ID NO: 120.

2. The siRNA of claim 1, wherein the first and second RNA strands forming the RNA duplex are covalently linked by a single-stranded hairpin.

3. The siRNA of claim 1, wherein the siRNA further comprises non-nucleotide material.

4. The siRNA of claim 1, wherein the first and second RNA strands are stabilized against nuclease degradation.

5. A pharmaceutical composition comprising an effective amount of an isolated siRNA comprising a sense RNA strand and an antisense RNA strand, wherein the sense and the antisense RNA strands form an RNA duplex, and wherein the sense RNA strand comprises a nucleotide sequence that consists of SEQ ID NO: 119, and the antisense strand comprises a nucleotide sequence that consists of SEQ ID NO: 120.

6. The pharmaceutical composition of claim 5, wherein the first and second RNA strands are stabilized against nuclease degradation.

7. A method of treating an angiogenic disease in a subject comprising: administering to a subject an effective amount of a short interfering ribonucleic acid (siRNA) comprising a sense RNA strand and an antisense RNA strand, wherein the sense and the antisense RNA strands form an RNA duplex, and wherein the sense RNA strand comprises a nucleotide sequence identical to a target sequence of about 19 to about 25 contiguous nucleotides in human vascular endothelial growth factor (VEGF) mRNA, and wherein the sense RNA strand comprises a nucleotide sequence that consists of SEQ ID NO: 119, and the antisense strand comprises a nucleotide sequence that consists of SEQ ID NO: 120, such that angiogenesis associated with the angiogenic disease is inhibited.

8. The method of claim 7, wherein the angiogenic disease comprises a tumor associated with a cancer.

9. The method of claim 8, wherein the cancer is selected from the group consisting of breast cancer, lung cancer, head and neck cancer, brain cancer, abdominal cancer, colon cancer, colorectal cancer, esophagus cancer, gastrointestinal cancer, glioma, liver cancer, tongue cancer, neuroblastoma, osteosarcoma, ovarian cancer, pancreatic cancer, prostate cancer, retinoblastoma, Wilm's tumor, multiple myeloma, skin cancer, lymphoma, and blood cancer.

10. The method of claim 7, wherein the angiogenic disease is selected from the group consisting of diabetic retinopathy, age-related macular degeneration, and inflammatory diseases.

11. The method of claim 10, wherein the inflammatory disease is psoriasis or rheumatoid arthritis.

12. The method of claim 7, wherein the angiogenic disease is age-related macular degeneration.

13. The method of claim 7, wherein the pharmaceutical composition is administered in combination with a pharmaceutical agent for treating the angiogenic disease, which pharmaceutical agent is different from the short interfering ribonucleic acid (siRNA).

14. The method of claim 7, wherein the angiogenic disease is cancer, and the pharmaceutical agent comprises a chemotherapeutic agent.

15. The method of claim 14, wherein the chemotherapeutic agent is selected from the group consisting of cisplatin, carboplatin, cyclophosphamide, 5-fluorouracil, adriamycin, daunorubicin, and tamoxifen.

16. The method of claim 7, wherein the pharmaceutical composition is administered to a subject in combination with another therapeutic method designed to treat the angiogenic disease.

17. The method of claim 7, wherein the angiogenic disease is cancer, and the pharmaceutical composition is administered in combination with radiation therapy, chemotherapy or surgery.

18. A method of inhibiting expression of human vascular endothelial growth factor (VEGF) comprising: administering to a subject an effective amount of a short interfering ribonucleic acid (siRNA) comprising a sense RNA strand and an antisense RNA strand, wherein the sense and the antisense RNA strands form an RNA duplex, and wherein the

sense RNA strand comprises a nucleotide sequence identical to a target sequence of about 19 to about 25 contiguous nucleotides in human vascular endothelial growth factor (VEGF) mRNA and wherein the sense RNA strand comprises a nucleotide sequence that consists of SEQ ID NO: 119, and the antisense strand comprises a nucleotide sequence that consists of SEQ ID NO: 120.

19. The method of claim 18, wherein the effective amount comprises from about 1 nM to about 100 nM of the short interfering ribonucleic acid (siRNA).

20. The method of claim 18, wherein the pharmaceutical composition further comprises a delivery reagent.

21. The method of claim 18, wherein the delivery agent is selected from the group consisting of lipofectin, lipofectamine, cellfectin, polycations, and liposomes.

22. The method of claim 21, wherein the delivery agent is a liposome.

23. The method of claim 22, wherein the liposome comprises a ligand which targets the liposome to cells at or near the site of angiogenesis.

24. The method of claim 23, wherein the ligand binds to receptors on tumor cells or vascular endothelial cells.

25. The method of claim 23, wherein the ligand comprises a monoclonal antibody.

26. The method of claim 22, wherein the liposome is modified with an opsonization-inhibition moiety.

27. The method of claim 26, wherein the opsonization-inhibiting moiety comprises a PEG, PPG, or derivatives thereof.

28. The method of claim 18, wherein the short interfering ribonucleic acid (siRNA) is expressed from a recombinant plasmid.

29. The method of claim 18, wherein the short interfering ribonucleic acid (siRNA) is expressed from a recombinant viral vector.

30. The method of claim 29, wherein the recombinant viral vector comprises an adenoviral vector, an adeno-associated viral vector, a lentiviral vector, a retroviral vector, or a herpes virus vector.

31. The method of claim 30, wherein the recombinant viral vector is pseudotyped with surface proteins from vesicular stomatitis virus, rabies virus, Ebola virus, or Mokola virus.

32. The method of claim 29, wherein the recombinant viral vector comprises an adeno-associated viral vector.

33. The method of claim 18, wherein the pharmaceutical composition is administered by an enteral administration route.

34. The method of claim 33, wherein the enteral administration route is selected from the group consisting of oral, rectal, and intranasal.

35. The method of claim 18, wherein the pharmaceutical composition is administered by a parenteral administration route.

36. The method of claim 35, wherein the parenteral administration route is selected from the group consisting of intravascular administration, peri- and intra-tissue injection, subcutaneous injection or deposition, subcutaneous infusion, and direct application at or near the site of neovascularization.

37. The method of claim 35, wherein the intravascular administration is selected from the group consisting of intravenous bolus injection, intravenous infusion, intra-arterial bolus injection, intra-arterial infusion and catheter instillation into the vasculature.

38. A method of degrading human vascular endothelial growth factor (VEGF) mRNA comprising: administering to a subject an effective amount of a short interfering ribonucleic acid (siRNA) comprising a sense RNA strand and an antisense RNA strand, wherein the sense and the antisense RNA strands form an RNA duplex, and wherein the sense RNA strand comprises a nucleotide sequence identical to a target sequence of about 19 to about 25 contiguous nucleotides in human vascular endothelial growth factor (VEGF) mRNA and wherein the sense RNA strand comprises a nucleotide sequence that consists of SEQ ID NO: 119, and the antisense strand comprises a nucleotide sequence that consists of SEQ ID NO: 120.

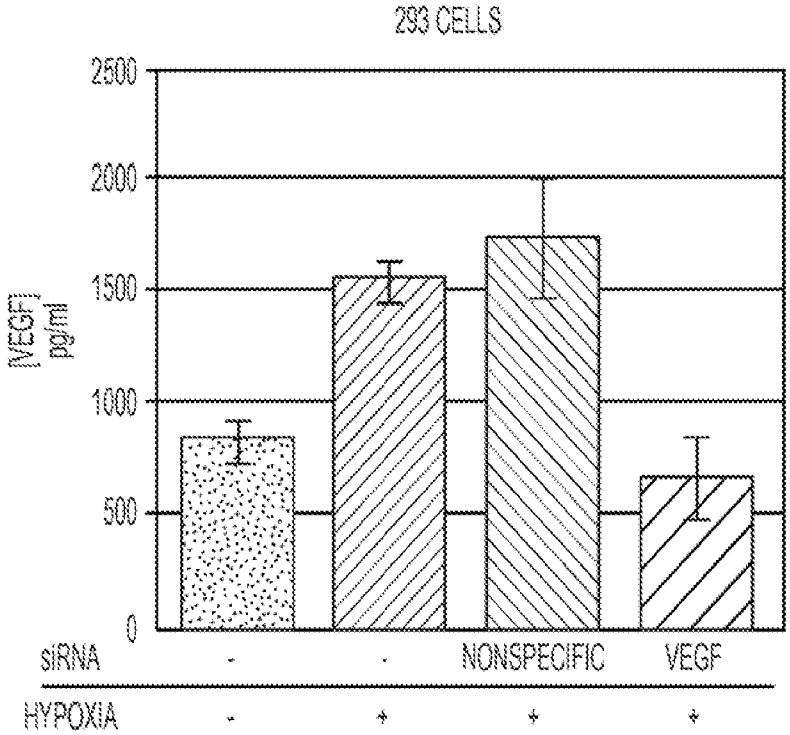
293 CELLS
2500
2000
1500
1000
500
0
[VEGF] pg/ml
siRNA - - NONSPECIFIC VEGF
HYPOXIA - + + +

FIG. 1A

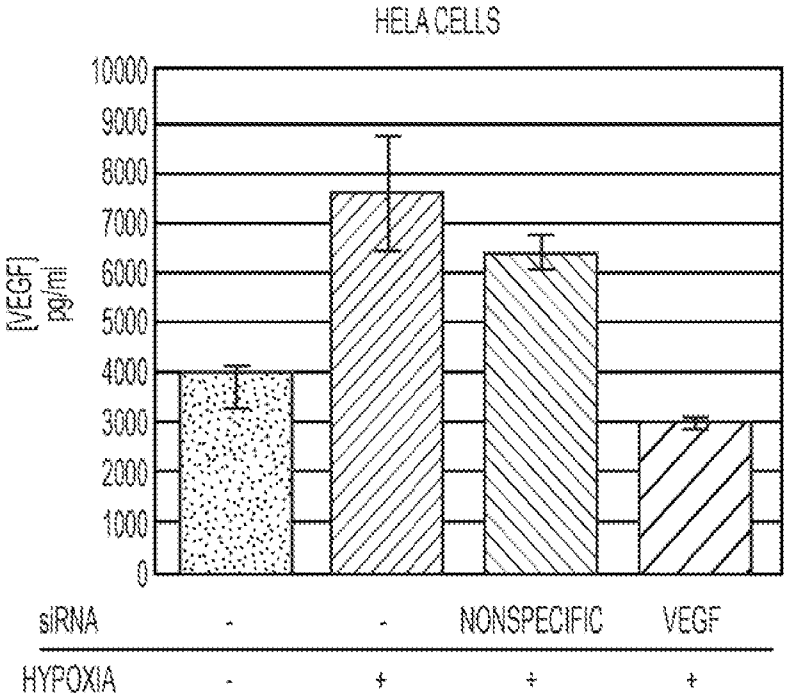
HELA CELLS
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
[VEGF] pg/ml
siRNA - - NONSPECIFIC VEGF
HYPOXIA - + + +

FIG. 1B

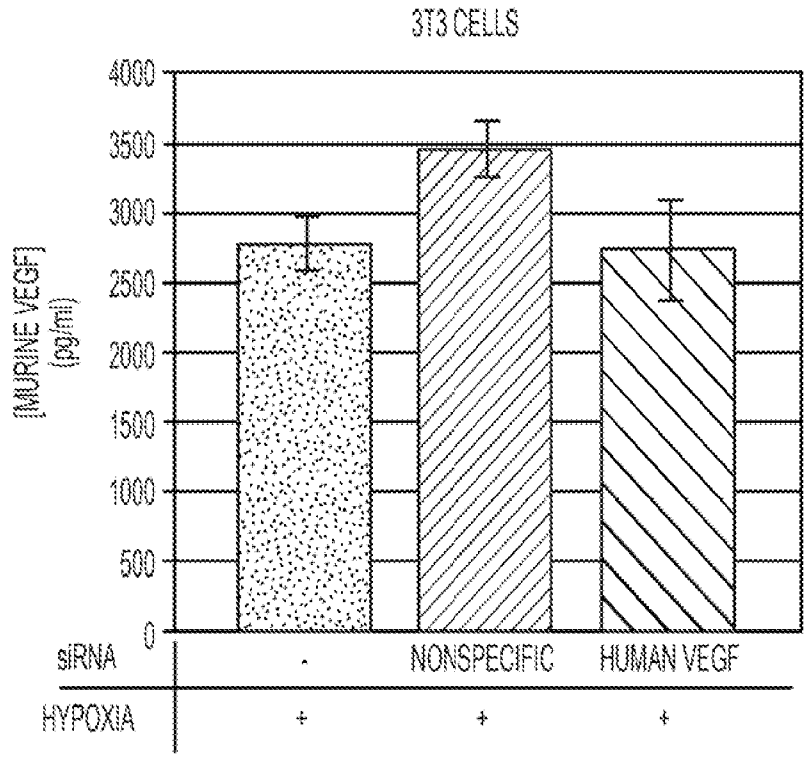
3T3 CELLS
[MURINE VEGF] (pg/ml)
4000
3500
3000
2500
2000
1500
1000
500
0
siRNA
-
NONSPECIFIC
HUMAN VEGF
HYPOXIA
+
+
+

FIG. 2

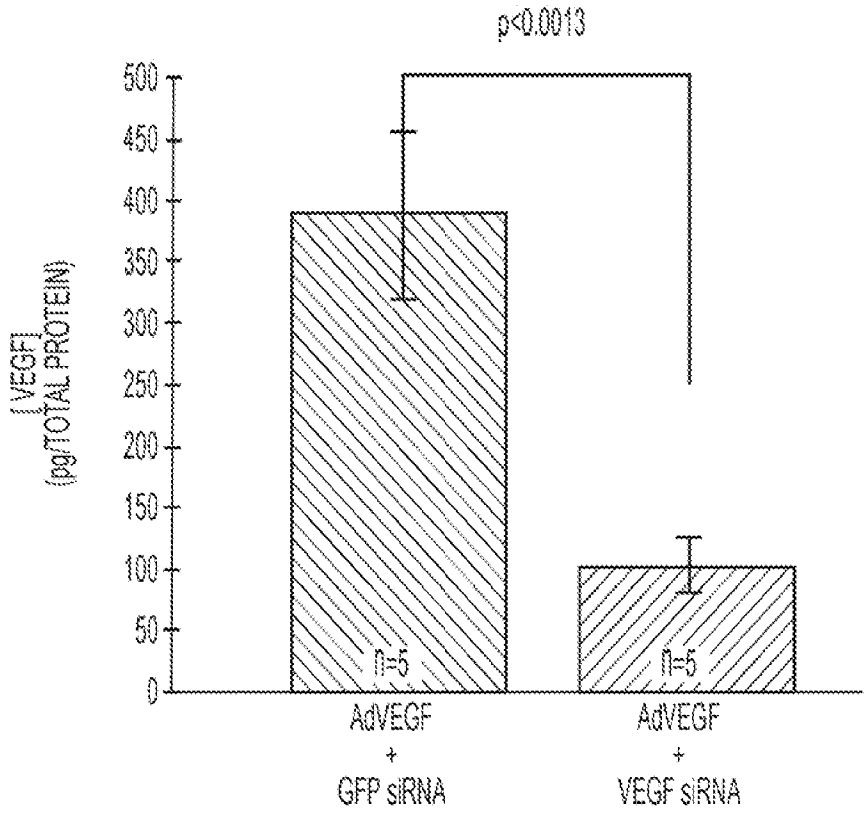
p<0.0013
[VEGF]
(pg/TOTAL PROTEIN)
500
450
400
350
300
250
200
150
100
50
0
n=5
n=5
AdVEGF
+
GFP siRNA
AdVEGF
+
VEGF siRNA

FIG. 3

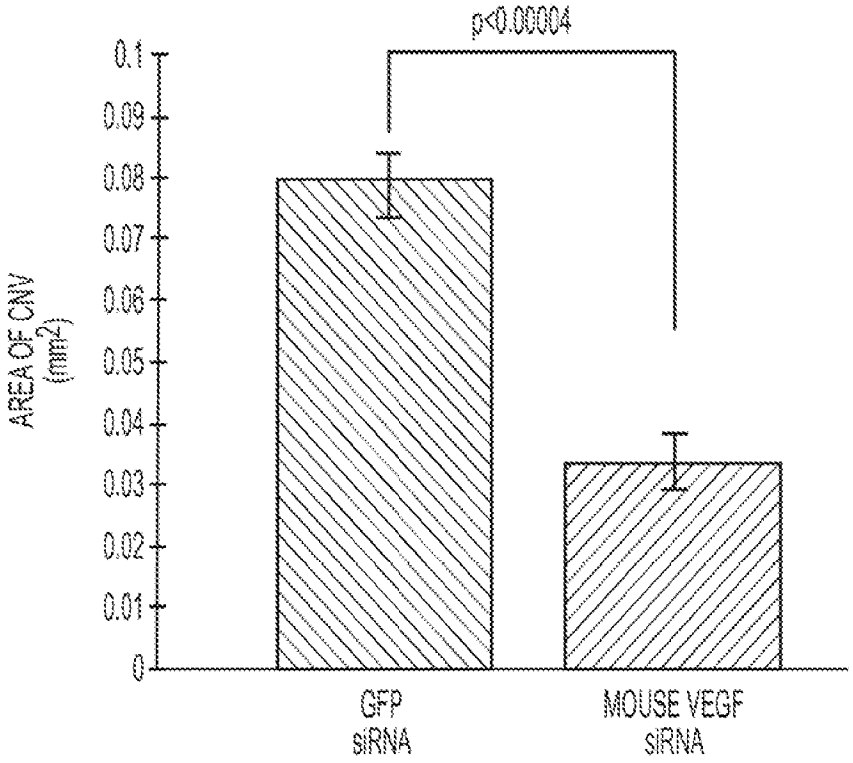
p<0.00004
AREA OF CNV (mm2)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
GFP siRNA
MOUSE VEGF siRNA

FIG. 4

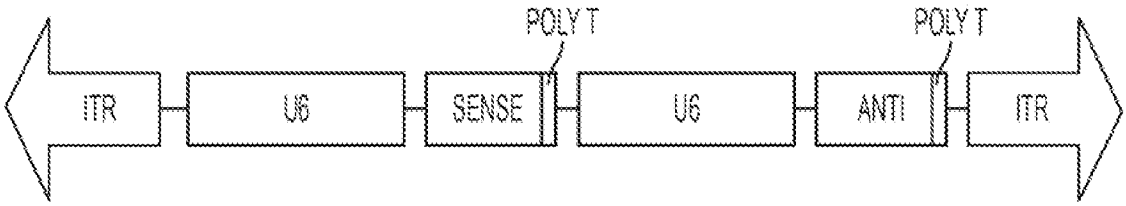

FIG. 5

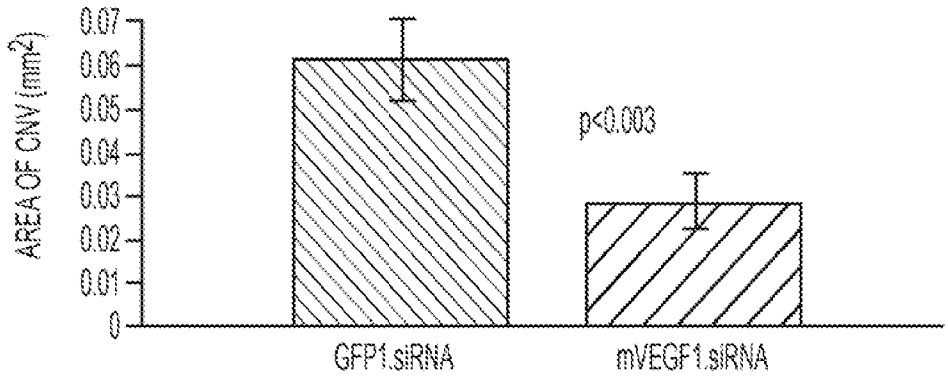

FIG. 6A

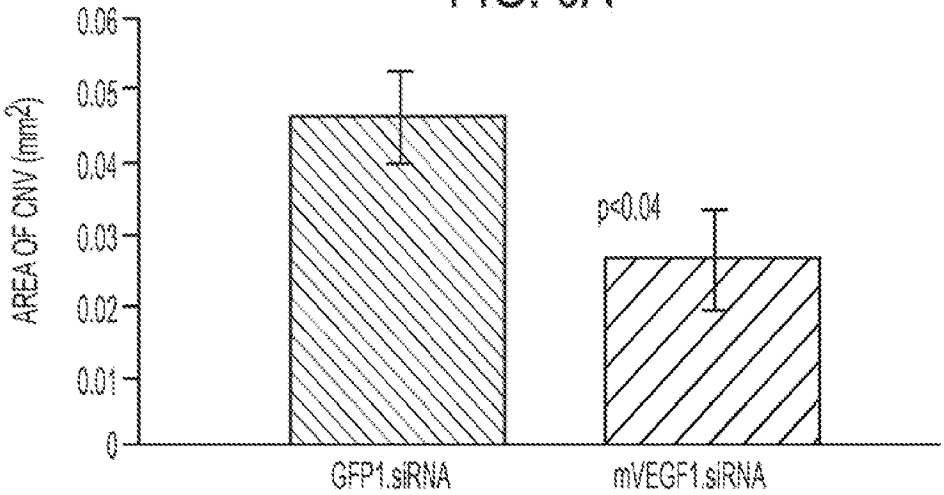

FIG. 6B

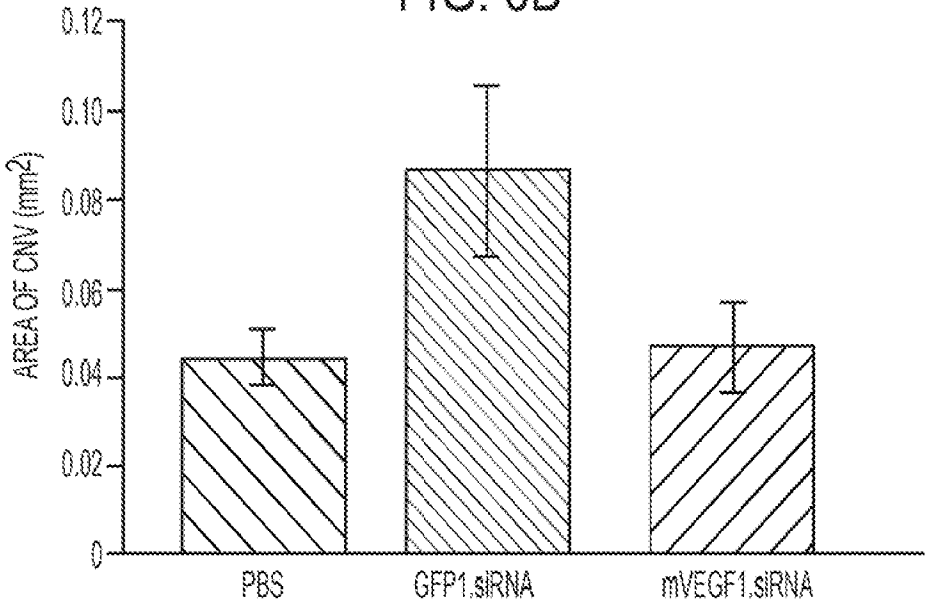

FIG. 6C

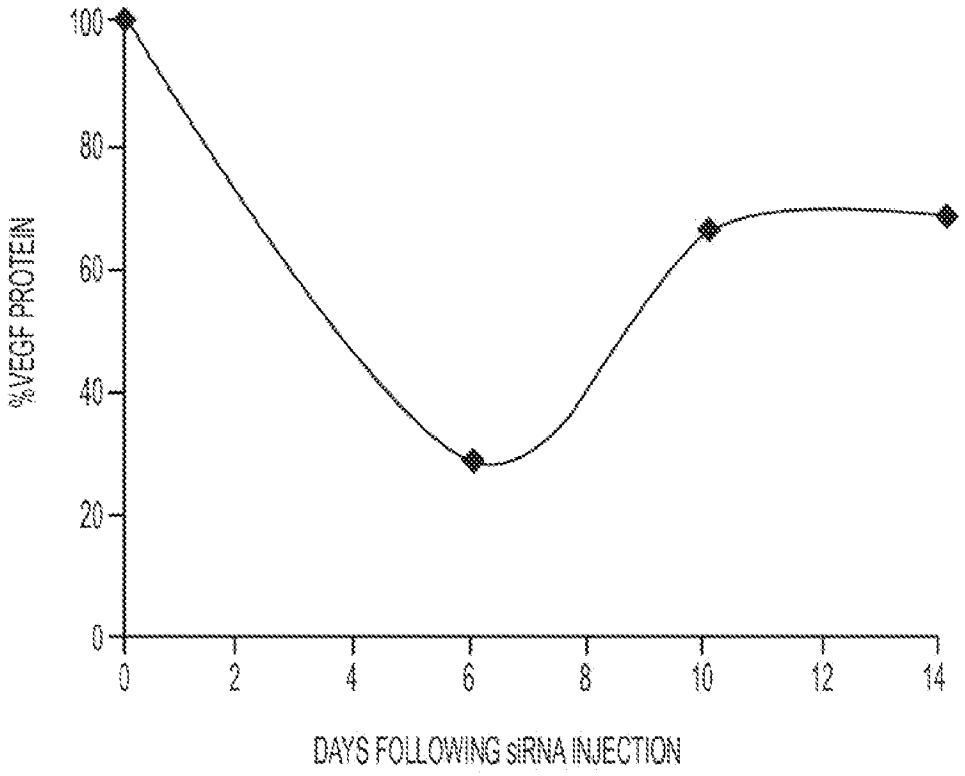
100
80
60
40
20
0
% VEGF PROTEIN
0
2
4
6
8
10
12
14
DAYS FOLLOWING siRNA INJECTION

FIG. 7

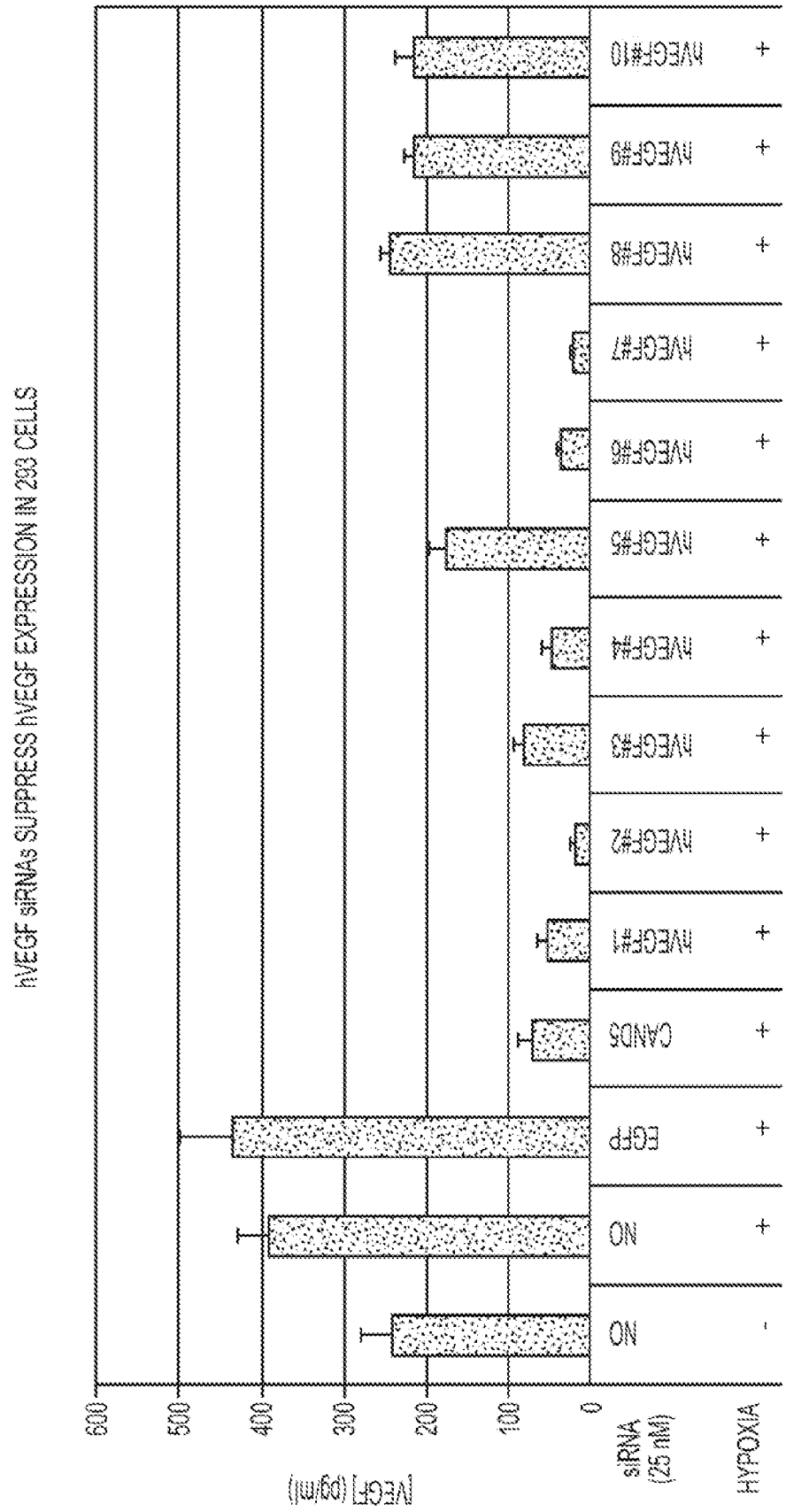
hVEGF siRNAs SUPPRESS hVEGF EXPRESSION IN 293 CELLS
VEGF (pg/ml)
600
500
400
300
200
100
0
siRNA (25 nM)
HYPOXIA
NO
-
NO
+
EGFP
+
CAND5
+
hVEGF#1
+
hVEGF#2
+
hVEGF#3
+
hVEGF#4
+
hVEGF#5
+
hVEGF#6
+
hVEGF#7
+
hVEGF#8
+
hVEGF#9
+
hVEGF#10
+

FIG. 8

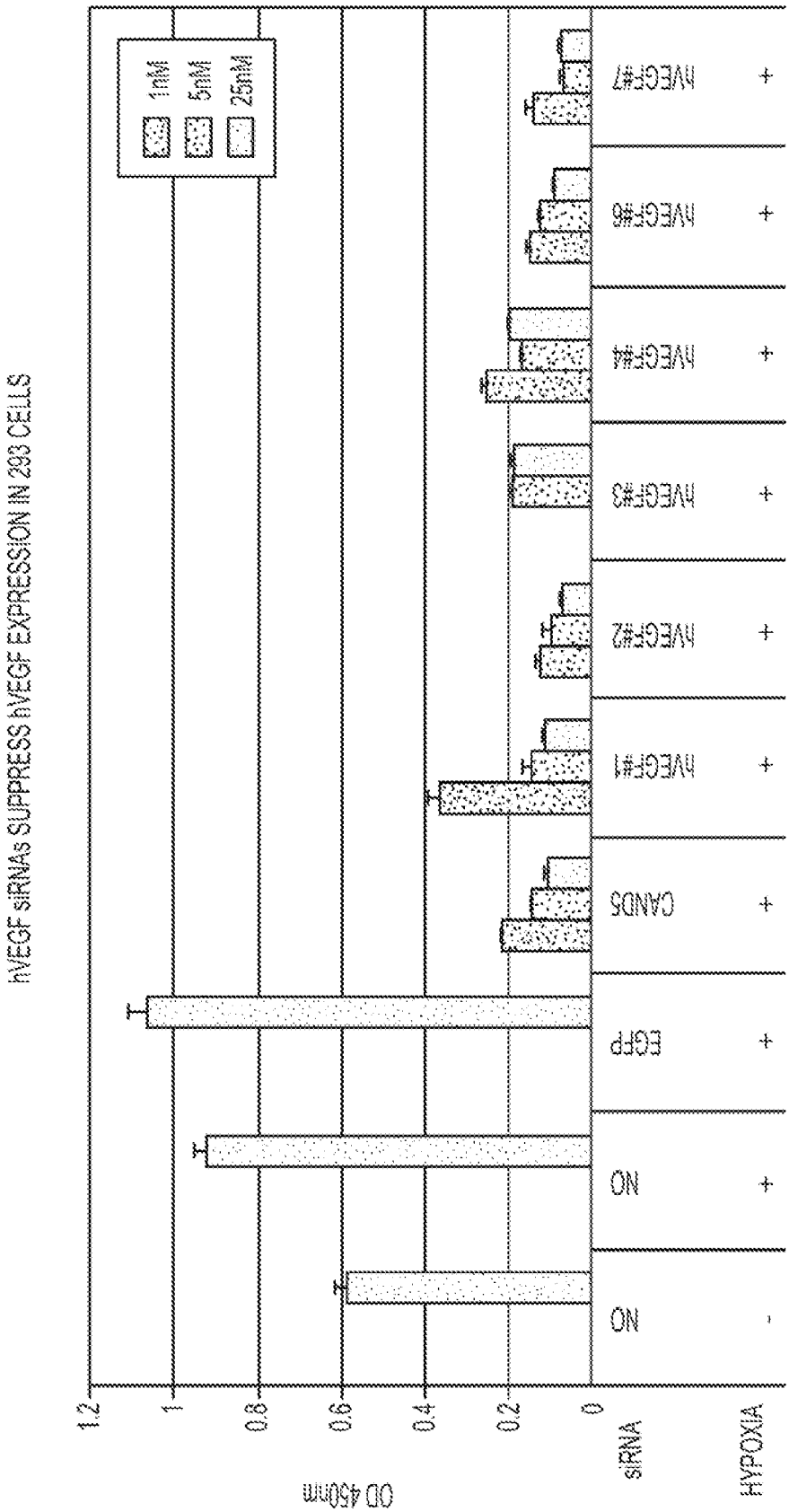
hVEGF siRNAs SUPPRESS hVEGF EXPRESSION IN 293 CELLS
1nM
5nM
25nM
OD 450nm
1.2
1
0.8
0.6
0.4
0.2
0
siRNA
HYPOXIA
ON
ON
EGFP
CAND5
hVEGF#1
hVEGF#2
hVEGF#3
hVEGF#4
hVEGF#6
hVEGF#7
-
+
+
+
+
+
+
+
+

FIG. 9

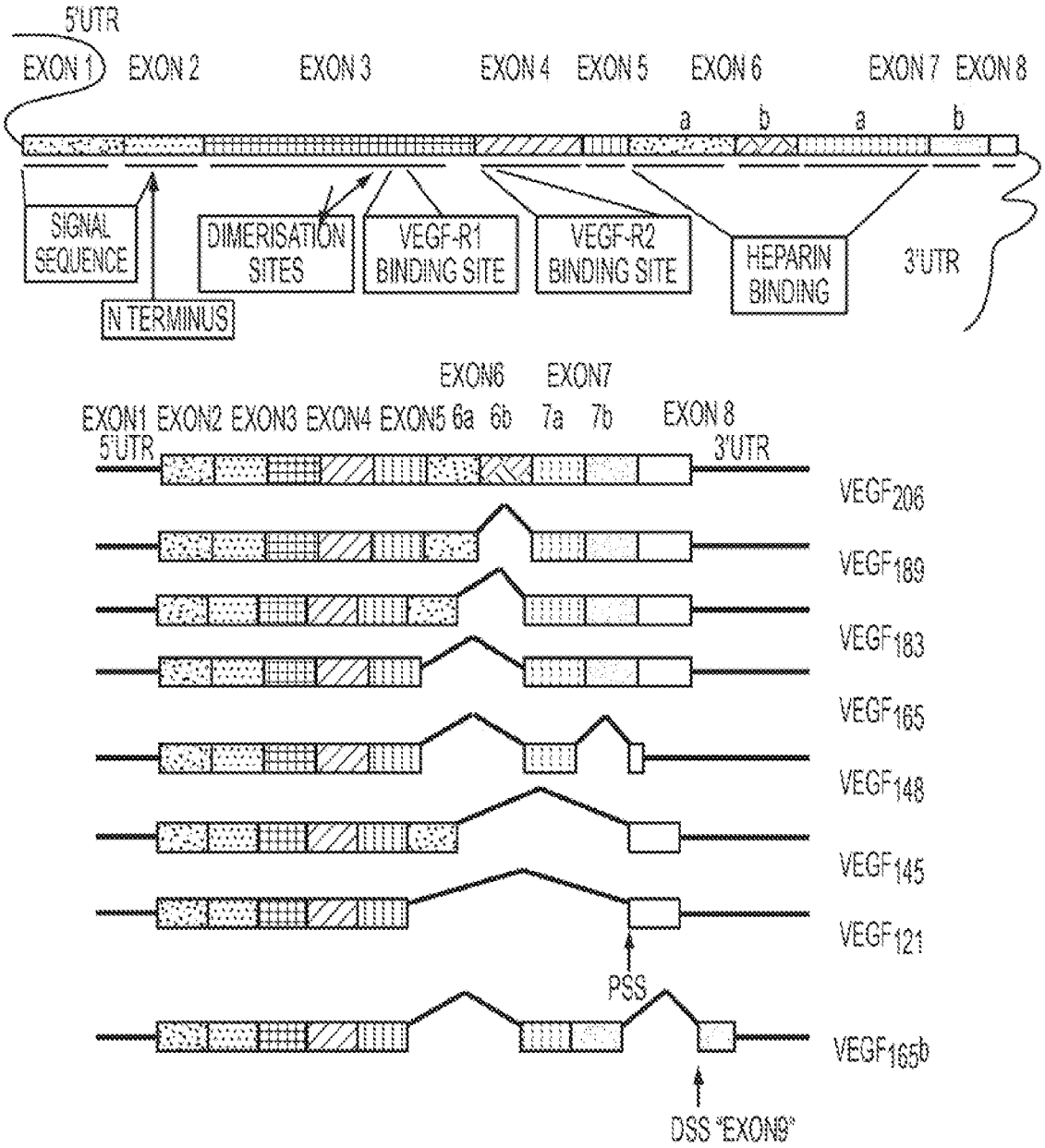
5'UTR
EXON 1
EXON 2
EXON 3
EXON 4
EXON 5
EXON 6
EXON 7
EXON 8
a
b
a
b
SIGNAL SEQUENCE
N TERMINUS
DIMERISATION SITES
VEGF-R1 BINDING SITE
VEGF-R2 BINDING SITE
HEPARIN BINDING
3'UTR
EXON6
EXON7
EXON1 EXON2 EXON3 EXON4 EXON5 6a 6b 7a 7b
EXON 8
5'UTR
3'UTR
VEGF206
VEGF189
VEGF183
VEGF165
VEGF148
VEGF145
VEGF121
PSS
VEGF165b
DSS "EXON8"

FIG. 10

```
                                                    Exon 7/8 boundry
nt        518
VEGF 165   Aaggcgaggcagcttgagttaaacgaacgtacttgcagatgtgacaagccgaggcggtga
VEGF 165B  Aaggcgaggcagcttgagttaaacgaacgtacttgcag--------------------

VEGF 165   gccgggcaggaggaaggagcctccctcagggtttcgggaaccagatctctcaccaggaaa
VEGF 165B  ----------------------------------------atctctcaccaggaaa

VEGF 165   gactgatacagaacgatcgatacagaaaccac
VEGF 165B  gactgatacagaacgatcgatacagaaaccac
```

FIG. 11

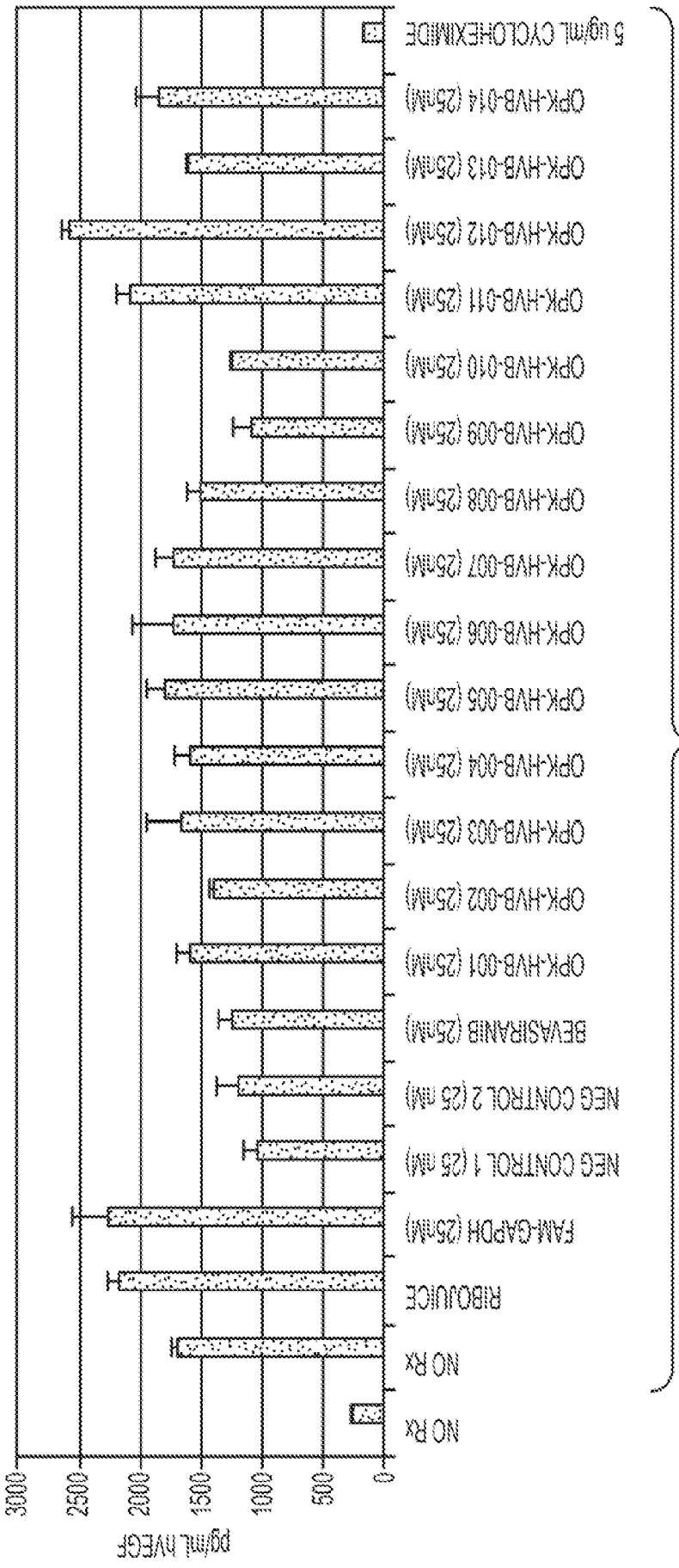
pg/mL hVEGF
3000
2500
2000
1500
1000
500
0
NO Rx
NO Rx
RIBOLUICE
FAM-GAPDH (25nM)
NEG CONTROL 1 (25 nM)
NEG CONTROL 2 (25 nM)
BEVASIRANIB (25nM)
OPK-HVB-001 (25nM)
OPK-HVB-002 (25nM)
OPK-HVB-003 (25nM)
OPK-HVB-004 (25nM)
OPK-HVB-005 (25nM)
OPK-HVB-006 (25nM)
OPK-HVB-007 (25nM)
OPK-HVB-008 (25nM)
OPK-HVB-009 (25nM)
OPK-HVB-010 (25nM)
OPK-HVB-011 (25nM)
OPK-HVB-012 (25nM)
OPK-HVB-013 (25nM)
OPK-HVB-014 (25nM)
5 ug/mL CYCLOHEXIMIDE
10 ng/mL TGFβIII

FIG. 12

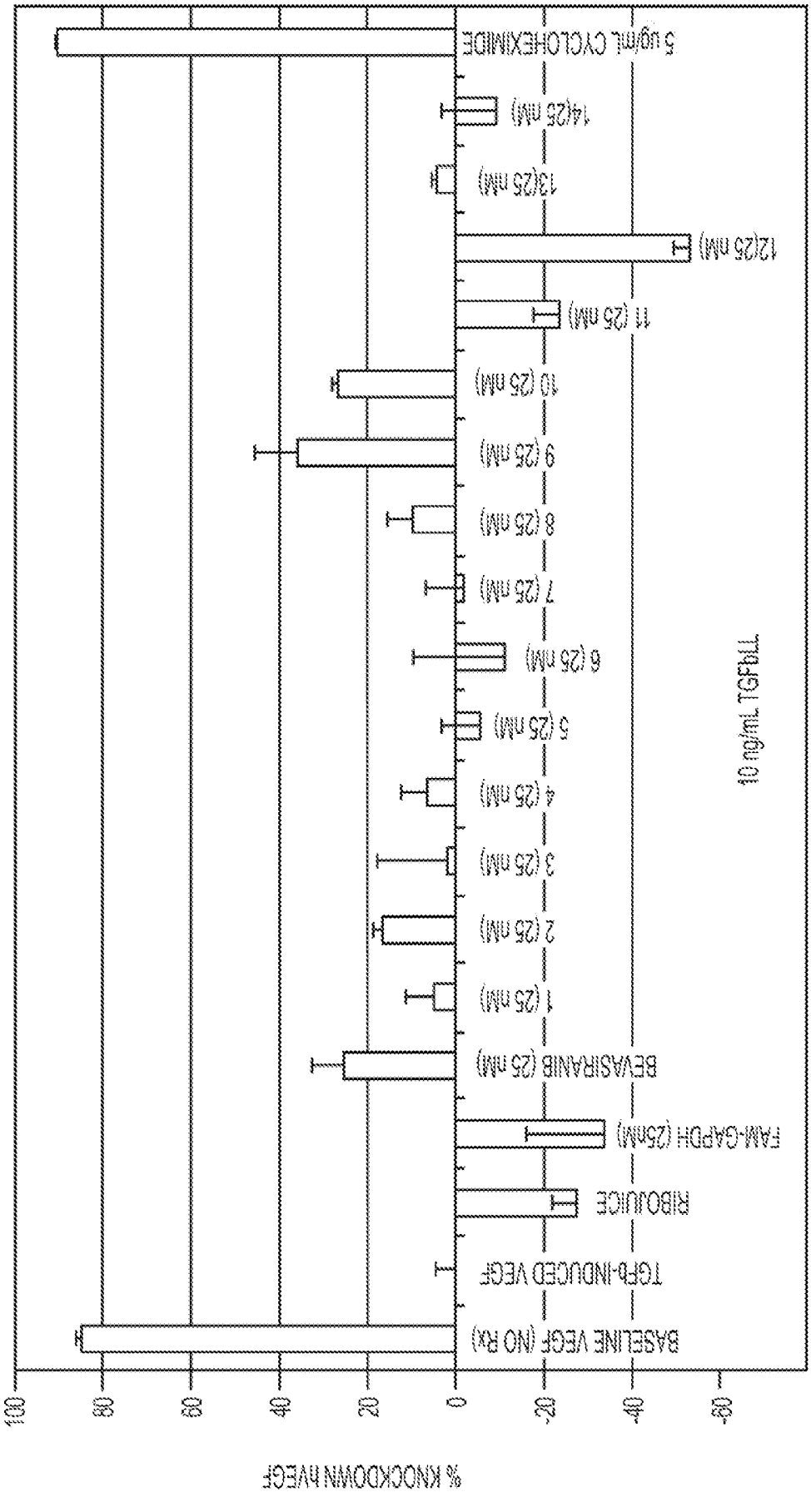
% KNOCKDOWN hVEGF
100
80
60
40
20
0
-20
-40
-60
BASELINE VEGF (NO Rx)
TGFb-INDUCED VEGF
RIBOJUICE
FAM-GAPDH (25nM)
BEVASIRANIB (25 nM)
1 (25 nM)
2 (25 nM)
3 (25 nM)
4 (25 nM)
5 (25 nM)
6 (25 nM)
7 (25 nM)
8 (25 nM)
9 (25 nM)
10 (25 nM)
11 (25 nM)
12(25 nM)
13(25 nM)
14(25 nM)
5 ug/mL CYCLOHEXIMIDE
10 ng/mL TGFbLL

FIG. 13

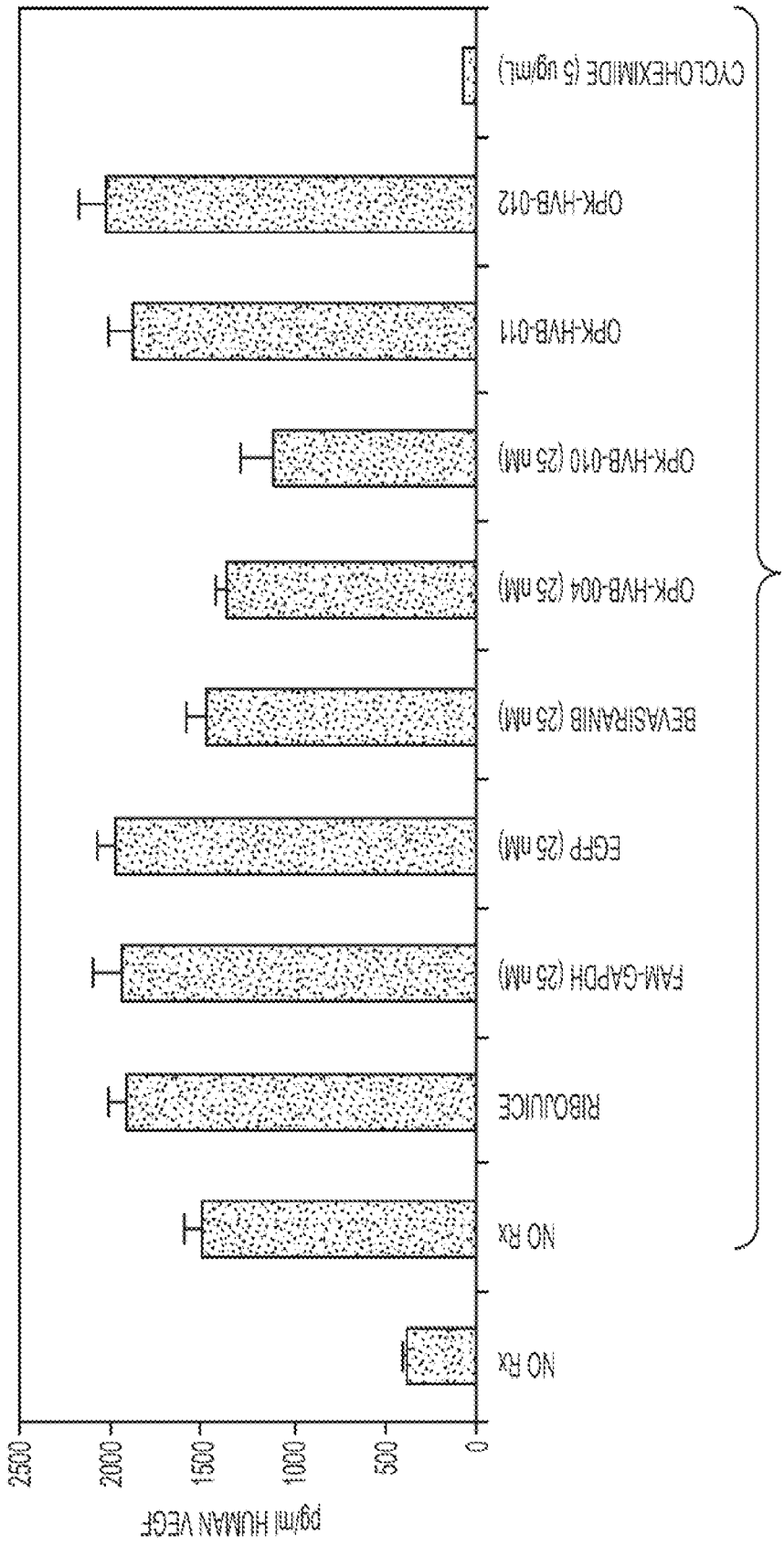
pg/ml HUMAN VEGF
2500
2000
1500
1000
500
0
NO Rx
NO Rx
RIBOJUICE
FAM-GAPDH (25 nM)
EGFP (25 nM)
BEVASIRANIB (25 nM)
OPK-HVB-004 (25 nM)
OPK-HVB-010 (25 nM)
OPK-HVB-011
OPK-HVB-012
CYCLOHEXIMIDE (5 ug/mL)
10 ng/mL TGFβIII

FIG. 14

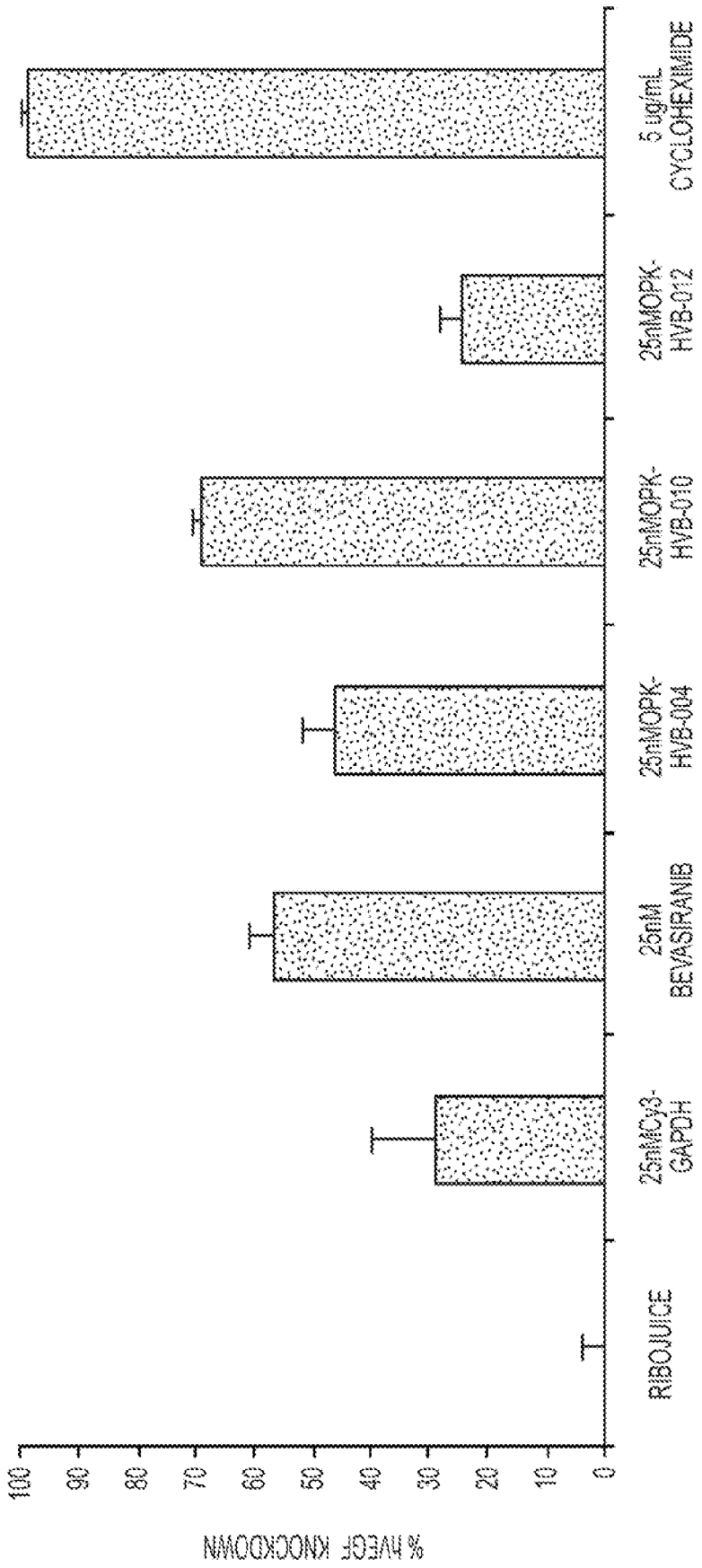
% VEGF KNOCKDOWN
100
90
80
70
60
50
40
30
20
10
0
RIBOJUICE
25nMCy3-GAPDH
25nM BEVASIRANIB
25nMOPK-HVB-004
25nMOPK-HVB-010
25nMOPK-HVB-012
5 ug/mL CYCLOHEXIMIDE

FIG. 15

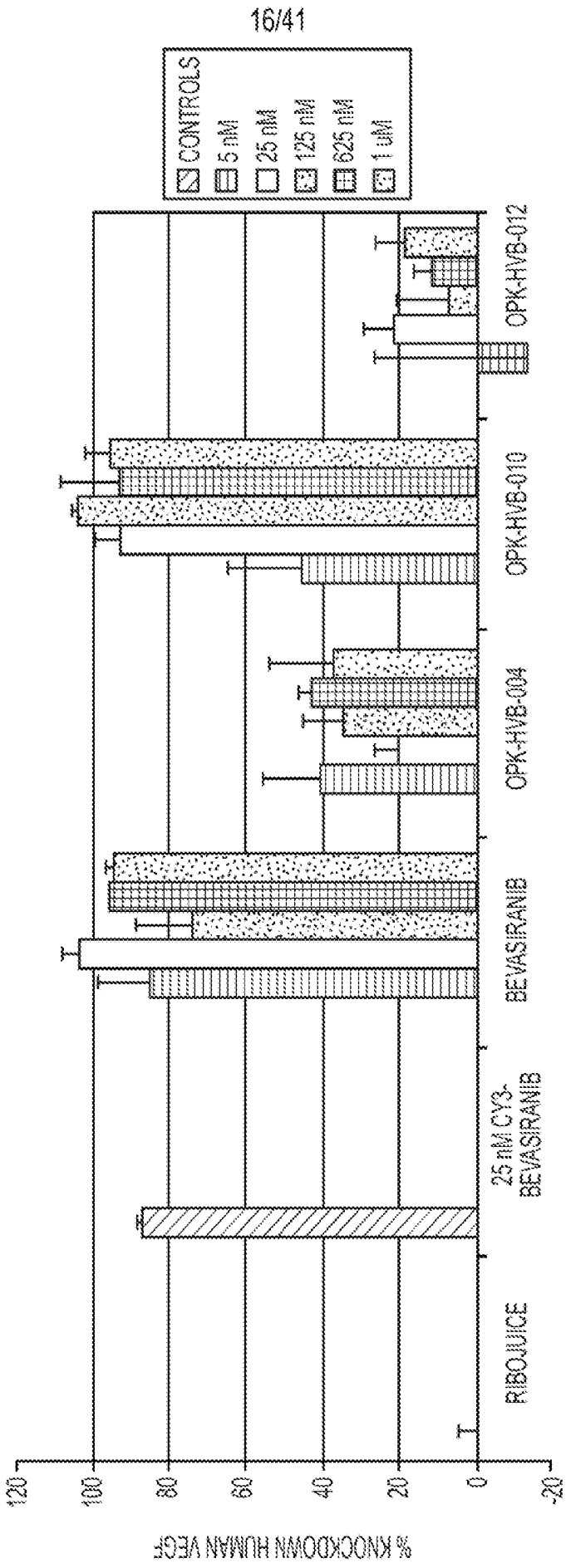
CONTROLS
5 nM
25 nM
125 nM
625 nM
1 uM
OPK-HVB-012
OPK-HVB-010
OPK-HVB-004
BEVASIRANIB
25 nM CY3-BEVASIRANIB
RIBOJUICE
120
100
80
60
40
20
0
-20
% KNOCKDOWN HUMAN VEGF

FIG. 16

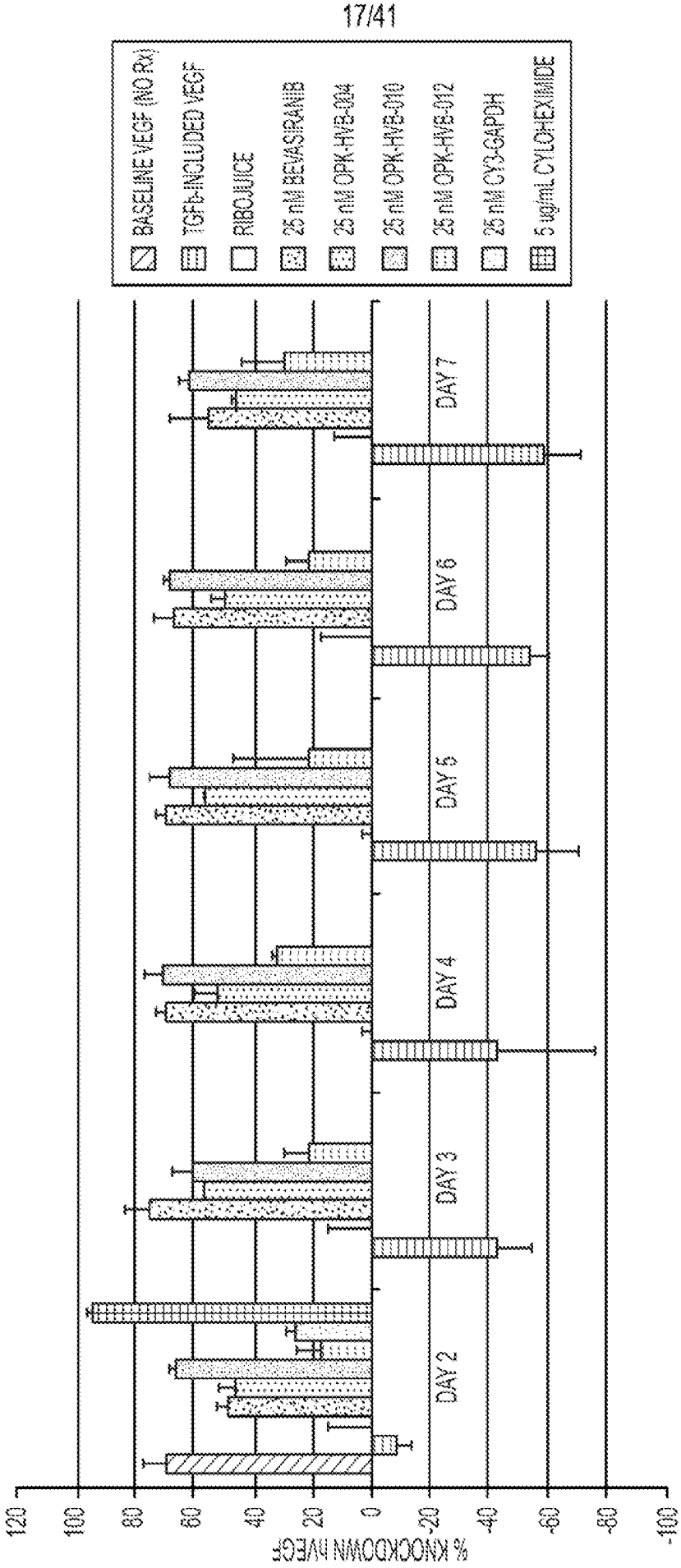
BASELINE VEGF (NO Rx)
TGFb-INCLUDED VEGF
RIBOJUICE
25 nM BEVASIRANIB
25 nM OPK-HVB-004
25 nM OPK-HVB-010
25 nM OPK-HVB-012
25 nM CYC-GAPDH
5 ug/mL CYLOHEXIMIDE
DAY 2
DAY 3
DAY 4
DAY 5
DAY 6
DAY 7
120
100
80
60
40
20
0
-20
-40
-60
-80
-100
% KNOCKDOWN hVEGF
DAYS POST-TRANSFECTION

FIG. 17

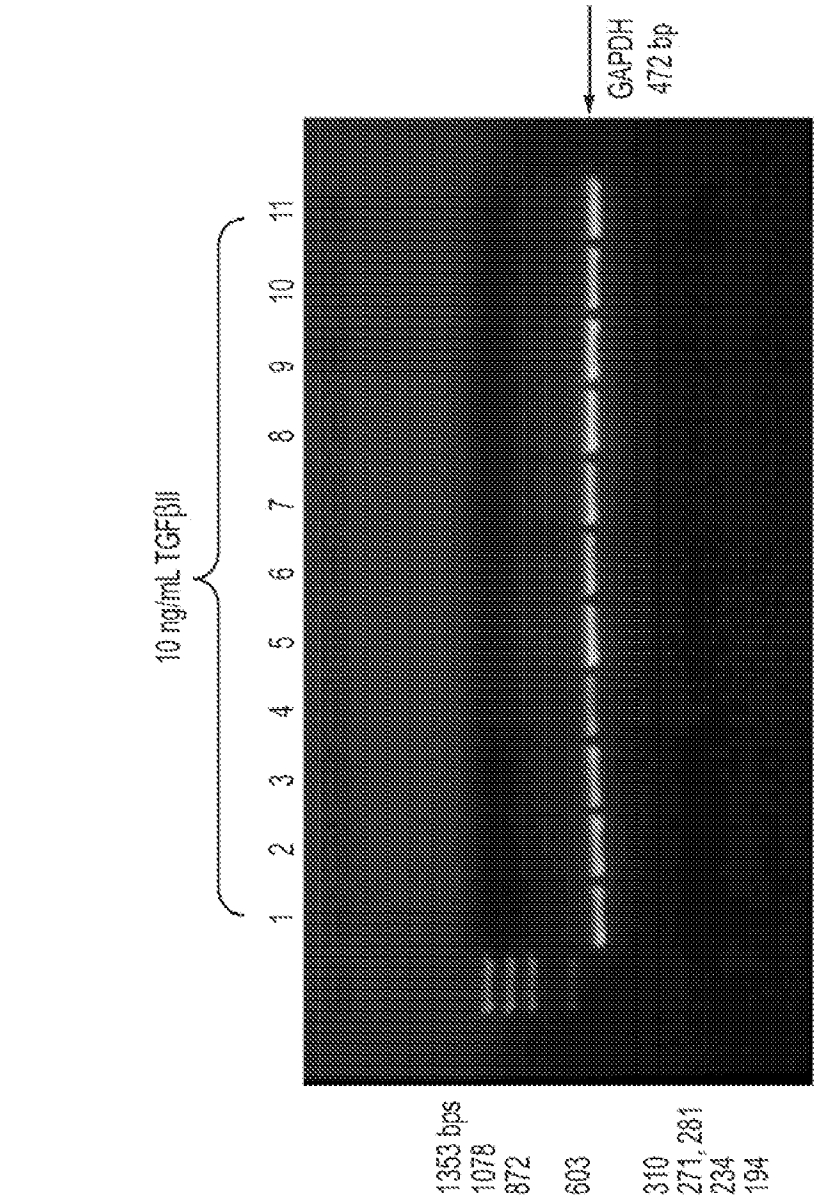
GAPDH
472 bp
10 ng/mL TGFβIII
1 2 3 4 5 6 7 8 9 10 11
1353 bps
1078
872
603
310
271, 281
234
194
LANE 1: NO Rxn
LANE 2: NO Rxn
LANE 3: RIBOJUICE
LANE 4: 25 nM FAM-GAPDH siRNA
LANE 5: 25 nM EGFP siRNA
LANE 6: 25 nM BEVASIRANIB
LANE 7: 25 nM OPX-HVB-004
LANE 8: 25 nM OPX-HVB-010
LANE 9: 25 nM OPX-HVB-011
LANE 10: 25 nM OPX-HVB-012
LANE 11: 5 µg/mL CYCLOHEXIMIDE

FIG. 18

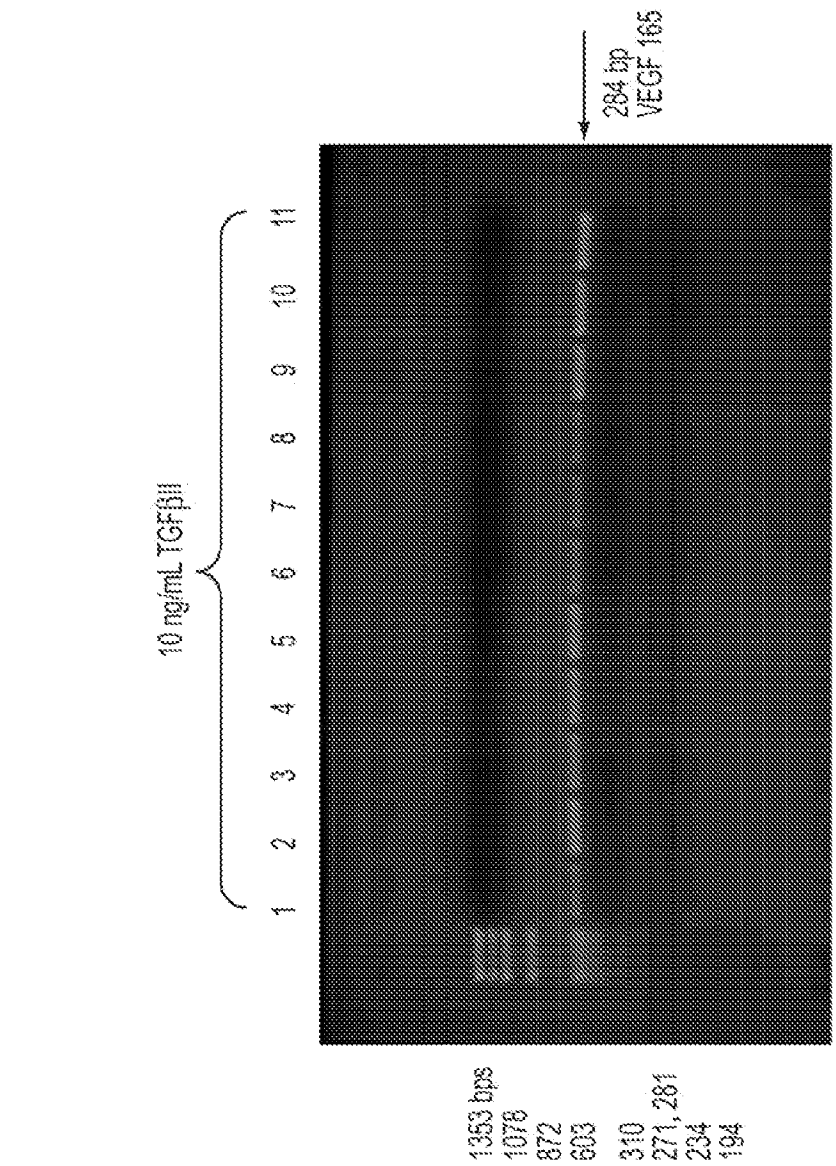
284 bp
VEGF 165
10 ng/mL TGFβIII
1 2 3 4 5 6 7 8 9 10 11
1353 bps
1078
872
603
310
271, 281
234
194
LANE 1: NO Rxn
LANE 2: NO Rxn
LANE 3: RIBOJUICE
LANE 4: 25 nM FAM-GAPDHsiRNA
LANE 5: 25 nM EGFP siRNA
LANE 6: 25 nM BEVASIRANIB
LANE 7: 25 nM OPX-HVB-004
LANE 8: 25 nM OPX-HVB-010
LANE 9: 25 nM OPX-HVB-011
LANE 10: 25 nM OPX-HVB-012
LANE 11: 5 µg/mL CYCLOHEXIMIDE

FIG. 19

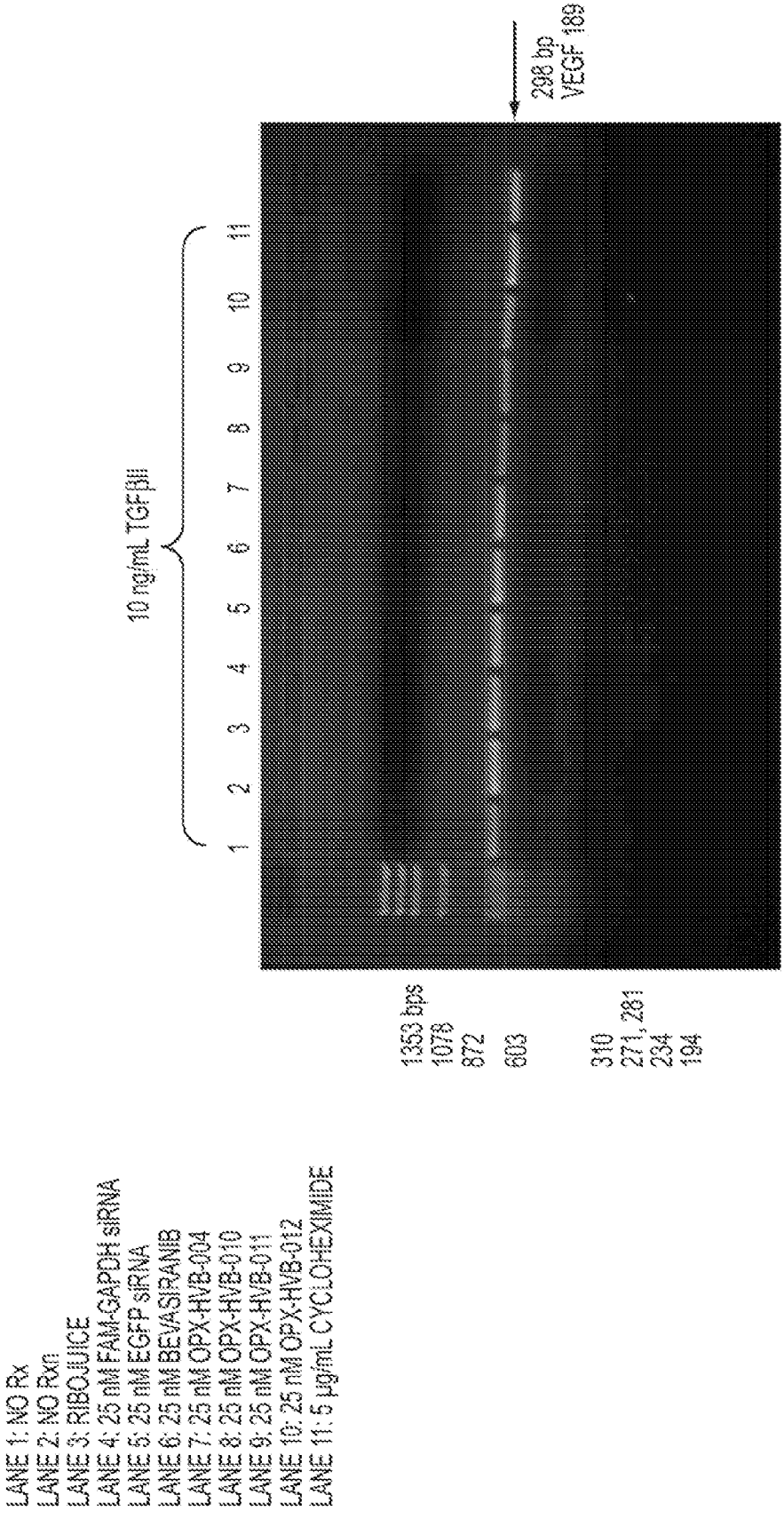
298 bp
VEGF 189
10 ng/mL TGFβIII
1 2 3 4 5 6 7 8 9 10 11
1353 bps
1078
872
603
310
271, 281
234
194
LANE 1: NO Rx
LANE 2: NO Rxn
LANE 3: RIBOJUICE
LANE 4: 25 nM FAM-GAPDH siRNA
LANE 5: 25 nM EGFP siRNA
LANE 6: 25 nM BEVASIRANIB
LANE 7: 25 nM OPX-HVB-004
LANE 8: 25 nM OPX-HVB-010
LANE 9: 25 nM OPX-HVB-011
LANE 10: 25 nM OPX-HVB-012
LANE 11: 5 µg/mL CYCLOHEXIMIDE

FIG. 20

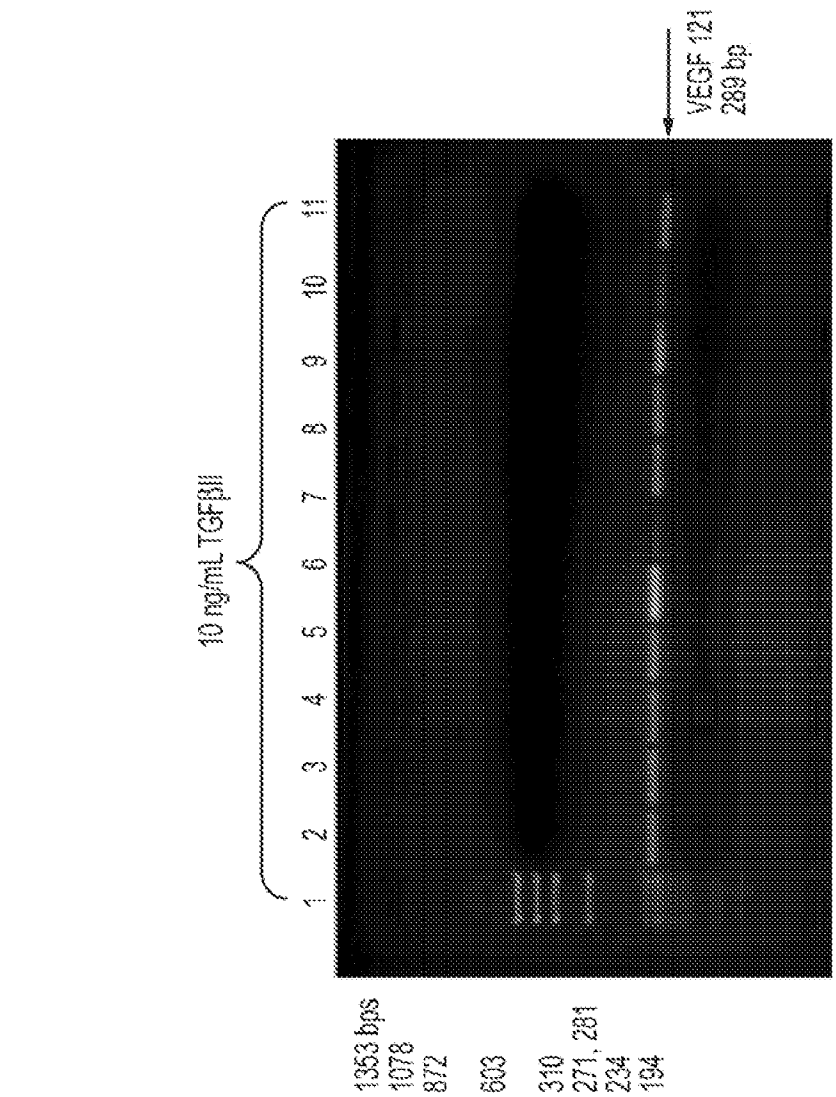
VEGF 121
289 bp
10 ng/mL TGFβIII
1 2 3 4 5 6 7 8 9 10 11
1353 bps
1078
872
603
310
271, 281
234
194
LANE 1: NO Rx
LANE 2: NO Rxn
LANE 3: RIBOJUICE
LANE 4: 25 nM FAM-GAPDH siRNA
LANE 5: 25 nM EGFP siRNA
LANE 6: 25 nM BEVASIRANIB
LANE 7: 25 nM OPX-HVB-004
LANE 8: 25 nM OPX-HVB-010
LANE 9: 25 nM OPX-HVB-011
LANE 10: 25 nM OPX-HVB-012
LANE 11: 5 µg/mL CYCLOHEXIMIDE

FIG. 21

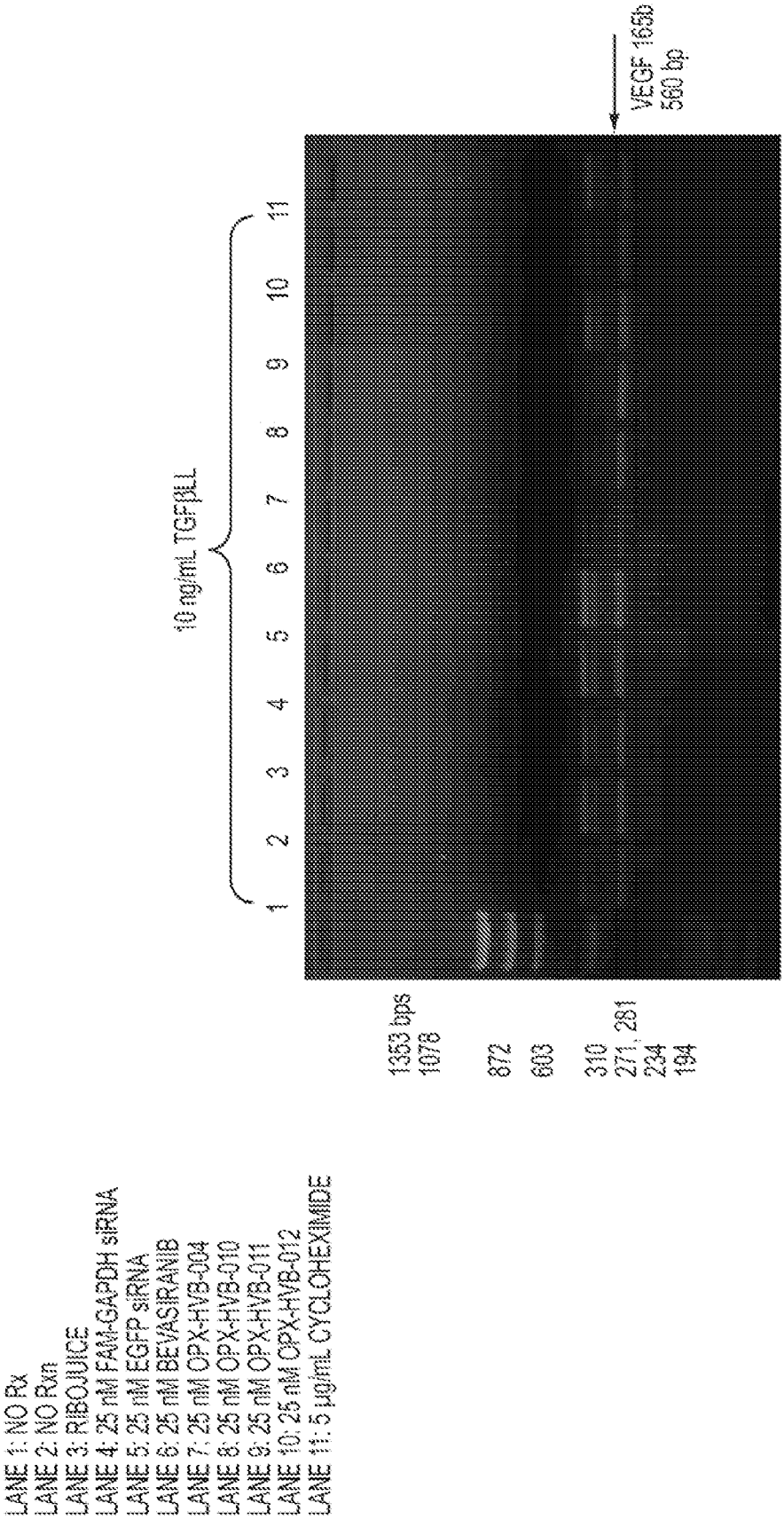
VEGF 165b
560 bp
10 ng/mL TGFβLL
1 2 3 4 5 6 7 8 9 10 11
1353 bps
1078
872
603
310
271, 281
234
194
LANE 1: NO Rx
LANE 2: NO Rxn
LANE 3: RIBOJUICE
LANE 4: 25 nM FAM-GAPDH siRNA
LANE 5: 25 nM EGFP siRNA
LANE 6: 25 nM BEVASIRANIB
LANE 7: 25 nM OPX-HVB-004
LANE 8: 25 nM OPX-HVB-010
LANE 9: 25 nM OPX-HVB-011
LANE 10: 25 nM OPX-HVB-012
LANE 11: 5 µg/mL CYCLOHEXIMIDE

FIG. 22

CYTOKINE PROFILE OF ARPE19 CELLS FOLLOWING TREATMENT WITH siRNAs

CYTOKINE	POLY I:C	BEVASIRANIB	OPK-HVB-004	OPK-HVB-010	OPK-HVB-009	OPK-HVB-012
IFN-α	-	-	-	-	-	-
IFN-β	-	-	-	-	-	-
IFN-γ	-	-	-	-	-	-
IL-8	+	-	+	-	-	+
IL-6	+	+	+	-	-	+
TNFα	+	-	-	-	-	-
ICAM	+	-	-	-	-	-
IL-12	-	-	-	-	-	-
MCP-1	+	-	+	-	-	+

FIG. 23

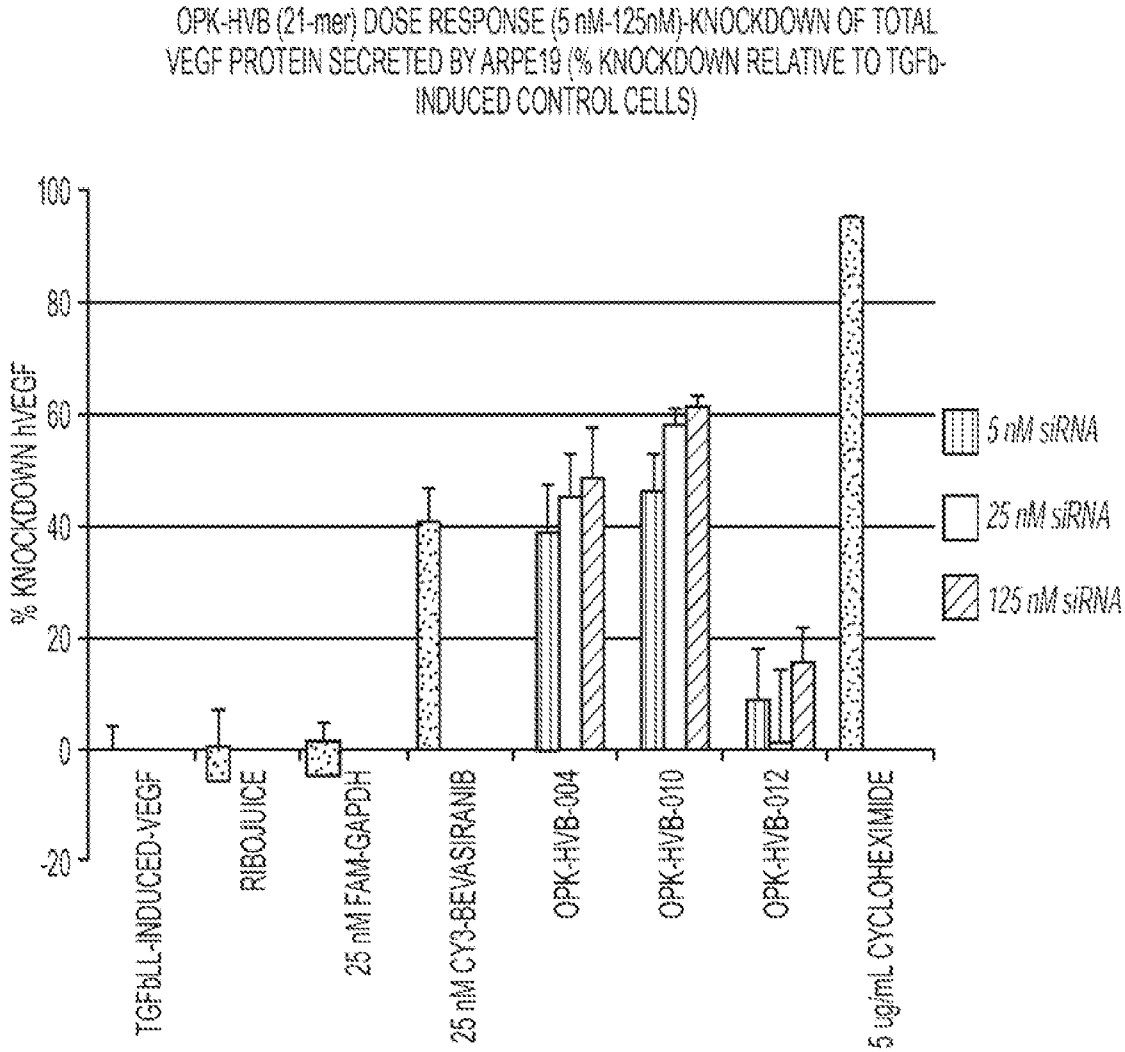
OPK-HVB (21-mer) DOSE RESPONSE (5 nM-125nM)-KNOCKDOWN OF TOTAL VEGF PROTEIN SECRETED BY ARPE19 (% KNOCKDOWN RELATIVE TO TGFb-INDUCED CONTROL CELLS)
% KNOCKDOWN hVEGF
100
80
60
40
20
0
-20
5 nM siRNA
25 nM siRNA
125 nM siRNA
TGFbLL-INDUCED-VEGF
RIBOJUICE
25 nM FAM-GAPDH
25 nM CY3-BEVASIRANIB
OPK-HVB-004
OPK-HVB-010
OPK-HVB-012
5 ug/mL CYCLOHEXIMIDE

FIG. 24

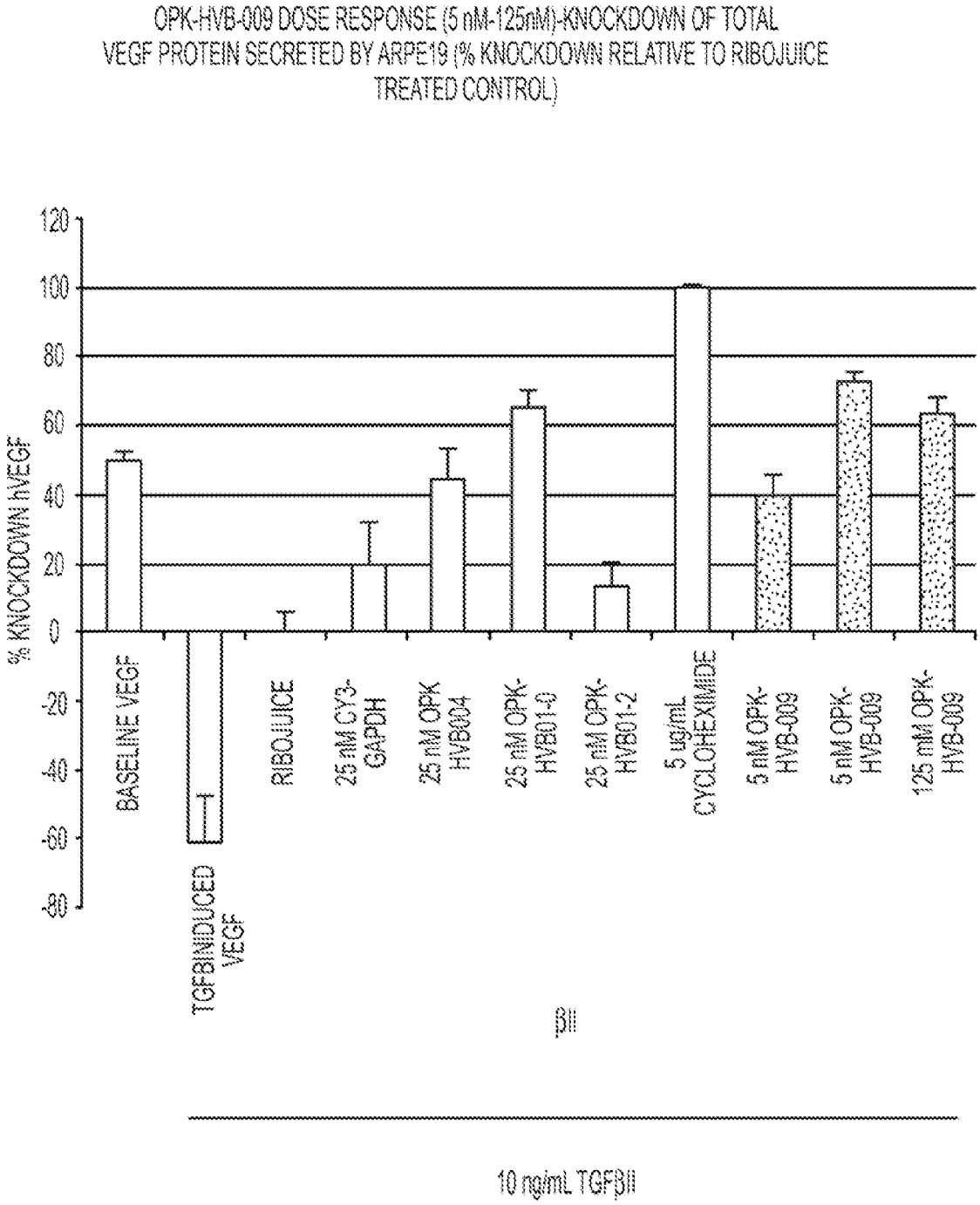
OPK-HVB-009 DOSE RESPONSE (5 nM-125nM)-KNOCKDOWN OF TOTAL VEGF PROTEIN SECRETED BY ARPE19 (% KNOCKDOWN RELATIVE TO RIBOJUICE TREATED CONTROL)
% KNOCKDOWN hVEGF
120
100
80
60
40
20
0
-20
-40
-60
-80
BASELINE VEGF
TGFBINDUCED VEGF
RIBOJUICE
25 nM CY3-GAPDH
25 nM OPK HVB004
25 nM OPK-HVB01-0
25 nM OPK-HVB01-2
5 ug/mL CYCLOHEXIMIDE
5 nM OPK-HVB-009
5 nM OPK-HVB-009
125 nM OPK-HVB-009
βII
10 ng/mL TGFβII

FIG. 25

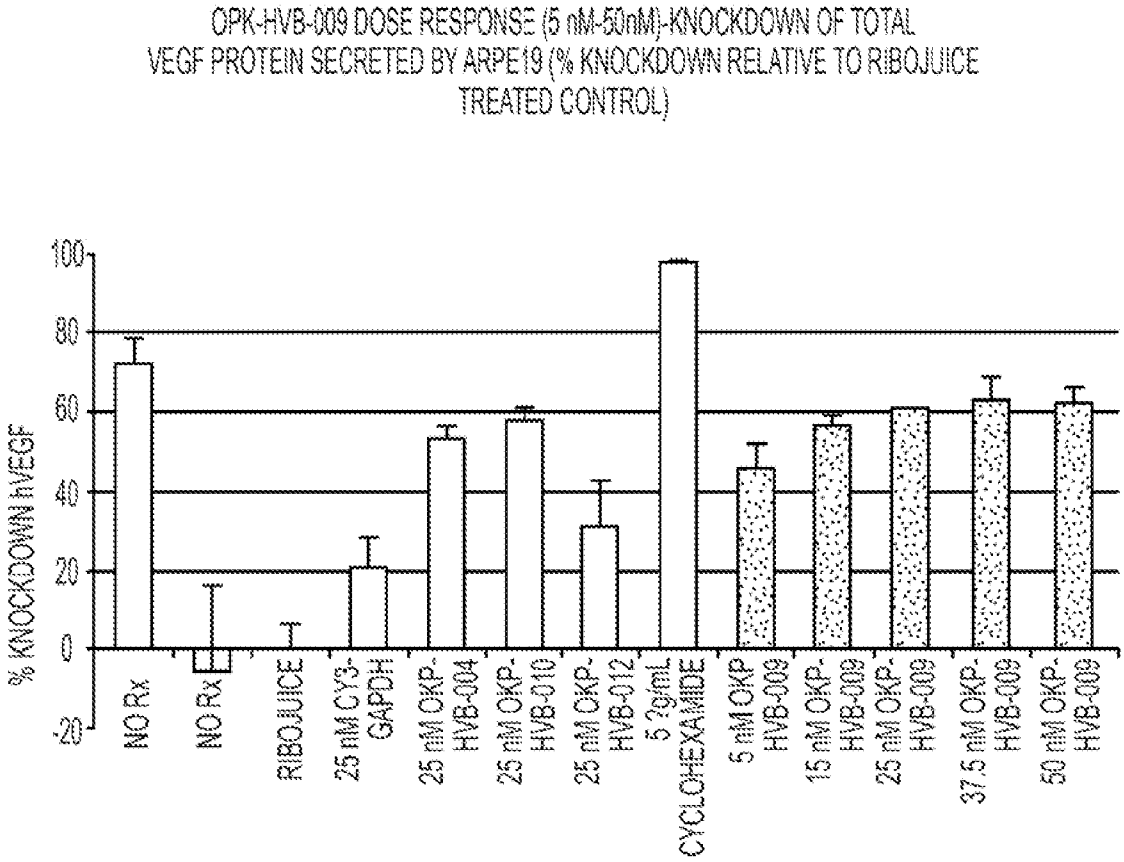
OPK-HVB-009 DOSE RESPONSE (5 nM-50nM)-KNOCKDOWN OF TOTAL VEGF PROTEIN SECRETED BY ARPE19 (% KNOCKDOWN RELATIVE TO RIBOJUICE TREATED CONTROL)
% KNOCKDOWN hVEGF
100
80
60
40
20
0
-20
NO Rx
NO Rx
RIBOJUICE
25 nM CY3-GAPDH
25 nM OKP-HVB-004
25 nM OKP-HVB-010
25 nM OKP-HVB-012
5 ?g/mL CYCLOHEXAMIDE
5 nM OKP HVB-009
15 nM OKP-HVB-009
25 nM OKP-HVB-009
37.5 nM OKP-HVB-009
50 nM OKP-HVB-009

FIG. 26

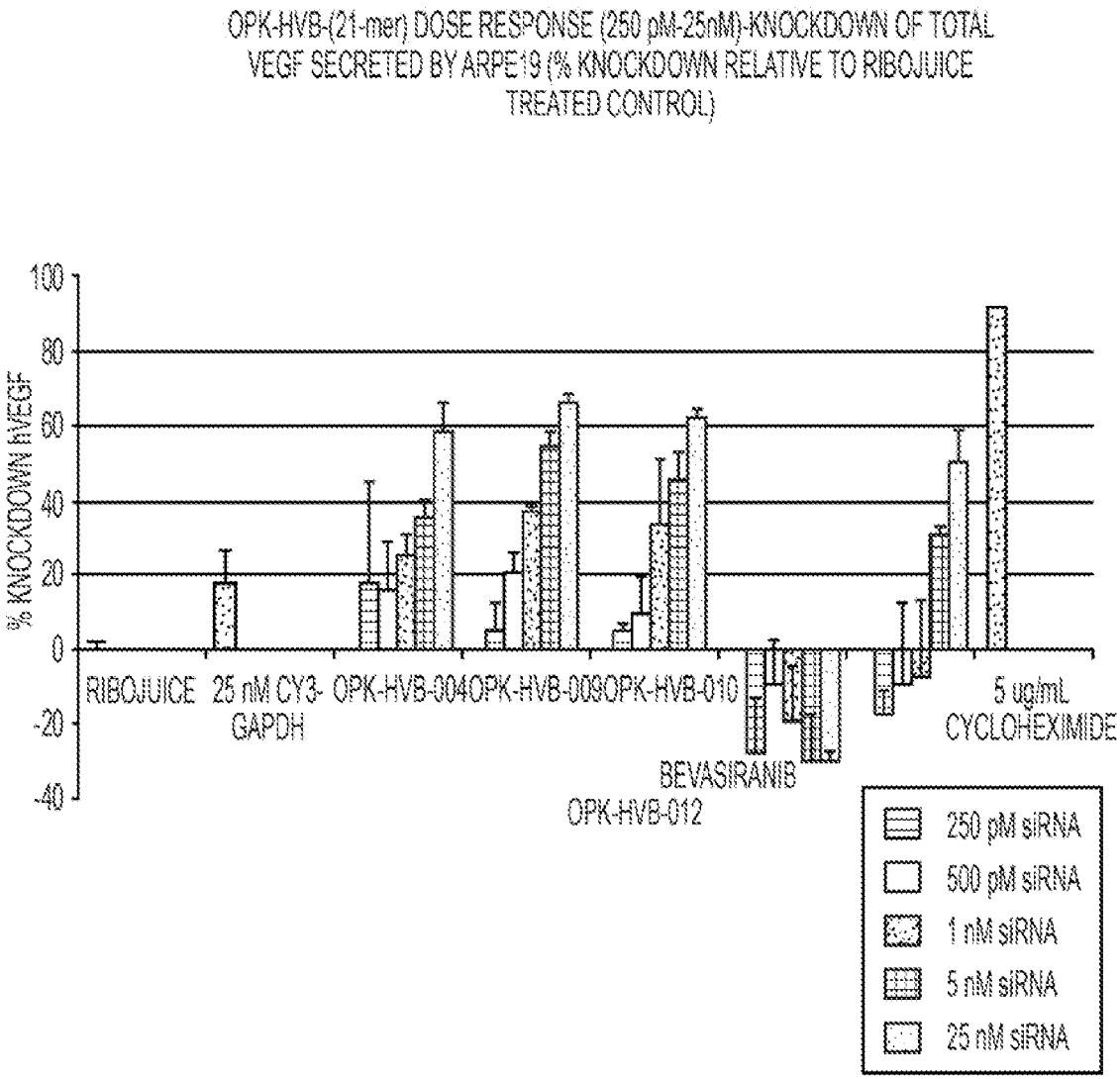
OPK-HVB-(21-mer) DOSE RESPONSE (250 pM-25nM)-KNOCKDOWN OF TOTAL VEGF SECRETED BY ARPE19 (% KNOCKDOWN RELATIVE TO RIBOJUICE TREATED CONTROL)
% KNOCKDOWN hVEGF
100
80
60
40
20
0
-20
-40
RIBOJUICE
25 nM CY3-GAPDH
OPK-HVB-004
OPK-HVB-009
OPK-HVB-010
BEVASIRANIB
OPK-HVB-012
5 ug/mL CYCLOHEXIMIDE
250 pM siRNA
500 pM siRNA
1 nM siRNA
5 nM siRNA
25 nM siRNA

FIG. 27

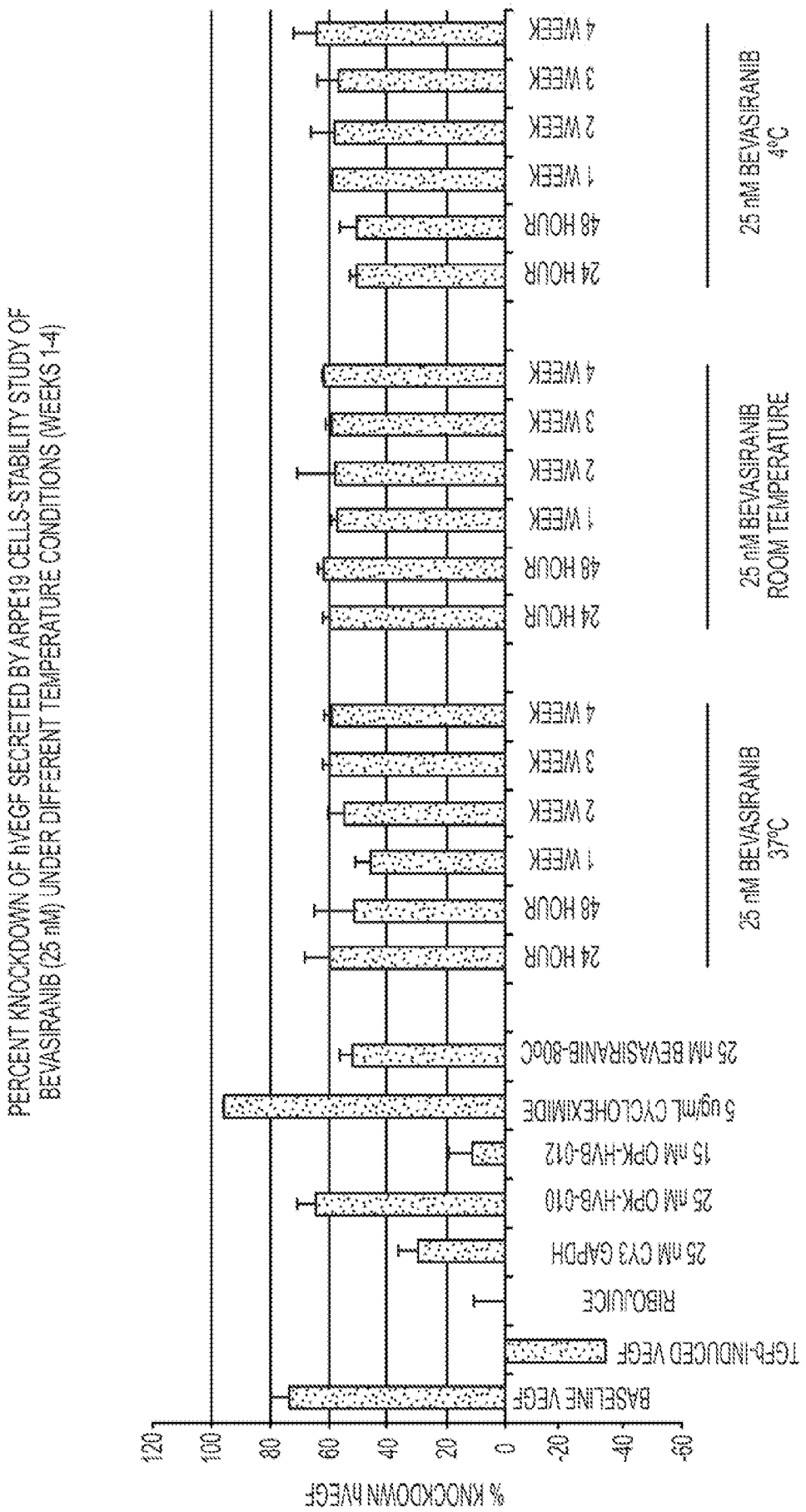
PERCENT KNOCKDOWN OF hVEGF SECRETED BY ARPE19 CELLS-STABILITY STUDY OF BEVASIRANIB (25 nM) UNDER DIFFERENT TEMPERATURE CONDITIONS (WEEKS 1-4)
% KNOCKDOWN hVEGF
120
100
80
60
40
20
0
-20
-40
-60
BASELINE VEGF
TGFb-INDUCED VEGF
RIBOJUICE
25 nM CY3 GAPDH
25 nM OPK-HVB-010
15 nM OPK-HVB-012
5 ug/mL CYCLOHEXIMIDE
25 nM BEVASIRANIB-80oC
24 HOUR
48 HOUR
1 WEEK
2 WEEK
3 WEEK
4 WEEK
25 nM BEVASIRANIB 37°C
24 HOUR
48 HOUR
1 WEEK
2 WEEK
3 WEEK
4 WEEK
25 nM BEVASIRANIB ROOM TEMPERATURE
24 HOUR
48 HOUR
1 WEEK
2 WEEK
3 WEEK
4 WEEK
25 nM BEVASIRANIB 4°C

FIG. 28

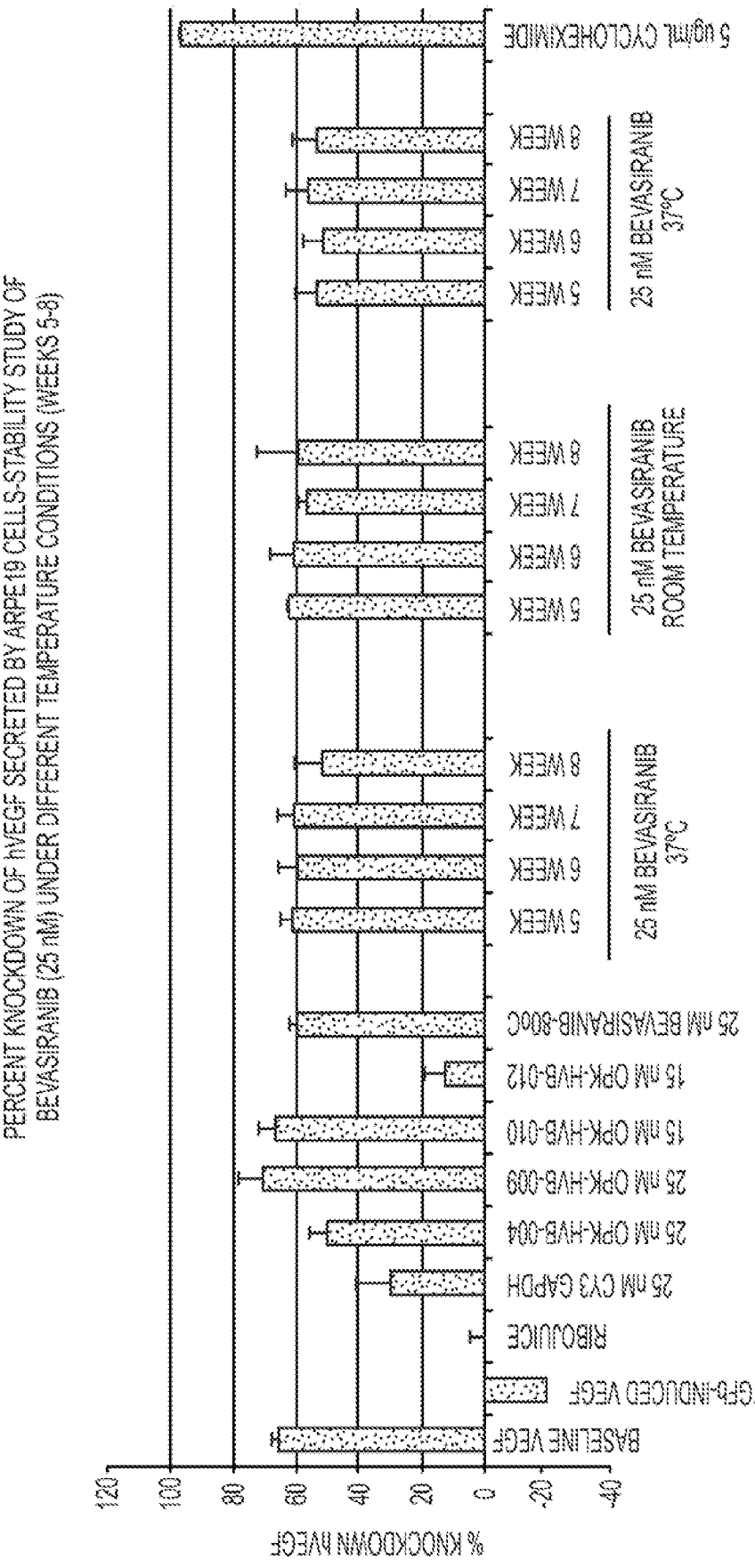
PERCENT KNOCKDOWN OF hVEGF SECRETED BY ARPE19 CELLS-STABILITY STUDY OF BEVASIRANIB (25 nM) UNDER DIFFERENT TEMPERATURE CONDITIONS (WEEKS 5-8)
% KNOCKDOWN hVEGF
120
100
80
60
40
20
0
-20
-40
BASELINE VEGF
TGFb-INDUCED VEGF
RIBOJUICE
25 nM CY3 GAPDH
25 nM OPK-HVB-004
25 nM OPK-HVB-009
15 nM OPK-HVB-010
15 nM OPK-HVB-012
25 nM BEVASIRANIB-80oC
25 nM BEVASIRANIB 37°C
5 WEEK
6 WEEK
7 WEEK
8 WEEK
25 nM BEVASIRANIB ROOM TEMPERATURE
5 WEEK
6 WEEK
7 WEEK
8 WEEK
25 nM BEVASIRANIB 37°C
5 WEEK
6 WEEK
7 WEEK
8 WEEK
5 ug/mL CYCLOHEXIMIDE

FIG. 29

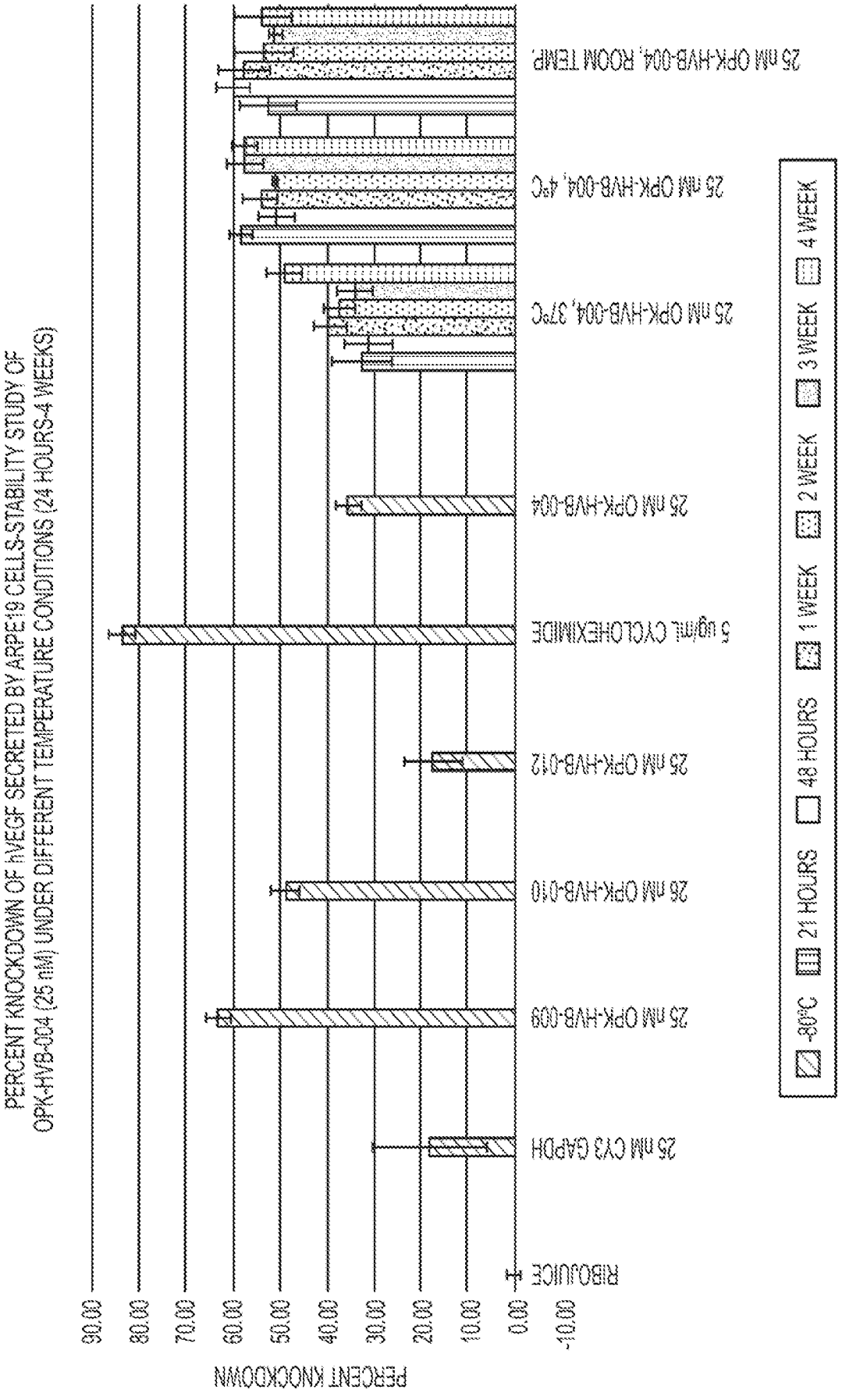
PERCENT KNOCKDOWN OF hVEGF SECRETED BY ARPE19 CELLS-STABILITY STUDY OF OPK-HVB-004 (25 nM) UNDER DIFFERENT TEMPERATURE CONDITIONS (24 HOURS-4 WEEKS)
PERCENT KNOCKDOWN
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
-10.00
RIBOJUICE
25 nM CY3 GAPDH
25 nM OPK-HVB-009
25 nM OPK-HVB-010
25 nM OPK-HVB-012
5 ug/mL CYCLOHEXIMIDE
25 nM OPK-HVB-004
25 nM OPK-HVB-004, 37°C
25 nM OPK-HVB-004, 4°C
25 nM OPK-HVB-004, ROOM TEMP.
-80°C
21 HOURS
48 HOURS
1 WEEK
2 WEEK
3 WEEK
4 WEEK

FIG. 30

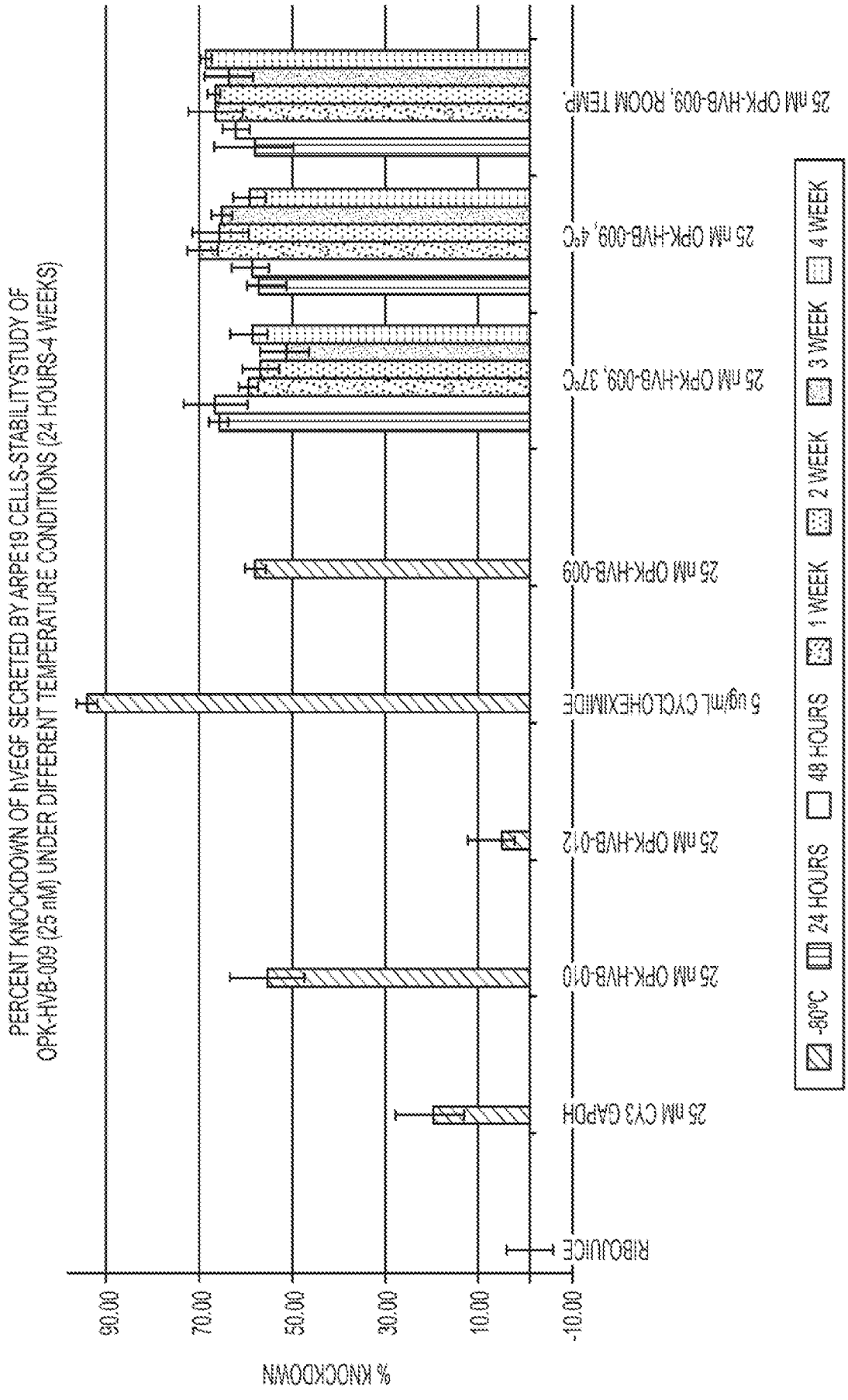
PERCENT KNOCKDOWN OF hVEGF SECRETED BY ARPE19 CELLS-STABILITYSTUDY OF OPK-HVB-009 (25 nM) UNDER DIFFERENT TEMPERATURE CONDITIONS (24 HOURS-4 WEEKS)
% KNOCKDOWN
90.00
70.00
50.00
30.00
10.00
-10.00
RIBOJUICE
25 nM CY3 GAPDH
25 nM OPK-HVB-010
25 nM OPK-HVB-012
5 ug/mL CYCLOHEXIMIDE
25 nM OPK-HVB-009
25 nM OPK-HVB-009, 37°C
25 nM OPK-HVB-009, 4°C
25 nM OPK-HVB-009, ROOM TEMP.
-80°C
24 HOURS
48 HOURS
1 WEEK
2 WEEK
3 WEEK
4 WEEK

FIG. 31

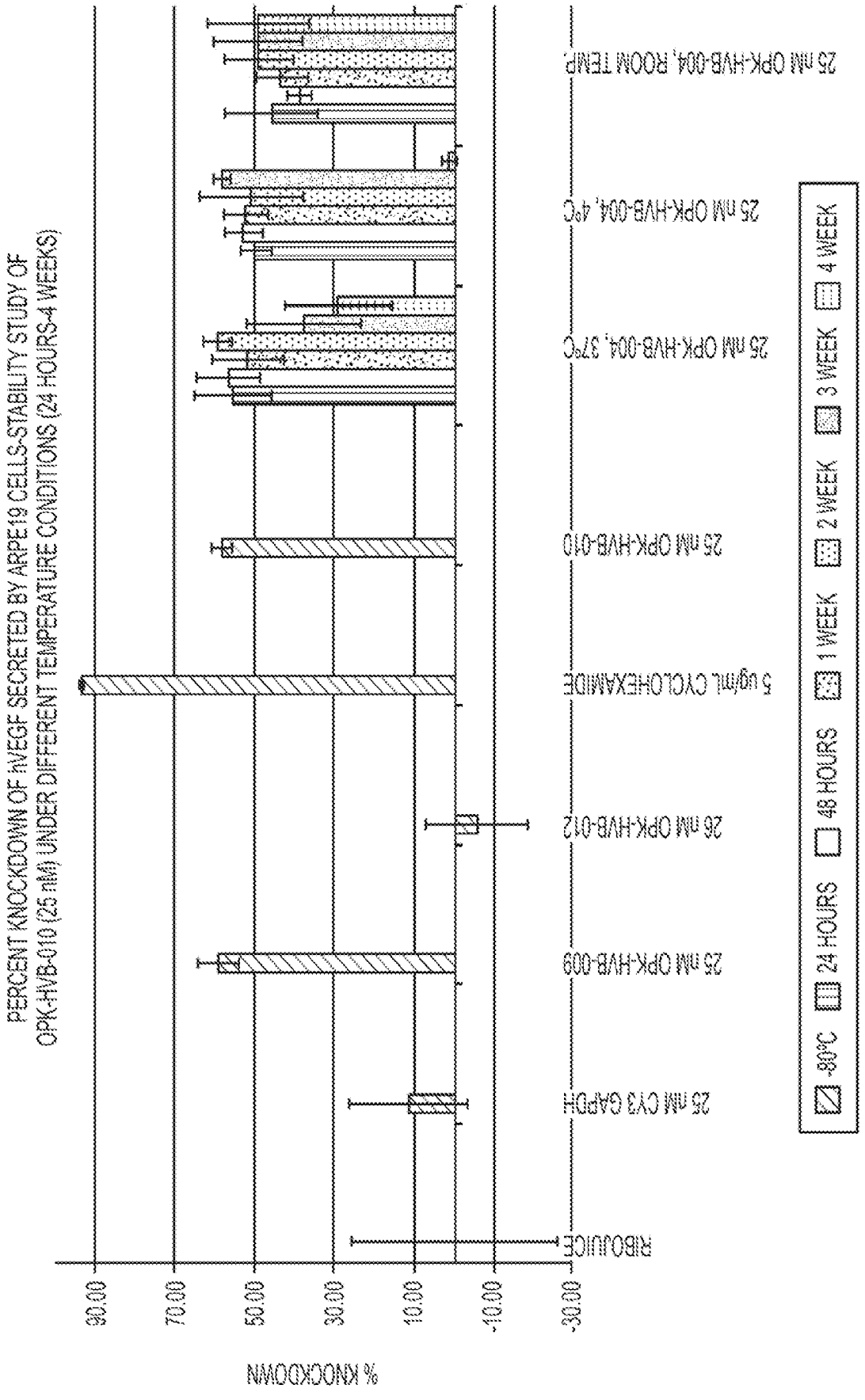
PERCENT KNOCKDOWN OF hVEGF SECRETED BY ARPE19 CELLS-STABILITY STUDY OF OPK-HVB-010 (25 nM) UNDER DIFFERENT TEMPERATURE CONDITIONS (24 HOURS-4 WEEKS)
% KNOCKDOWN
90.00
70.00
50.00
30.00
10.00
-10.00
-30.00
RIBOJUICE
25 nM CY3 GAPDH
25 nM OPK-HVB-009
25 nM OPK-HVB-012
5 ug/mL CYCLOHEXAMIDE
25 nM OPK-HVB-010
25 nM OPK-HVB-004, 37C
25 nM OPK-HVB-004, 4C
25 nM OPK-HVB-004, ROOM TEMP
-80°C
24 HOURS
48 HOURS
1 WEEK
2 WEEK
3 WEEK
4 WEEK

FIG. 32

Homology between terminal codons encoding human,mouse and rat VEGF

Human	gaacgtactt gcaga	tgt	gac	aag	ccg	agg	cgg	tga
		C	D	K	P	R	R	
		Cys	Asp	Lys	Pro	Arg	Arg	
Rat	gaacgtactt gcaga	tgt	gac	aag	cc[A]	agg	cgg	tga
		C	D	K	P	R	R	
		Cys	Asp	Lys	Pro	Arg	Arg	
Mouse	gaacgtactt gcaga	tgt	gac	aag	cc[A]	agg	cgg	tga
		C	D	K	P	R	R	
		Cys	Asp	Lys	Pro	Arg	Arg	

SiRNA	Target Sequence
OPK-HVB-004	GTACTTGCAGATGTGACAA
OPK-HVB-009	TGCAGATGTGACAAGCC[G]A
OPK-HVB-010	GCAGATGTGACAAGCC[G]AG
OPK-HVB-012	AGATGTGACAAGCC[G]AGGC

FIG. 33

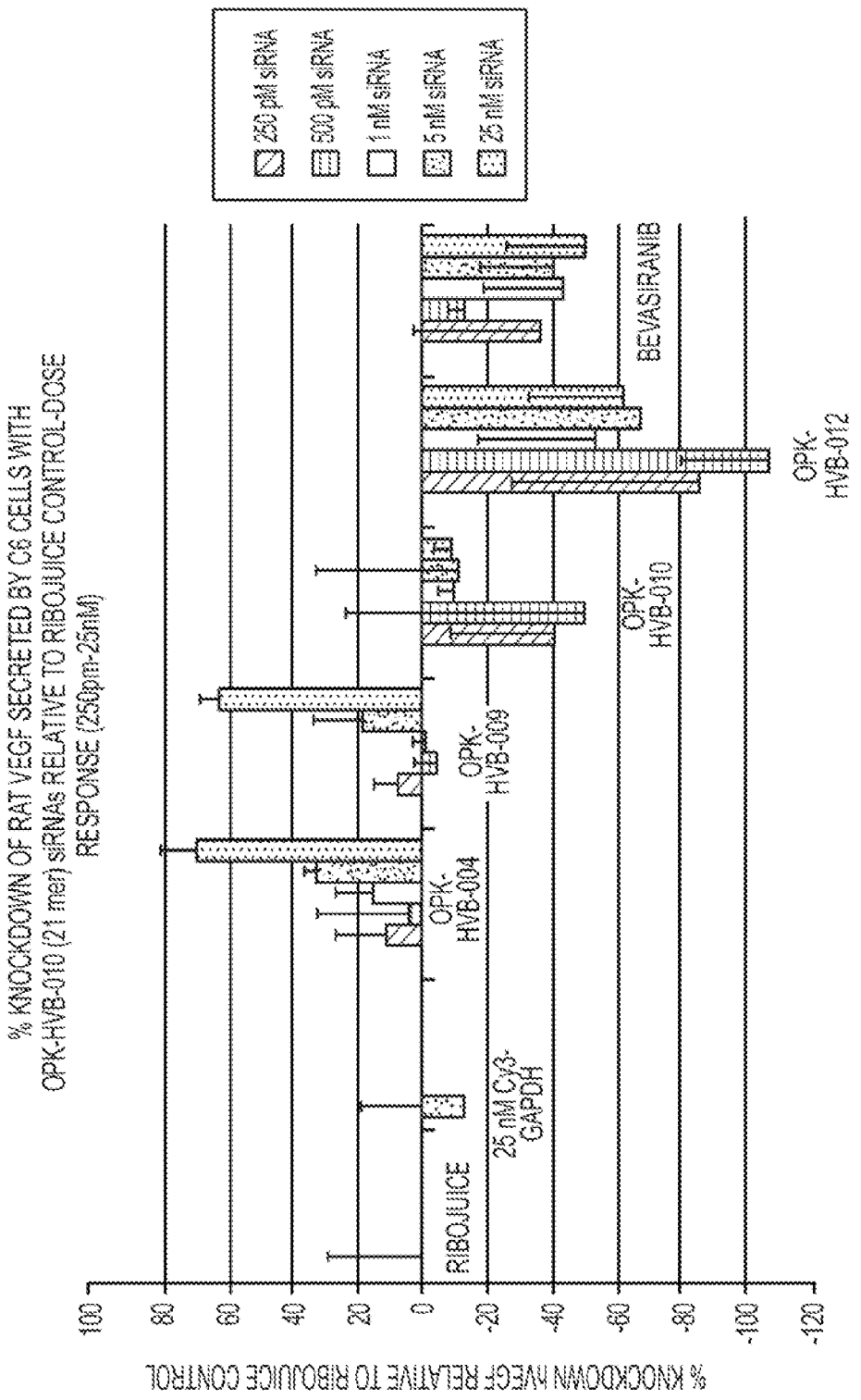
% KNOCKDOWN OF RAT VEGF SECRETED BY C6 CELLS WITH
OPK-HVB-010 (21 mer) siRNAs RELATIVE TO RIBOJUICE CONTROL-DOSE
RESPONSE (250pm-25nM)
250 pM siRNA
500 pM siRNA
1 nM siRNA
5 nM siRNA
25 nM siRNA
RIBOJUICE
25 nM Cy3-GAPDH
OPK-HVB-004
OPK-HVB-009
OPK-HVB-010
BEVASIRANIB
OPK-HVB-012
100
80
60
40
20
0
-20
-40
-60
-80
-100
-120
% KNOCKDOWN VEGF RELATIVE TO RIBOJUICE CONTROL

FIG. 34

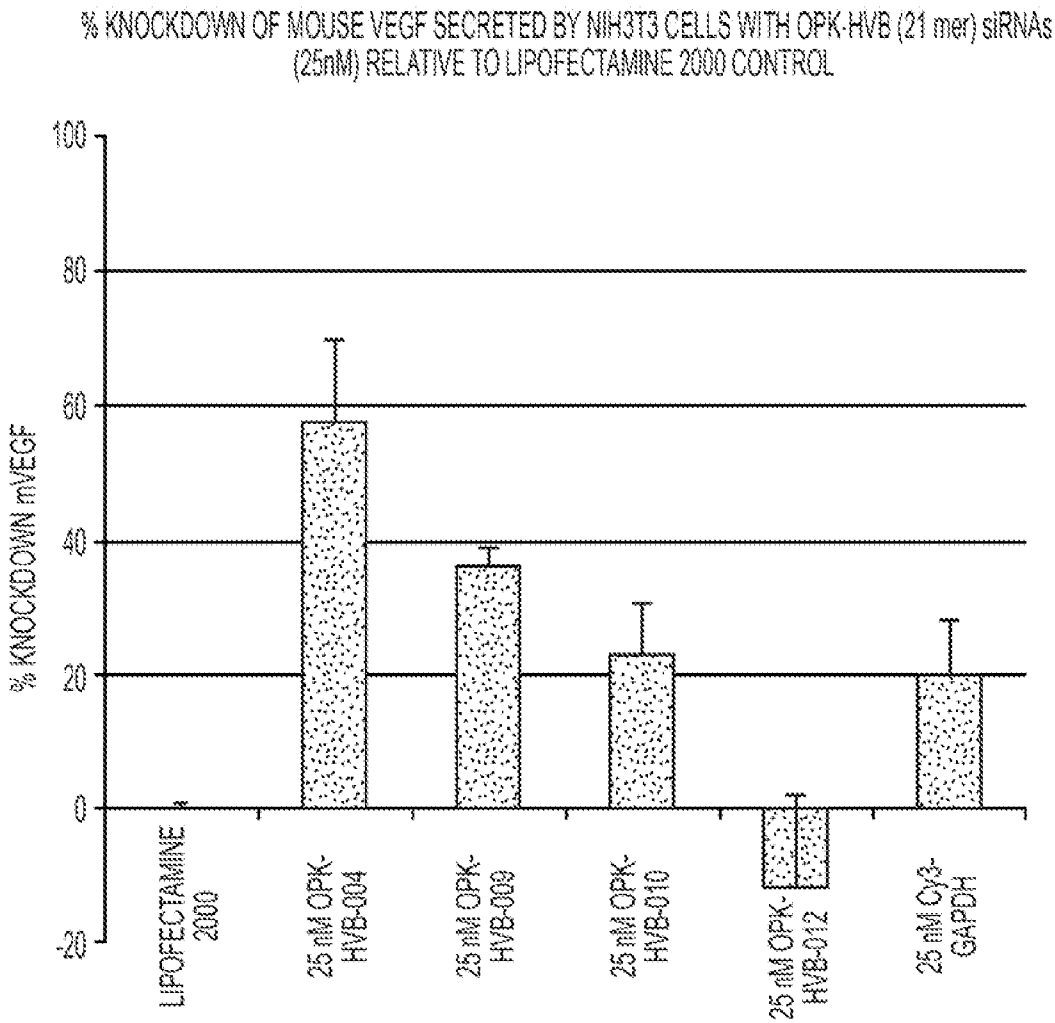
% KNOCKDOWN OF MOUSE VEGF SECRETED BY NIH3T3 CELLS WITH OPK-HVB (21 mer) siRNAs (25nM) RELATIVE TO LIPOFECTAMINE 2000 CONTROL
% KNOCKDOWN mVEGF
100
80
60
40
20
0
-20
LIPOFECTAMINE 2000
25 nM OPK-HVB-004
25 nM OPK-HVB-009
25 nM OPK-HVB-010
25 nM OPK-HVB-012
25 nM Cy3-GAPDH

FIG. 35

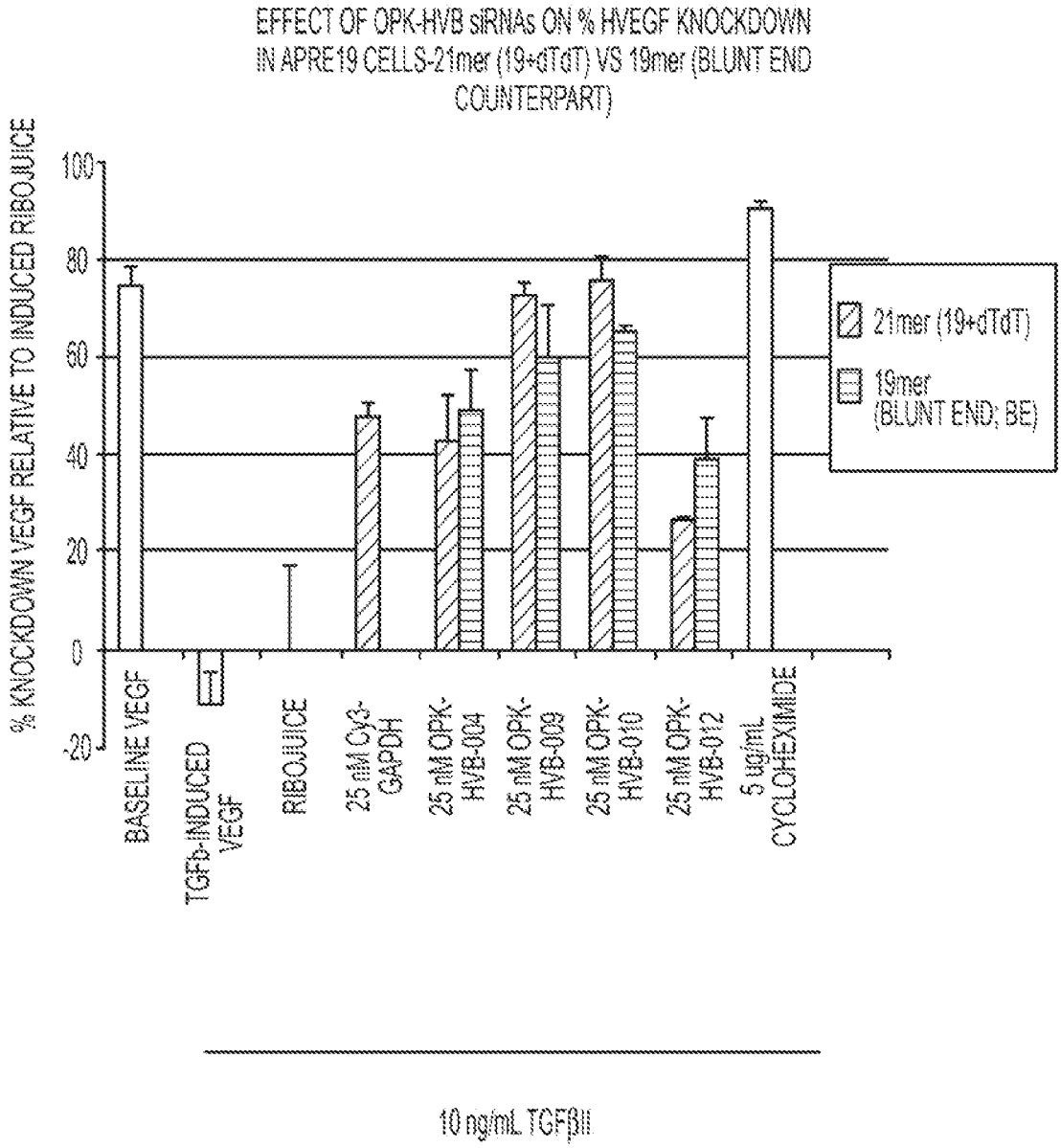
EFFECT OF OPK-HVB siRNAs ON % HVEGF KNOCKDOWN IN APRE19 CELLS-21mer (19+dTdT) VS 19mer (BLUNT END COUNTERPART)
% KNOCKDOWN VEGF RELATIVE TO INDUCED RIBOJUICE
100
80
60
40
20
0
-20
21mer (19+dTdT)
19mer (BLUNT END; BE)
BASELINE VEGF
TGFb-INDUCED VEGF
RIBOJUICE
25 nM Cy3-GAPDH
25 nM OPK-HVB-004
25 nM OPK-HVB-009
25 nM OPK-HVB-010
25 nM OPK-HVB-012
5 ug/mL CYCLOHEXIMIDE
10 ng/mL TGFβII

FIG. 36

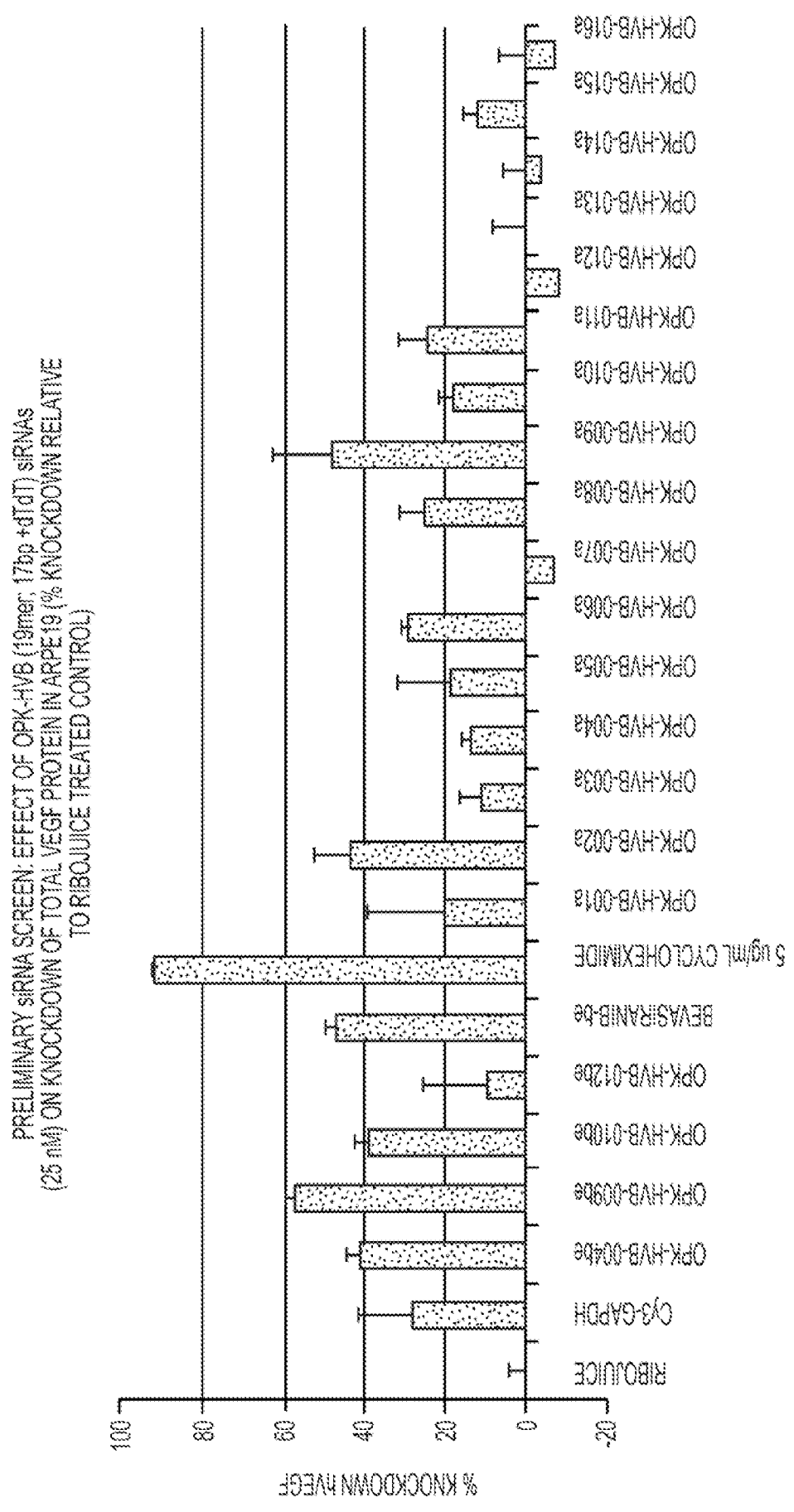
PRELIMINARY siRNA SCREEN: EFFECT OF OPK-HVB (19mer, 17bp +dTdT) siRNAs
(25 nM) ON KNOCKDOWN OF TOTAL VEGF PROTEIN IN ARPE19 (% KNOCKDOWN RELATIVE
TO RIBOJUICE TREATED CONTROL)
% KNOCKDOWN hVEGF
100
80
60
40
20
0
-20
RIBOJUICE
Cy3-GAPDH
OPK-HVB-004be
OPK-HVB-009be
OPK-HVB-010be
OPK-HVB-012be
BEVASIRANIB-be
5 ug/mL CYCLOHEXIMIDE
OPK-HVB-001a
OPK-HVB-002a
OPK-HVB-003a
OPK-HVB-004a
OPK-HVB-005a
OPK-HVB-006a
OPK-HVB-007a
OPK-HVB-008a
OPK-HVB-009a
OPK-HVB-010a
OPK-HVB-011a
OPK-HVB-012a
OPK-HVB-013a
OPK-HVB-014a
OPK-HVB-015a
OPK-HVB-016a

FIG. 37

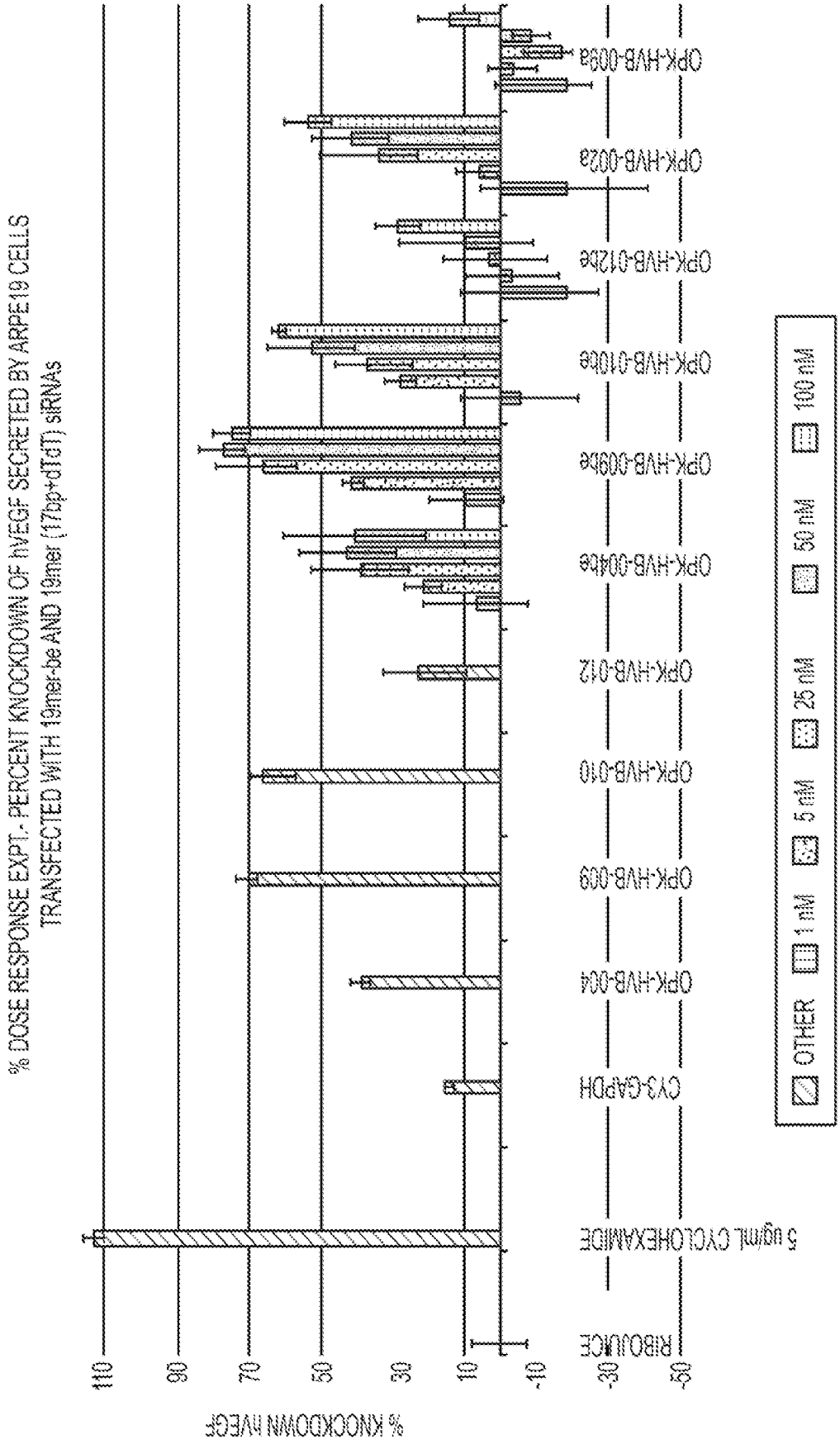
% DOSE RESPONSE EXPT.- PERCENT KNOCKDOWN OF hVEGF SECRETED BY ARPE19 CELLS
TRANSFECTED WITH 19mer-be AND 19mer (17bp+dTdT) siRNAs
% KNOCKDOWN hVEGF
110
90
70
50
30
10
-10
-30
-50
OPK-HVB-009a
OPK-HVB-002a
OPK-HVB-012be
OPK-HVB-010be
OPK-HVB-009be
OPK-HVB-004be
OPK-HVB-012
OPK-HVB-010
OPK-HVB-009
OPK-HVB-004
CY3-GAPDH
5 ug/mL CYCLOHEXAMIDE
RIBOJUICE
OTHER
1 nM
5 nM
25 nM
50 nM
100 nM

FIG. 38

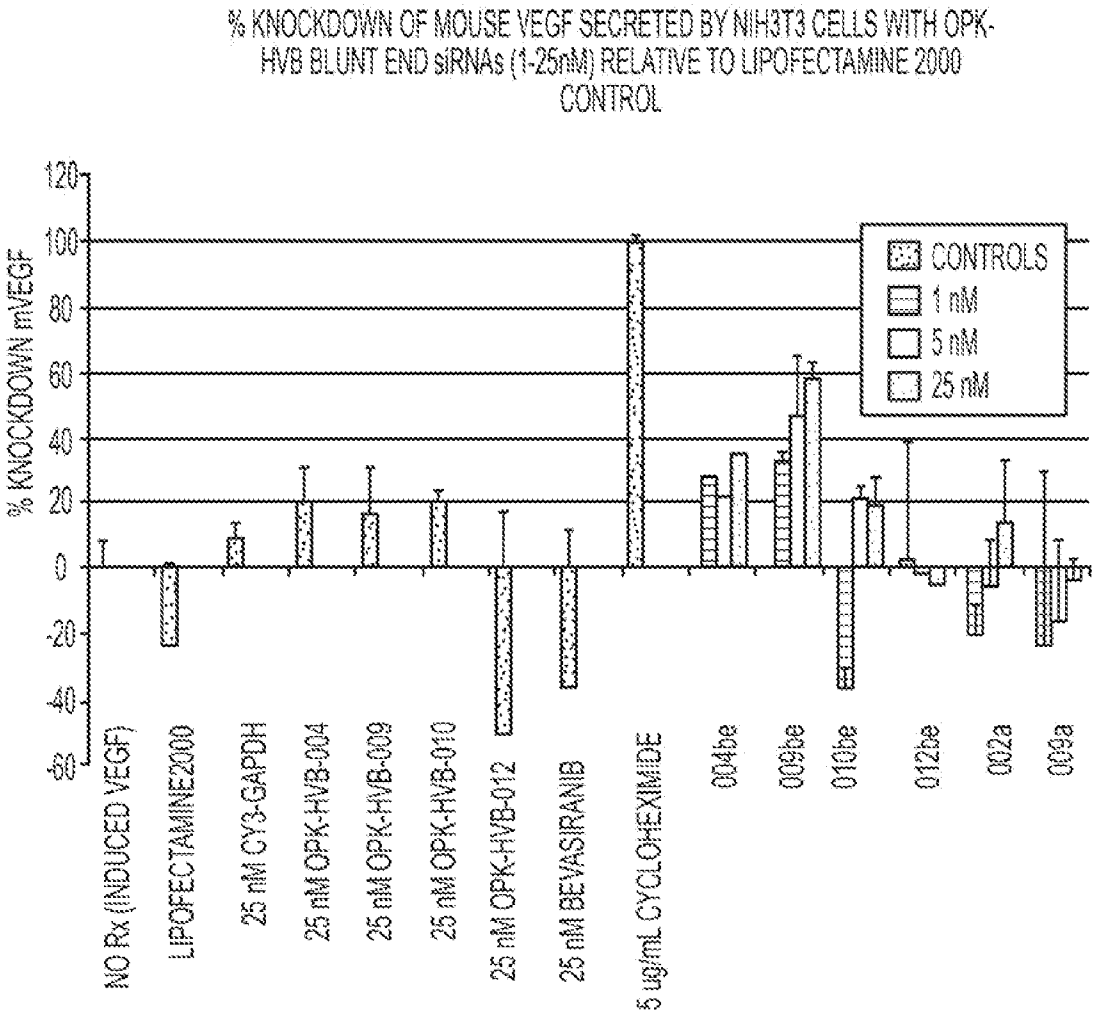
% KNOCKDOWN OF MOUSE VEGF SECRETED BY NIH3T3 CELLS WITH OPK-HVB BLUNT END siRNAs (1-25nM) RELATIVE TO LIPOFECTAMINE 2000 CONTROL
% KNOCKDOWN mVEGF
120
100
80
60
40
20
0
-20
-40
-60
CONTROLS
1 nM
5 nM
25 nM
NO Rx (INDUCED VEGF)
LIPOFECTAMINE2000
25 nM CY3-GAPDH
25 nM OPK-HVB-004
25 nM OPK-HVB-009
25 nM OPK-HVB-010
25 nM OPK-HVB-012
25 nM BEVASIRANIB
5 ug/mL CYCLOHEXIMIDE
004be
009be
010be
012be
002a
009a

FIG. 39

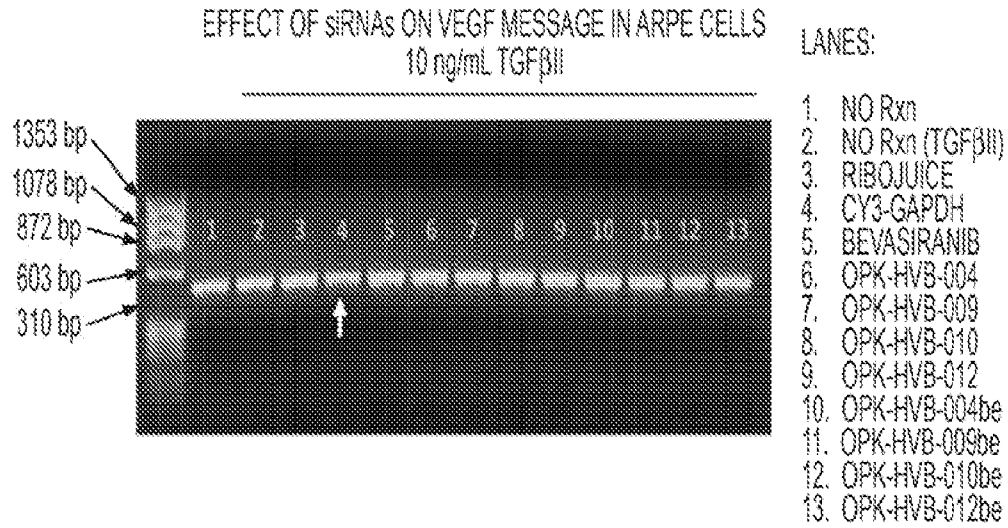

FIG. 40A

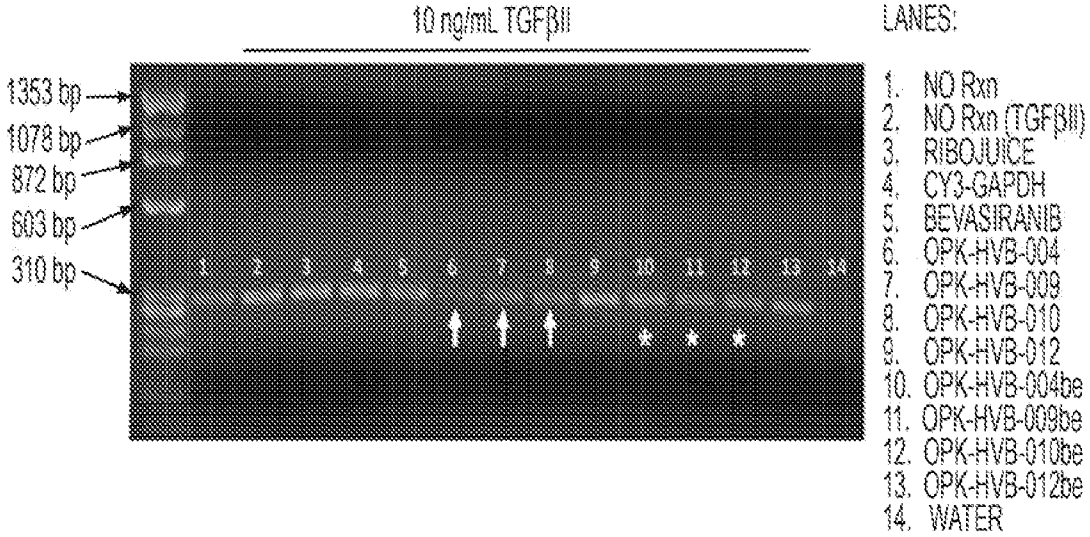

FIG. 40B

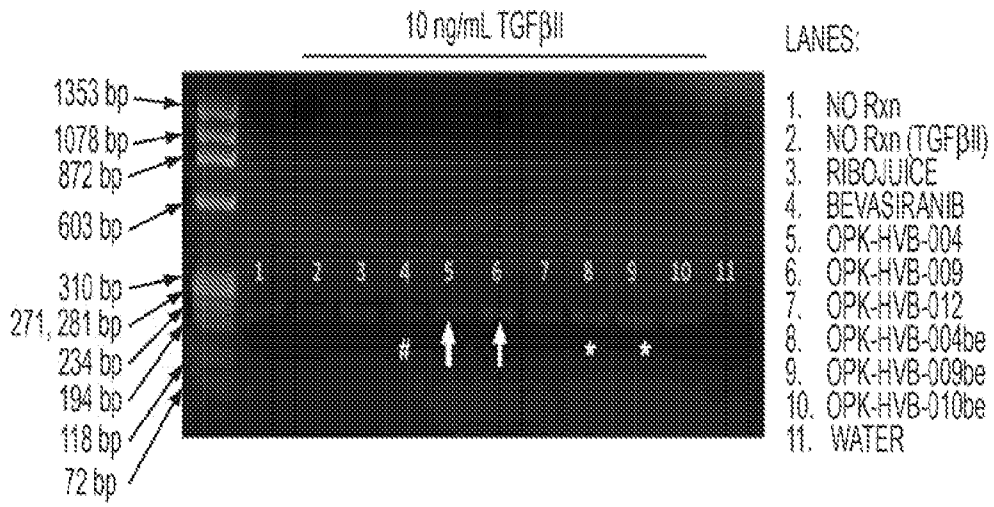

FIG. 40C

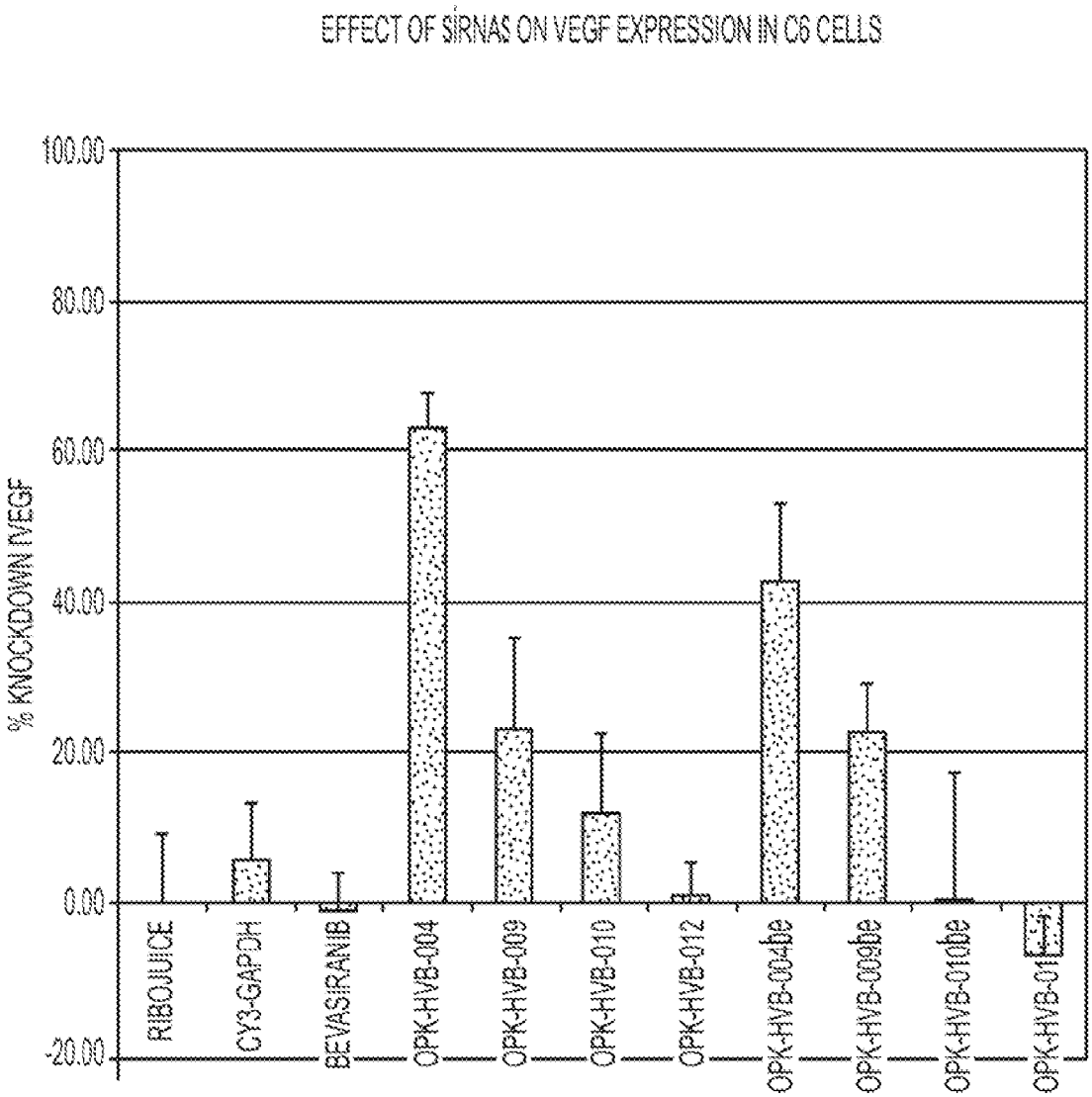
EFFECT OF SIRNAS ON VEGF EXPRESSION IN C6 CELLS
% KNOCKDOWN IVEGF
100.00
80.00
60.00
40.00
20.00
0.00
-20.00
RIBOJUICE
CY3-GAPDH
BEVASIRANIB
OPK-HVB-004
OPK-HVB-009
OPK-HVB-010
OPK-HVB-012
OPK-HVB-004be
OPK-HVB-009be
OPK-HVB-010be
OPK-HVB-01

FIG. 41

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/59090

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 15/113 (2011.01)
USPC - 514/44a; 536/24.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC: 514/44a; 536/24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 514/44a; 536/24.5 (text search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic data bases: PubWEST (PGPB, EPAB, JPAB, USPT); Google Scholar, GenCore sequence search (NT)
Search terms: Vascular endothelial growth factor (VEGF), siRNA, RNA duplex, single-stranded hairpin, nuclease degradation, angiogenic, cancer, age-related macular degeneration, psoriasis, administer, pharmaceutical composition, SEQ ID NOs: 77,7

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 7,148,342 B2 (TOLENTINO et al.) 12 December 2006 (12.12.2006). Especially col 3 ln 9-15, col 4 ln 9-12, col 5 ln 40-44, col 9 38-40, col 10 ln 45-59, col 12 40-44, col 13 ln 6-67, col 14 ln 58-61, col 15 ln 20-27, claims 1 and 2, SEQ ID NO: 77,78.	1-38
X	US 2005/0281861 Al (HUGHES et al.) 22 December 2005 (22.12.2005). Especially para [0045], [0049-0051], [0197].	1, 2, 5, 7, 18, 38
X	US 2009/0226531 A1 (LYONS et al.) 10 September 2009 (10.09.2009) para [0021], [0022], [0031], [0036], [0042]; SEQ ID NO: 3, 4.	1, 5, 7, 18

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 March 2011 (02.03.2011)	21 MAR 2011
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/59090

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☐ in the international application as filed

   ☒ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: