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(54) **Title**: COMPOSITIONS AND METHODS USING siRNA MOLECULES FOR TREATMENT OF GLIOMAS

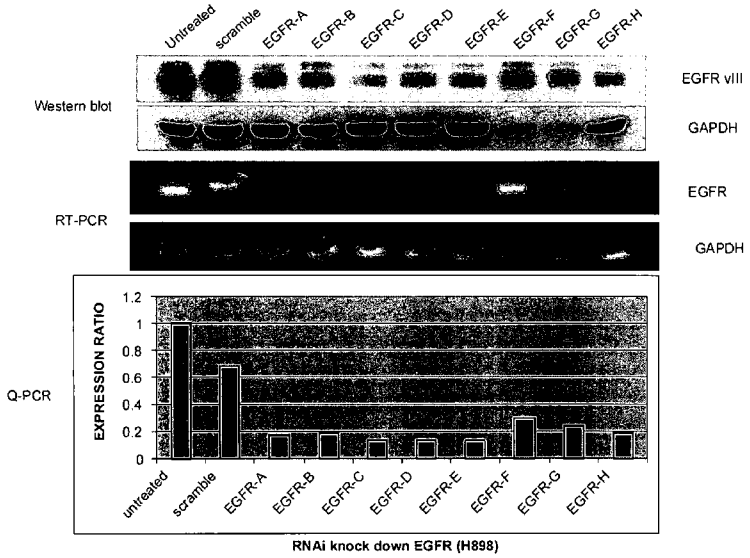

Figure 2

(57) **Abstract**: The present invention provides small interfering RNA (siRNA) molecules, compositions containing the molecules, and methods of using the compositions to treat gliomas.

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COMPOSITIONS AND METHODS USING siRNA MOLECULES FOR TREATMENT OF GLIOMAS

This application claims priority to U.S. Provisional Patent Application No. 61/055,966, filed May 24, 2008, which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

The invention relates to RNA interference and, in particular, the use of small interfering RNA (siRNA) molecules for the treatment of cancer, such as gliomas.

BACKGROUND

Major advances in molecular biology, cellular biology, and genomics have substantially improved our understanding of cancer. Now, these advances are being translated into therapy. Targeted therapy directed at specific molecular alterations is already creating a shift in the treatment of cancer patients. Glioblastoma multiforme (GBM), the most common brain cancer of adults, is highly suited for this new approach. GBM is among the most aggressive and deadly of neoplasms. Despite decades of aggressive surgical treatment, chemotherapy, radiotherapy, and extensive basic science and clinical research focused on combating this disease, the prognosis remains virtually unchanged, with survival rates still measured in months. The current genetic understanding of GBM has led to the identification of crucial intracellular molecules and their associated signaling pathways as potential therapeutic targets. Multiple genes are being identified as critical to the development of GBM and other similar tumors, such as gliomas and other astrocytomas.

Targets involved in GBM

GBM, like most malignant tumors, exhibits multiple genetic abnormalities, including aberrant activation of intracellular signal-transduction pathways that regulate processes such as proliferation, angiogenesis, and apoptosis. For example, vascular endothelial growth factor (VEGF) is needed to promote tumorigenesis and angiogenesis in GBM (7). In addition, over 40% of all GBMs exhibit amplification, mutation, or rearrangement of the epidermal growth factor receptor (EGFR) (2). As is common in malignant neoplasms, EGFR overexpression leads to aberrant activation of crucial downstream targets, including the PI3K/AKT signaling pathway, which mediates cell survival, and the mitogenic RAS/MAPK

cascade. Recent studies implicate a mutant variant of EGFR, EGFRvIII, in the activation of other receptor tyrosine kinases (RTKs), such as MET, thereby providing multiple inputs to these downstream signaling pathways for cellular proliferation and evasion of apoptosis. This redundant signaling could partially account for the modest response of GBMs to RTK inhibitors, such as erlotinib and gefitinib. In addition, GBM is frequently associated with elevated levels of O^6-methylguanine-DNA-methlytransferase (AGT), a DNA repair enzyme that enhances neoplastic resistance to chemotherapeutics such as Temozolomide (TMZ).

Matrix metalloproteinases (MMPs) enhance tumor cell invasion by degrading extracellular matrix proteins, by activating signal transduction cascades that promote motility, and by solubilizing extracellular matrix-bound growth factors. MMP-9 and MMP-2 promote GBM invasion *in vitro* and in xenograft models, and their inhibition dramatically reduces the invasive phenotype (1). The serine/threonine kinase Raf-1 is involved in the regulation of tumor cell survival, proliferation and metastasis formation, and has therefore emerged as a promising target for cancer therapy. Raf-1 silencing appears as a potential therapeutic strategy to inhibit brain tumor angiogenesis and thereby outgrowth of highly vascularized glioblastoma multiforme (28). RNA interference targeting TGF-beta 1, 2 results in a glioma cell phenotype that is more sensitive to immune cell lysis and less motile in vitro and nontumorigenic in nude mice (29). The mammalian target of rapamycin (mTOR) activity is required for the survival of some cells within these GBMs, and mTOR appears required for the maintenance of astrocytic character in the surviving cells (30). A small molecule inhibitor of Cox-2 enhanced glioblastoma radiosensitivity, reduced tumor cell viability and prolonged survival of implanted glioblastoma mice by inhibition of tumor angiogenesis and causing extensive tumor necrosis (31).

RNA interference (RNAi) and Small Interfering RNA (siRNA)

RNA interference (RNAi) is a sequence-specific RNA degradation process that provides a relatively easy and direct way to knock down, or silence, theoretically any gene. In naturally occurring RNAi, a double-stranded RNA (dsRNA) is cleaved by an RNase III/helicase protein, Dicer, into small interfering RNA (siRNA) molecules, a dsRNA of 19-27 nucleotides (nt) with 2-nt overhangs at the 3' ends. These siRNAs are incorporated into a multicomponent-ribonuclease called RNA-induced silencing complex (RISC). One strand of siRNA remains associated with RISC and guides the complex toward a cognate RNA that has sequence complementary to the guider ss-siRNA in RISC. This siRNA-directed endonuclease digests the RNA, thereby inactivating it. Recent studies have revealed that

chemically synthesized 21-27-nt siRNAs exhibit RNAi effects in mammalian cells, and the thermodynamic stability of siRNA hybridization (at terminals or in the middle) plays a central role in determining the molecule's function. These and other characteristics of RISC, siRNA molecules, and RNAi have been described.

Applications of RNAi in mammalian cells in the laboratory or, potentially, in therapeutic settings, use either chemically synthesized siRNAs or endogenously expressed molecules. The endogenous siRNA is first expressed as small hairpin RNAs (shRNAs) by an expression vector (plasmid or virus vector) and is then processed by Dicer into siRNAs. It is thought that siRNAs hold great promise to be therapeutics for human diseases, especially those caused by viral infections. Certain siRNA therapeutics are described in PCT application PCT/US2005/003858 for "Compositions and Methods for Combination RNAi Therapeutics", which is incorporated herein by reference in its entirety.

Importantly, it is presently not possible to predict with any degree of confidence which of many possible candidate siRNA sequences potentially targeting a genomic sequence (e.g., oligonucleotides of about 16-30 base pairs) will in fact exhibit effective siRNA activity. Instead, individual, specific candidate siRNA polynucleotide or oligonucleotide sequences must be generated and tested to determine whether the intended interference with expression of a targeted gene has occurred. Accordingly, no routine method exists for designing an siRNA polynucleotide that is, with certainty, capable of specifically altering the expression of a given mRNA.

SUMMARY OF THE INVENTION

The present invention provides an isolated small interfering RNA (siRNA) molecule that binds to a single-stranded RNA molecule, wherein the single-stranded RNA molecule comprises an mRNA that encodes at least part of a peptide or protein whose activity promotes tumorigenesis, angiogenesis, or cell proliferation in the brain or spinal cord of a mammal, or wherein the single stranded RNA molecule comprises an miRNA whose activity promotes tumorigenesis, angiogenesis, or cell proliferation in the brain or spinal cord of a mammal.

The invention also provides a composition comprising at least three of the siRNA molecules. In one embodiment, the molecules bind to multiple, different target sequences in the single-stranded RNA molecule or in different single stranded RNA molecules.

The invention further provides compositions comprising one or more of the siRNA molecules in a pharmaceutically acceptable carrier. These compositions may include, or be used with, a therapeutic agent that prevents or hinders tumorigenesis, angiogenesis, or cell proliferation in the brain or spinal cord of a mammal.

The invention also provides a method for treating a glioma in a subject comprising administering to the subject an effective amount of a composition of the invention. In one embodiment, the subject is a human. In another embodiment, the glioma is a glioblastoma multiforme (GBM).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Identification of siRNA duplexes silencing *MGMT* expression. The total RNA and protein samples were collected after H80TR cells were transfected with corresponding siRNA duplexes, followed by RT-PCR, quantitative PCR and Western blot analyses. Selection of potent siRNA duplexes targeting both human and mouse MGMT genes.

Figure 2. Identification of siRNA duplexes silencing *EGFR* expression. The total RNA and protein samples were collected after H898 cells were transfected with corresponding siRNA duplexes, followed by RT-PCR, quantitative PCR and Western blot analyses. Selection of potent siRNA duplexes targeting both human and mouse *EGFR*.

Figure 3. Identification of potent siRNA duplexes silencing *VEGF-A* expression. The total RNA and protein samples were collected after 293 cells and F3 cells were transfected with corresponding siRNA duplexes, followed by quantitative PCR analyses. Selection of potent siRNA duplexes targeting both human and mouse *VEGF*.

Figure 4. Selection of potent siRNA for MMP-2 knockdown. On the day before transfection, 2.5×10^5 PC-3 cells were plated on the wells of 6-well plates in 2 ml of culture medium without antibiotics. On the next day, the cells were transfected with siRNA - hMMP-2 #1, #2, and #3 and Luc siRNA at different concentrations—0.5 μg/well, 1.0 μg/well, and 2.0 μg/well. For each transfection sample (in duplicate), oligomer-Lipofectamine 2000 complexes were prepared:

250 μl of Optimem I Reduced Serum Medium + 1.4 μl siRNA (0.5 μg) (28 pmole/well)
250 μl of Optimem I Reduced Serum Medium + 2.8 μl siRNA (1 μg) (56 pmole/well)
250 μl of Optimem I Reduced Serum Medium + 5.6 μl (siRNA (2 μg) (112 pmole/well)

Small interfering ultimobranchial body cells were used for negative control (0.5 μg/well – 28 pmole). After 6 hours, the medium was changed. mRNA was isolated by using an RNAqueous-4PCR kit.

Figure 5. Selection of potent MMP-9 siRNA. mRNA knockdown after transfection of the PC-3 cells with siRNA (hMMP-9). On the day before transfection, 2.5×10^5 PC-3 cells were plated on the wells of 6-well plates in 2 ml of culture medium without antibiotics. On the next day, cells were transfected with siRNA hMMP-9 #1, siRNA hMMP-9 #2, siRNA hMMP-9 #3, and siRNA Luc at different concentrations—0.9 μg/well (50 pmole), 1.8 μg/well (100 pmole), and 2.7 μg/well (150 pmole). After 6 hours, the medium was changed. Cells were incubated at 37°C for 48 hours.

Figure 6. Selection of potent PDGF siRNA using the same procedures as above.

Figure 7. Comparison of silencing potencies between 25mer and 21mer siRNA duplexes. The most potent 25 mer and 21mer siRNA were selected first from each set of 6 duplexes. Then comparison was carried out with two tumor cell lines expressing human VEGF protein (DLD-1, colon carcinoma and MBA-MD-435, breast carcinoma) using in vitro transfection with Lipo2000 (Invitrogen, CA) followed by RT-PCR analyses. At either 0.3μg or 2.0μg doses, 25mer siRNA demonstrated stronger inhibitory activity than 21mer siRNA, especially at 2.0μg. We found that 25mer duplexes with blunt ends are the most potent inhibitors, up to 60% either MBA-MD-435 or DLD-1 cells and in tumor bearing in animals. We have tested a 25mer siRNA duplex targeting human VEGF gene, hVEGF-25c (sense: 5'-CACAACAAAUGUGAAUGCAGACCAA-3'; Antisense:5'-UUGGUCUGCAUUCACAUUUGUUGUG-3'), comparing to a 21mer siRNA duplex which has been tested many times as one of the most potent VEGF specific inhibitory duplexes, hVEGF-21a (sense: 5'-UCGAGACCCUGGUGGACAUTT-3'; antisense: 5'-AUGUCCACCAGGGUCUCGATT-3'), in the cell culture followed with Q-RT-PCR analysis. Figure 7 demonstrates that the 25 mer blunt end siRNA is more potent than the 21mer sticky end siRNA, which supports using 25mer siRNA duplexes in the proposed study of multi-targeted siRNA cocktail therapeutics for cancer treatment.

Figure 8. Selection of potent siRNA targeting RAF-1. (A) The lower panel illustrates selection of eight 25 mer siRNA duplexes with control siRNA were transfected into human MDA-MB-231 cells and mouse CT26 cells. 24 hr later, mRNA were collected and subject to Q-RT-PCR with the standard control gene target Rigs15. Based on the gene silencing

activity observed, the most potent Raf1-siRNA was selected as a component of a future siRNA cocktail for both in vitro and in vivo study.

RAF-1-siRNA: 5'-GCCUGCUGCUCCUCGGCUGCGGAUA-3'.

Figure 9. Selection of potent siRNA targeting mTOR. (A) The lower panel illustrates selection of eight 25 mer siRNA duplexes with control siRNA were transfected into human MDA-MB-231 cells and mouse CT26 cells. 24 hr later, mRNA were collected and subject to Q-RT-PCR with the standard control gene target Rigs15.. Based on the gene silencing activity observed, the most potent mTOR-siRNA was selected as a component of a future siRNA cocktail for both in vitro and in vivo study.

mTOR-siRNA: 5'-GGUCUGGUGCCUGGUCUGAUGAUGU-3'.

Figure 10. RT-PCR analysis for selection of potent siRNA targeting TGFβ1 The silencing activities of three siRNA oligos targeting the TGFβ-1 gene expression was demonstrated through gel electorphoresis analysis. A potent siRNA oligo was indentified based on the silencing activities in both human and moue cells.

hmTGFβ1: sense, 5'-CCCAAGGGCUACCAUGCCAACUUCU-3', antisense,5'-AGAAGUUGGCAUGGUAGCCCUUGGG-3'.

Figure 11. RT-PCR analysis for selection of potent siRNA targeting Cox-2 The silencing activities of three siRNA oligos targeting the Cox-2 gene expression was demonstrated through gel electorphoresis analysis. A potent siRNA oligo was indentified based on the silencing activities in both human and moue cells.

hmCox-2: sense, 5'-GGUCUGGUGCCUGGUCUGAUGAUGU-3', antisense, 5'-ACAUCAUCAGACCAGGCACCAGACC-3'.

Figure 12. HKP for in vivo siRNA Delivery (A) Polymeric Nanoparticle-siRNA Systems Histidine-Lysine polymer (HKP) mixed with siRNA duplexes resulted in HKP-siRNA nanoparticles. Structures of two species of HKP, H3K4b and PT73, were showed with the scanning electron microscope (SEM) image of HKP-siRNA nanoparticle. (B) Cocktail siRNAs are more potent than single siRNA for anti-angiogenesis efficacy of siRNA cocktail on ocular NV. Local delivery of HKP-siRNA cocktail (black bar) significantly minimized angiogenesis areas in mouse eyes at P4 (**$P < 0.01$, $n = 8$) compared to single gene targeted HKP-siRNA: VEGF (grey), VEGFR1 and VEGFR2 (stripe and dot) at P4 ((* $P < 0.05$). At P7, VEGF and VEGFR1 specific siRNA lost the inhibitory activities, while the cocktail siRNA are still demonstrated its activity.

Figure 13. HKP for Systemic Tumor siRNA Delivery Accumulation of intravenously (IV) delivered HKP-encapsulated siRNA molecules in established head and neck squamous cancer 1483 xenograft tumors. A: Images of mouse tissues and tumors with Alexa Fluor 555 labeled fluorescent siRNA CT-2 deposits following tail vein injection of HKP-siRNA. The tissues were harvested at the indicated time, freshly frozen, sectioned, and analyzed by fluorescent microscopy. Magnification: 400x. B: Accumulation of fluorescent CT-2 (Red deposits indicated with arrows) in the tumors in proximity to blood vessels (Brown; CD31 immunostaining). Magnification: 400x. Inset: Detail showing labeled siRNA in tumor tissue.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides siRNA molecules, compositions containing the molecules, and methods of using the compositions to treat a glioma in a subject. As used herein, an "siRNA molecule" is a duplex oligonucleotide, that is a short, double-stranded oligonucleotide, that interferes with the expression of a gene in a cell that produces RNA, after the molecule is introduced into the cell. Such molecules are constructed by techniques known to those skilled in the art. Such techniques are described in U.S. Pat. Nos. 5, 898,031, 6,107,094, 6,506,559, 7,056,704 and in European Pat. Nos. 1214945 and 1230375, which are incorporated herein by reference in their entireties.

The siRNA molecule of the invention is an isolated siRNA molecule that binds to a single stranded RNA molecule, which is a messenger RNA (mRNA) that encodes at least part of a peptide or protein whose activity promotes tumorigenesis, angiogenesis, or cell proliferation in the brain or spinal cord of a mammal, or which is a micro-RNA (miRNA) whose activity promotes tumorigenesis, angiogenesis, or cell proliferation in the brain or spinal cord of a mammal. In one embodiment, the molecule is an oligonucleotide with a length of about 19 to about 35 base pairs. In another embodiment, the molecule is an oligonucleotide with a length of about 19 to about 27 base pairs. In still another embodiment, the molecule is an oligonucleotide with a length of about 21 to about 25 base pairs. In all of these embodiments, the molecule may have blunt ends at both ends, or sticky ends at both ends, or a blunt end at one end and a sticky end at the other.

The siRNA molecule can be made of naturally occurring ribonucleotides, i.e., those found in living cells, or one or more of its nucleotides can be chemically modified by techniques known in the art. In addition to being modified at the level of one or more of its

individual nucleotides, the backbone of the oligonucleotide also can be modified. Additional modifications include the use of small molecules (e.g. sugar molecules), amino acid molecules, peptides, cholesterol and other large molecules for conjugation onto the siRNA molecules.

In one embodiment, the siRNA molecule binds to an mRNA that encodes at least part of a peptide or protein whose activity promotes tumorigenesis, angiogenesis, or cell proliferation in the brain or spinal cord of a mammal. Such may be the case when the mRNA molecule encodes a protein in a pro-tumorigenic pathway, pro-angiogenesis pathway, pro-cell proliferation pathway, or anti-apoptotic pathway. For example, the protein can be a VEGF pathway protein, EGFR pathway protein, MGMT pathway protein, RAF pathway protein, MMP pathway protein, mTOR pathway protein, TGFβ pathway protein, or Cox-2 pathway protein. In one embodiment, the protein is one of the following: VEGF, EGFR, PI3K, AKT, AGT, RAF1, RAS, MAPK, ERK, MGMT, MMP-2, MMP-9, PDGF, PDGFR, IGF-1, HGF, mTOR, Cox-2, or TGFβ1. In another embodiment, the protein is VEGF, EGFR, MGMT, MMP-2, MMP-9, or PDGF. In still another embodiment, the protein is RAF1, mTOR, Cox-2, or TGFβ1.

In one embodiment, the siRNA molecule binds to both a human mRNA molecule and a homologous mouse mRNA molecule. That is, the human and mouse mRNA molecules encode proteins that are substantially the same in structure or function. Therefore, the efficacy and toxicity reactions observed in the mouse disease models (e.g. tumor models) will allow us to have a good understanding about what is going to happen in humans. More importantly, the siRNA molecules tested in the mouse model are good candidates for human pharmaceutical agents. The human/mouse homology design of an siRNA drug agent can eliminate the toxicity and adverse effect of those species specificities observed in monoclonal antibody drugs.

In another embodiment, the siRNA molecule binds to an miRNA whose activity promotes tumorigenesis, angiogenesis, or cell proliferation in the brain or spinal cord of a mammal. As used herein, an miRNA is a short, single-stranded RNA molecule that down-regulates gene expression through a loose homology binding to the 3' end of the untranslated region of a particular gene target. Such molecules are transcribed from DNA, but are not translated into a polypeptide. It has been associated with certain diseases (e.g. glioblastoma).

Similarly, siRNA molecules can be designed to bind to other single-stranded RNA molecules that can regulate gene expression through mechanisms other than the RNAi effect.

The siRNA molecules of the invention are used to treat one or more gliomas in the brain or spinal cord of a subject. In practice, a plurality of the molecules are used. The siRNA molecules may bind to a peptide or protein that causes or promotes the growth of a glioma in the subject. In one embodiment, the subject is a mammal, such as a mouse, non-human primate, or human. In another embodiment, the subject is a human. In one embodiment, the glioma is an astrocytoma. In another embodiment, it is a type of astrocytoma known as a glioblastoma multiforme (GBM). The molecules are delivered to the subject in pharmaceutically acceptable carriers known to those skilled in the by techniques known to those skilled in the art.

The invention also includes compositions of a plurality of the siRNA molecules, where each one targets a different RNA nucleotide sequence, which can be on the same RNA target molecule, different RNA target molecules, or any combination thereof. These compositions, in combination with pharmaceutically acceptable carriers, such as those described herein, are sometimes called siRNA cocktails. Thus, the invention provides multi-targeted siRNA cocktails for the treatment of gliomas.

All possible combinations of types of molecules and targets are included in the invention. For example, the targeted RNA molecules may encode or regulate the expression of one or more proteins in the subject. The proteins can be in the same or different pathways. The pathways, categories of proteins, and specific proteins are the ones identified previously herein. In one embodiment, the composition comprises two or more different siRNA molecules, each binding to a different RNA target sequence. In another embodiment, the composition comprises three different siRNA molecules, each binding to a different RNA target sequence. In still another embodiment, the composition comprises more than three different siRNA molecules, each binding to a different RNA target sequence. In one embodiment, the siRNA molecules target one or more of the mRNA molecules that are transcribed from one or more of the gene sequences listed in Tables 1-6 herein. In another embodiment, the siRNA molecules are selected from those listed in Tables 7-12 herein.

In yet another embodiment, the composition comprises at least one siRNA (sense: 5'-CUGUAGACACACCCACCCACAUACA-3', antisense: 5'-UGUAUGUGGGUGGGUGUGUCUACAG-3') or (sense 5'-CCCUGGUGGACAUCUUCCAGGAGUA-3', antisense 5'-UACUCCUGGAAGAUGUCCACCAGGG-3') that binds to an mRNA molecule that encodes both a human and a mouse VEGF protein, at least one siRNA molecule (sense: 5'-

CCAUCGAUGUCUACAUGAUCAUGGU-3', antisense: 5'-ACCAUGAUCAUGUAGACAUCGAUGG-3') or (sense 5'-GAUCAUGGUCAAGUGCUGGAUGAUA-3', antisense 5'-UAUCAUCCAGCACUUGACCAUGAUC-3') that binds to an mRNA molecule that encodes both a human and mouse EGFR protein, and at least one siRNA molecule (sense: 5'-GCUGAAGGUUGUGAAAUUCGGAGAA-3', antisense: 5'-UUCUCCGAAUUUCACAACCUUCAGC-3') or (sense 5'-GCUGCUGAAGGUUGUGAAAUUCGGA-3', antisense 5'-UCCGAAUUUCACAACCUUCAGCAGC-3') that binds to an mRNA molecule that encodes both a human and mouse MGMT protein.

As previously mentioned, the siRNA cocktails of the invention comprise two or more different siRNA molecules of the invention in a pharmaceutically acceptable carrier. Such carriers are generally known to those skilled in the art and include saline, sugars, polypeptides, polymers, lipids, creams, gels, micelle materials, and metal nanoparticles. In one embodiment, the carrier comprises at least one of the following: a glucose solution, a polycationic binding agent, a cationic lipid, a cationic micelle, a cationic polypeptide, a hydrophilic polymer grafted polymer, a non-natural cationic polymer, a cationic polyacetal, a hydrophilic polymer grafted polyacetal, a ligand functionalized cationic polymer, a ligand functionalized-hydrophilic polymer grafted polymer, and a ligand functionalized liposome. In another embodiment, the polymers comprise a biodegradable histidine-lysine polymer, a biodegradable polyester, such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and poly(lactic-co-glycolic acid) (PLGA), a polyamidoamine (PAMAM) dendrimer, a cationic lipid (such as DOTAP), or a PEGylated PEI. In still another embodiment, the carrier is a histidine-lysine copolymer that forms a nanoparticle with the siRNA molecule, wherein the diameter of the nanoparticle is about 100nm to about 500 nm. In a further embodiment, the ligand comprises one or more of an RGD peptide, such as H-ACRGDMFGCA-OH, an RVG peptide, such as H- YTIWMPENPRPGTPCDIFTNSRGKRASNG-OH, or a FROP peptide, such as H-EDYELMDLLAYL-OH.

The invention also provides a nanoparticle comprising the siRNA molecule of the invention, a carrier, such as one or more of those described herein, and a targeting ligand. Examples of targeting ligands include EGF receptor ligands, IL13 ligand, hepatocyte growth factor ligand, single chain monoclonal antibodies, RGD peptide ligands, and RVG peptide

ligands. In one embodiment, the nanoparticle comprises an RGD peptide ligand. In another embodiment, it comprises an RVG peptide ligand.

These nanoparticles may be used to prepare the siRNA cocktails previously described herein. Thus, the invention also includes a composition comprising 3 or more of the nanoparticles described herein.

The invention also provides a method of treating a subject with a glioma by administering to the subject an effective amount of one or more of the siRNA molecules of the invention or the compositions of the invention. The subject is a mammal. In one embodiment, the mammal is a mouse or rat. In another embodiment, it is a non-human primate. In another embodiment, the subject is a human patient. The glioma may be characterized at least in part by neovascularization and inflammation in the subject's brain or spinal cord. In one embodiment, the glioma is an astrocytoma. In another embodiment, the astrocytoma is a glioblastome multiforme.

The compositions are administered by techniques known to those skilled in the art. In one embodiment, the composition comprises at least three siRNA molecules at a ratio determined by the potency of each siRNA molecule and the therapeutic needs of the subject. In another embodiment, the composition comprises three different siRNA molecules at a ratio of 1:1:1, 1:1.5:0.5, or 0.5:0.5:2.

The compositions of the invention can be used with an effective amount of other anti-cancer therapeutic agents. These include ones that impede or block tumorigenesis, angiogenesis, or cell proliferation in the brain or spinal cord of a mammal. In one embodiment, the agent impedes or blocks the activity of a peptide or protein whose activity promotes tumorigenesis, angiogenesis, or cell proliferation in the brain or spinal cord of the mammal. For example, it may impede or block the activity of a peptide or protein that causes or promotes the growth of a glioma. In one embodiment, it impedes or blocks the activity of a protein that is a pro-tumorigenic pathway protein, a pro-angiogenesis pathway protein, a pro-cell proliferation pathway protein, or an anti-apoptotic pathway protein. Such proteins include, but are not limited to, a VEGF pathway protein, EGFR pathway protein, MGMT pathway protein, RAF pathway protein, MMP pathway protein, mTOR pathway protein, TGFβ pathway protein, or Cox-2 pathway protein. Particular examples of proteins that may be targeted by the therapeutic agent are VEGF, EGFR, PI3K, AKT, RAF1, RAS, MAPK, ERK, MGMT, MMP-2, MMP-9, PDGF, PDGFR, IGF-1, HGF, mTOR, Cox-2, and TGFβ.

In one embodiment of the invention, the therapeutic agent is selected from the group consisting of bevacizumab (trade name Avastin), sunitinib (trade name Sutent), sorafenib (trade name Nexavar), temsirolimus (trade name Torisel), and temozolomide (trade name Temodar). In one embodiment, a composition of the invention comprises temozolomide and siRNA molecules that inhibit the expression of MGMT and two of the following: EGFR, VEGF, PDFG, MMP-2, and MMP-9. In another embodiment, a composition of the invention comprises bevacizumab and siRNA molecules that inhibit the expression of two of the following: EGFR, PDFG, MMP-2, and MMP-9. In still another embodiment, a composition of the invention comprises temozolomide and siRNA molecules that inhibit the expression of EGFR, VEGF, and MGMT.

EXAMPLES

The following examples illustrate certain aspects and embodiments of the invention and should not be construed as limiting the scope thereof.

Designing siRNA inhibitors

Our experimental approach provides a novel approach to designing siRNA targeting sequences. It differs from other approaches in three important aspects:

(1) The sequences designed to be targeted by siRNA duplexes have homology to both human and mouse sequences of the same gene. That means that each of the designed siRNA duplexes will be able to knock down the same gene target in either human or mouse cells. For example, a potent siRNA specific to the VEGF gene will be able to knock down both human VEGF and mouse VEGF gene expression.

(2) The sequences were designed in three different lengths: 21-mer, 23-mer, and 25-mer. One consideration is that 23-mer and 25-mer are usually more potent than 21-mer siRNA, but 25-mer may have a greater potential for inducing an unwanted interferon response. Therefore, siRNA duplexes at various lengths may provide the best chance to achieve potent inhibition with less interferon response.

(3) The siRNA oligos can be obtained in either blunt end or sticky end form, according to the synthesis design and annealing. One consideration is that the sticky end siRNA oligos may be sensitive to degradation, and the blunt end siRNA oligos may activate the cellular interferon response.

As used herein, "oligonucleotides" and similar terms relate to short oligos composed of naturally occurring nucleotides as well as to oligos composed of synthetic or modified nucleotides, as described in the preceding section on RNAi and siRNA. The terms "polynucleotide" and "oligonucleotide" are used synonymously.

Design of siRNA sequences against targets

Tables 1-6 present the sequences of six genes targeted by specific siRNA sequences. To identify the most potent siRNA duplex targeting three of these genes, *EGFR*, *VEGF*, and *MGMT*, we generated EGFR, VEGF, and MGMT siRNAs and evaluated the effect of siRNA-mediated gene knockdown *in vitro*. They provide templates for the design of specific siRNAs.

Table 1: The targeted sequences of *VEGF-A* gene of both human and mouse

Organism	Gene	homology	Length	No.	Sense Sequences
Human	VEGF165	Human	21-mer	1	gtgtgcgcagacagtgctcca
Mouse		Mouse		2	ccaccatgccaagtggtccca
				3	cctggtggacatcttccagga
				4	gcacataggagagatgagctt
				5	caagatccgcagacgtgtaaa
				6	ggcgaggcagcttgagttaaa
				7	cttgagttaaacgaacgtact
				8	ggaaggagcctccctcagggt
				9	cactttgggtccggagggcga
				10	cagtattcttggttaatattt
			23-mer	1	gcctccgaaaccatgaactttct
				2	ctccaccatgccaagtggtccca
				3	cctggtggacatcttccaggagt
				4	cagcacataggagagatgagctt
				5	gcttgagttaaacgaacgtactt
				6	gttaaacgaacgtacttgcagat
				7	ggaaggagcctccctcagggttt
				8	ctccctcagggtttcgggaacca
				9	ctaatgttattggtgtcttcact
				10	gagaaagtgttttatatacggta
			25-mer	1	cctccgaaaccatgaactttctgct
				2	ccaccatgccaagtggtcccaggct
				3	ccctggtggacatcttccaggagta
				4	gatccgcagacgtgtaaatgttcct
				5	cgcagacgtgtaaatgttcctgcaa
				6	gtaaatgttcctgcaaaaacacaga
				7	cagcttgagttaaacgaacgtactt
				8	gttaaacgaacgtacttgcagatgt
				9	ccatgccaagtggtcccaggctgca
				10	ccctggtggacatcttccaggagta

Table 2: The targeted sequences of *EGFR* gene of both human and mouse

Organism	Gene	homology	Length	No.	Sense Sequences
Human	EGFR	Human	21-mer	1	ccctgactaccagcaggactt
Mouse		Mouse		2	ctgactaccagcaggacttct
				3	caggggatgaaagaatgcat
				4	gggggatgaaagaatgcattt
				5	gaattctccaaaatggcccga
				6	ccatcgatgtctacatgatca
				7	gatcatggtcaagtgctggat
				8	cgatgtctacatgatcatggt
				9	caaagtgcctatcaagtggat
				10	ctggatcccagaaggtgagaa
			23-mer	1	gacaaccctgactaccagcagga
				2	caaccctgactaccagcaggact
				3	ccctgactaccagcaggacttct
				4	caggggatgaaagaatgcattt
				5	ggatgaaagaatgcatttgccaa
				6	gaattctccaaaatggcccgaga
				7	cgatgtctacatgatcatggtca
				8	ctacatgatcatggtcaagtgct
				9	ggcaaagtgcctatcaagtggat
				10	ctctggatcccagaaggtgagaa
			25-mer	1	gacaaccctgactaccagcaggact
				2	ggggatgaaagaatgcatttgccaa
				3	ccatcgatgtctacatgatcatggt
				4	gatgtctacatgatcatggtcaagt
				5	gtctacatgatcatggtcaagtgct
				6	gatcatggtcaagtgctggatgata
				7	gatcacagattttgggctggccaaa
				8	cagattttgggctggccaaactgct
				9	cacagattttgggctggccaaactg
				10	ctctggatcccagaaggtgagaaag

Table 3: The targeted sequences of *MGMT* gene of both human and mouse

Organism	Gene	homology	Length	No.	Sense Sequences
Human	MGMT	Human	21-mer	1	caccagacaggtgttatggaa
Mouse		Mouse		2	cagacaggtgttatggaagct
				3	ggtgttatggaagctgctgaa
				4	ggaagctgctgaaggttgtga
				5	gctgctgaaggttgtgaaatt
				6	gaaggttgtgaaattcggaga
				7	cagcaattagcagccctggca
				8	cagcaattagcagccctggca
				9	cagccctggcaggcaacccca
				10	gccctggcaggcaaccccaaa
			23-mer	1	ccagacaggtgttatggaagct
				2	gacaggtgttatggaagctgct
				3	caggtgttatggaagctgctga
				4	gttatggaagctgctgaaggtt
				5	ggaagctgctgaaggttgtgaa
				6	gctgaaggttgtgaaattcgga
				7	gaaggttgtgaaattcggagaa
				8	cttaccagcaattagcagccct
				9	ccagcaattagcagccctggca
				10	gcagccctggcaggcaacccca
			25-mer	1	ccagacagguguuauggaagcugcu
				2	gacagguguuauggaagcugcugaa
				3	gguguuauggaagcugcugaagguu
				4	ggaagcugcugaagguugugaaauu
				5	gcugcugaagguugugaaauucgga
				6	gcugaagguugugaaauucggagaa
				7	cagcaauuagcagcccuggcaggca
				8	cuuaccagcaauuagcagcccuggc
				9	ccagcaauuagcagcccuggcaggc
				10	gcaauuagcagcccuggcaggcaac

Table 4: The targeted sequences of *MMP-2* gene of both human and mouse

Human	MMP-2	Human	21-mer	1	cccttgtttccgctgcatcca
Mouse		Mouse		2	catcatcaagttccccggcga
				3	gacaaagagttggcagtgcaa
				4	gcaacccagatgtggccaact
				5	caagcccaagtgggacaagaa
				6	gcccaagtgggacaagaacca
				7	caactttgagaaggatggcaa
				8	gatggcatcgctcagatccgt
				9	cctggatgccgtcgtggacct
					gccagggatctcttcaatgct
			23-mer	1	cccttgtttccgctgcatccaga
				2	ccatcatcaagttccccggcgat
				3	gacaaagagttggcagtgcaata
				4	ggcaacccagatgtggccaacta
				5	cgcaagcccaagtgggacaagaa
				6	gcccaagtgggacaagaaccaga
				7	ggacaagaaccagatcacataca
				8	caactttgagaaggatggcaagt
				9	ggcatcgctcagatccgtggtga
				10	ctggatgccgtcgtggacctgca
			25-mer	1	cccttgtttccgctgcatccagact
				2	ccatcatcaagttccccggcgatgt
				3	gagttggcagtgcaatacctgaaca
				4	gcaacccagatgtggccaactacaa
				5	gcaagcccaagtgggacaagaacca
				6	cccaagtgggacaagaaccagatca
				7	gacaagaaccagatcacatacagga
				8	ggcatcgctcagatccgtggtgaga
				9	gagcgtgaagtttggaagcatcaaa
				10	gagatcttcttcttcaaggaccggt

Table 5: The targeted sequences of *MMP-9* gene of both human and mouse

Organism	Gene	homology	Length	No.	Sense Sequences
Human	MMP-9	Human	21-mer	1	catccagtttggtgtcgcgga
Mouse		Mouse		2	ccagtttggtgtcgcggagca
				3	gcggagcacggagacgggtat
				4	cggagacgggtatcccttcga
				5	gagctgtgcgtcttcccccttc
			23-mer	1	gtcatccagtttggtgtcgcgga
				2	gcgcggagcacggagacgggtat
				3	ggagcacggagacgggtatccct
			25-mer	1	ccagtttggtgtcgcggagcacgga
				2	cgcgcgcggagcacggagacgggta
				3	cggagcacggagacgggtatcccttt
		Human only	21-mer	1s	CCACCACAACAUCACCUAUTT
				1a	AUAGGUGAUGUUGUGGUGGTT

Table 5: The targeted sequences of *MMP-9* gene of both human and mouse

Organism	Gene	homology	Length	No.	Sense Sequences
		s: sense		2s	GCCAGUUUCCAUUCAUCUUTT
		a: antisense		2a	AAGAUGAAUGGAAACUGGCTT
				3s	GCGCUGGGCUUAGAUCAUUTT
				3a	AAUGAUCUAAGCCCAGCGCTT
				4s	GCAUAAGGACGACGUGAAUTT
				4a	AUUCACGUCGUCCUUAUGCTT
				5s	CCUGCAACGUGAACAUCUUTT
				5a	AAGAUGUUCACGUUGCAGGTT
				6s	GGAACCAGCUGUAUUUGUUTT
				6a	AACAAAUACAGCUGGUUCCTT
				7s	GCCAGUUUGCCGGAUACAATT
				7a	UUGUAUCCGGCAAACUGGCTT
				8s	CCAGUUUGCCGGAUACAAATT
				8a	UUUGUAUCCGGCAAACUGGTT
				9s	GCCGGAUACAAACUGGUAUTT
				9a	AUACCAGUUUGUAUCCGGCTT
				10s	CCGGAUACAAACUGGUAUUTT
				10a	AAUACCAGUUUGUAUCCGGTT
		Human only	25-mer	1s	UGGCACCACCACAACAUCACCUAUU
				1a	AAUAGGUGAUGUUGUGGUGGUGCCA
		s: sense		2s	CACAACAUCACCUAUUGGAUCCAAA
		a: antisense		2a	UUUGGAUCCAAUAGGUGAUGUUGUG
				3s	GACGCAGACAUCGUCAUCCAGUUUG
				3a	CAAACUGGAUGACGAUGUCUGCGUC
				4s	GGAAACCCUGCCAGUUUCCAUUCAU
				4a	AUGAAUGGAAACUGGCAGGGUUUCC
				5s	CAUUCAUCUUCCAAGGCCAAUCCUA
				5a	UAGGAUUGGCCUUGGAAGAUGAAUG
				6s	ACGAUGCCUGCAACGUGAACAUCUU
				6a	AAGAUGUUCACGUUGCAGGCAUCGU
				7s	GCGGAGAUUGGGAACCAGCUGUAUU
				7a	AAUACAGCUGGUUCCCAAUCUCCGC
				8s	CGGAGAUUGGGAACCAGCUGUAUUU
				8a	AAAUACAGCUGGUUCCCAAUCUCCG
				9s	CAGUACCGAGAGAAAGCCUAUUUCU
				9a	AGAAAUAGGCUUUCUCUCGGUACUG
				10s	AAGCCUAUUUCUGCCAGGACCGCUU
				10a	AAGCGGUCCUGGCAGAAAUAGGCUU

Table 6: The targeted sequences of *PDGFa* gene of both human and mouse

Organism	Gene	homology	Length	No.	Sense Sequences
Human	PDGF a	Human	21-mer	1	caccctcctccgggccgcgct
Mouse		Mouse		2	ctcctccgggccgcgctccct
				3	gtactgaatttcgccgccaca
				4	ctgaatttcgccgccacagga
				5	ggagcgcccgccccgcggcct
				6	ctgctgctcctcggctgcgga
				7	gctgctcctcggctgcggata
				8	gatccacagcatccgggacct
				9	ccacagcatccgggacctcca
				10	catccgggacctccagcgact
			23-mer	1	gccaccctcctccgggccgcgct
				2	ccctcctccgggccgcgctccct
				3	gatggtactgaatttcgccgcca
				4	ctggagcgcccgccccgcggcct
				5	gcgcccgccccgcggcctcgcct
				6	gcctcgggacgcgatgaggacct
				7	ggcttgcctgctgctcctcggct
				8	gcctgctgctcctcggctgcgga
				9	cagatccacagcatccgggacct
				10	gaccaggacggtcatttacgaga
			25-mer	1	gcgccaccctcctccgggccgcgct
				2	caccctcctccgggccgcgctccct
				3	gggatggtactgaatttcgccgcca
				4	gatggtactgaatttcgccgccaca
				5	ggtactgaatttcgccgccacagga
				6	ggctggagcgcccgccccgcggcct
				7	gagcgcccgccccgcggcctcgcct
				8	ccagcgcctcgggacgcgatgagga
				9	gcgcctcgggacgcgatgaggacct
				10	gcctgctgctcctcggctgcggata

The following siRNA duplexes were designed to target human/mouse EGFR gene (BCER series, eight siRNAs), human/mouse VEGF gene (BCVF series, eight siRNAs) and human/mouse MGMT gene (MGMT series, eight siRNAs):

BCER siRNA, siRNA duplexes targeting EGFR genes (both mouse and human), 25 mer blunt ended:

BCER-a

Sense: 5′-CCAUCGAUGUCUACAUGAUCAUGGU-3′

Antisense: 5′-ACCAUGAUCAUGUAGACAUCGAUGG-3′

BCER-b

Sense: 5′-GAUGUCUACAUGAUCAUGGUCAAGU-3′

Antisense: 5′-ACUUGACCAUGAUCAUGUAGACAUC-4′

BCER-c:
Sense: 5′-GUCUACAUGAUCAUGGUCAAGUGCU-3′
Antisense: 5′-AGCACUUGACCAUGAUCAUGUAGAC-3′

BCER-d:
Sense: 5′-GAUCAUGGUCAAGUGCUGGAUGAUA-3′
Antisense: 5′-UAUCAUCCAGCACUUGACCAUGAUC-3′

BCER-e:
Sense: 5′-GAUCACAGAUUUUGGGCUGGCCAAA-3′
Antisense: 5′-UUUGGCCAGCCCAAAAUCUGUGAUC-3′

BCER-f:
Sense: 5′-CAGAUUUUGGGCUGGCCAAACUGCU-3′
Antisense: 5′-AGCAGUUUGGCCAGCCCAAAAUCUG-3′

BCER-g:
Sense: 5′-CACAGAUUUUGGGCUGGCCAAACUG-3′
Antisense: 5′-CAGUUUGGCCAGCCCAAAAUCUGUG-3′

BCER-h:
Sense: 5′-CUCUGGAUCCCAGAAGGUGAGAAAG-3′
Antisense: 5′-CUUUCUCACCUUCUGGGAUCCAGAG-3′

BCVF siRNA, siRNA duplexes targeting VEGF gene (both mouse and human) 25 mer blunt ended:

BCVF-a
Sense: 5′-CCAUGCCAAGUGGUCCCAGGCUGCA-3′
Antisense: 5′-UGCAGCCUGGGACCACUUGGCAUGG-3′

BCVF-b
Sense: 5′-CCAACAUCACCAUGCAGAUUAUGCG-3′
Antisense: 5′-CGCAUAAUCUGCAUGGUGAUGUUGG=3′

BCVF-c
Sense: 5′-CUGUAGACACACCCACCCACAUACA-3′
Antisense: 5′-UGUAUGUGGGUGGGUGUGUCUACAG-3′

BCVF-d
Sense: 5′-CACUUUGGGUCCGGAGGGCGAGACU-3′
Antisense: 5′-AGUCUCGCCCUCCGGACCCAAAGUG-3′

BCVF-e
Sense: 5′-CCAUGCCAAGUGGUCCCAGGCUGCA-3′
Antisense: 5′-UGCAGCCUGGGACCACUUGGCAUGG-3′

BCVF-f
Sense: 5′-CCCUGGUGGACAUCUUCCAGGAGUA-3′
Antisense: 5′-UACUCCUGGAAGAUGUCCACCAGGG-3′

BCVF-g
Sense: 5′-CGCAGACGUGUAAAUGUUCCUGCAA-3′
Antisense: 5′-UUGCAGGAACAUUUACACGUCUGCG-3′

BCVF-h
Sense: 5′-CCCUGGUGGACAUCUUCCAGGAGUA-3′
Antisense: 5′-UACUCCUGGAAGAUGUCCACCAGGG=3′

BCMM siRNA, siRNA duplexes targeting MGMT gene (both human and mouse) 25 mer blunt ended:

BCMM-a:
Sense: 5′-GGUGUUAUGGAAGCUGCUGAAGGUU-3′
Antisense: 5′-AACCUUCAGCAGCUUCCAUAACACC-3′

BCMM-b:
Sense: 5′-GGAAGCUGCUGAAGGUUGUGAAAUU-3′
Antisense: 5′-AAUUUCACAACCUUCAGCAGCUUCC-3′

BCMM-c:
Sense: 5′-GCUGCUGAAGGUUGUGAAAUUCGGA-3′
Antisense: 5′-UCCGAAUUUCACAACCUUCAGCAGC-3′

BCMM-d:
Sense: 5′-GCUGAAGGUUGUGAAAUUCGGAGAA-3′
Antisense: 5′-UUCUCCGAAUUUCACAACCUUCAGC-3′

BCMM-e:
Sense: 5′-CAGCAAUUAGCAGCCCUGGCAGGCA-3′
Antisense: 5′-UGCCUGCCAGGGCUGCUAAUUGCUG-3′

BCMM-f:
Sense: 5′-CUUACCAGCAAUUAGCAGCCCUGGC-3′
Antisense: 5′-GCCAGGGCUGCUAAUUGCUGGUAAG-3′

BCMM-g:
Sense: 5′-CCAGCAAUUAGCAGCCCUGGCAGGC-3′
Antisense: 5′-GCCUGCCAGGGCUGCUAAUUGCUGG-3′

BCMM-h:
Sense: 5′-GCAAUUAGCAGCCCUGGCAGGCAAC-3′
Antisense: 5′-GUUGCCUGCCAGGGCUGCUAAUUGC-3′

Example I. Identification of the most potent siRNA duplex targeting the above three genes

To demonstrate/validate the potency of siRNA against specific target genes.

To accomplish this objective, we generated EGFR, VEGF, and MGMT siRNAs and quantitatively evaluated the effect of siRNA-mediated gene knockdown *in vitro* using cell-culture-based assays. Gene knockdown was confirmed and evaluated by immunoblotting. We selected the most potent siRNA for each target gene for subsequent experiments. Using RT-PCR and Western blot analyses, we measured the gene expression silencing by each of the siRNA duplexes listed above, with optimized transfection followed by RNA and protein isolation. Figures 1-9 demonstrate the identified potent siRNA duplexes targeting each of the genes discussed.

Selection of potent siRNA sequences

A. Two siRNA duplexes were selected for *MGMT* knockdown:

MGMT-D	
Sense	5′-GCUGAAGGUUGUGAAAUUCGGAGAA-3′
Antisense	5′-UUCUCCGAAUUUCACAACCUUCAGC-3′
MGMT-C	
Sense	5′-GCUGCUGAAGGUUGUGAAAUUCGGA-3′
Antisense	5′-UCCGAAUUUCACAACCUUCAGCAGC-3′

B. Two siRNA duplexes were selected for *EGFR* knockdown:

EGFR-A	
Sense	5′-CCAUCGAUGUCUACAUGAUCAUGGU-3′
Antisense	5′-ACCAUGAUCAUGUAGACAUCGAUGG-3′
EGFR-D	
Sense	5′-GAUCAUGGUCAAGUGCUGGAUGAUA-3′
Antisense	5′-UAUCAUCCAGCACUUGACCAUGAUC-3′

C. Two siRNA duplexes were selected for human and mouse *VEGF* knockdown:

VEGF-C	
Sense	5′-CUGUAGACACACCCACCCACAUACA-3′
Antisense	5′-UGUAUGUGGGUGGGUGUGUCUACAG-3′
VEGF-H	
Sense	5′-CCCUGGUGGACAUCUUCCAGGAGUA-3′
Antisense	5′-UACUCCUGGAAGAUGUCCACCAGGG-3′

D. One siRNA duplex was selected for human and mouse *PDGF* knockdown:

hmPD-25-3:

sense: 5′-GCCUGCUGCUCCUCGGCUGCGGAUA-3′
antisense: 5′-UAUCCGCAGCCGAGGAGCAGCAGGC-3′

E. One siRNA duplex was selected for human and mouse *MMP-2* knockdown:

hmM2-25-3:
sense 5′-CCCAAGUGGGACAAGAACCAGAUCA-3′
antisense 5′-UGAUCUGGUUCUUGUCCCACUUGGG-3′

F. One siRNA duplex was selected for human and mouse *MMP-9* knockdown:

hmM9-25-1:
sense 5′-CCAGUUUGGUGUCGCGGAGCACGGA-3′
antisense 5′-UCCGUGCUCCGCGACACCAAACUGG-3′

SiRNA cocktail therapeutics for GBM

We designed a therapeutic siRNA cocktail targeting multiple-disease controlling genes for treating several types of brain cancer, including GBM, acting on multiple aspects of the diseases and reducing potential toxicity.

The siRNA cocktail has the following characteristics:

(1) The siRNA cocktail contains at least three siRNA duplexes targeting at least three genes at a ratio required by the therapy.

(2) The cocktail design for each combination follows the understanding of the role of each gene in a background of the system biology network, such as whether these genes are functioning in the same pathway or in different ones.

(3) The chemical property of each siRNA molecule in the cocktail is the same in terms of source of supply, manufacturing process, chemical modification, storage conditions, and formulation procedures.

(4) The individual siRNA molecules in the cocktail can be of different lengths, with either blunt or sticky end, as long as their potencies have been defined.

(5) Since an siRNA cocktail is targeting multiple genes and a single cell type usually does not express all those factors, the efficacy of the siRNA cocktail is tested in a relevant disease model, either a multiple cell model, a tissue model, or an animal model, after confirmation of the potency of each individual siRNA duplex in the cell culture.

(6) Each validated siRNA cocktail can be used for addressing one or more pathological conditions or for treating one or multiple types of diseases, such as

an siRNA cocktail for suppressing inflammation, siRNA cocktail for anti-angiogenesis activity and siRNA cocktail for autoimmune conditions.

(7) The siRNA cocktail is administrated through the same route of delivery in the same formulation, although the regimen of dosing for each cocktail will be defined based on either the experimental design or therapeutic requirements.

(8) Each siRNA cocktail can be applied either independently or in combination with other drug modalities, such as small molecule inhibitors, monoclonal antibodies, protein and peptides, and other siRNA cocktail drugs.

Table 7. VEGF-EGFR-MGMT siRNA cocktails

siRNA Cocktail Combinations (siRNA sense sequences)		
VEGF EGFR MGMT		Human and Mouse homologues
Cocktail 1	VEGF	5′-CUGUAGACACACCCACCCACAUACA-3′
	EGFR	5′-CCAUCGAUGUCUACAUGAUCAUGGU-3′
	MGMT	5′-GCUGAAGGUUGUGAAAUUCGGAGAA-3′
Cocktail 2	VEGF	5′-CUGUAGACACACCCACCCACAUACA-3′
	EGFR	5′-CCAUCGAUGUCUACAUGAUCAUGGU-3′
	MGMT	5′-GCUGCUGAAGGUUGUGAAAUUCGGA-3′
Cocktail 3	VEGF	5′-CUGUAGACACACCCACCCACAUACA-3′
	EGFR	5′-GAUCAUGGUCAAGUGCUGGAUGAUA-3′
	MGMT	5′-GCUGCUGAAGGUUGUGAAAUUCGGA-3′
Cocktail 4	VEGF	5′-CCCUGGUGGACAUCUUCCAGGAGUA-3′
	EGFR	5′-GAUCAUGGUCAAGUGCUGGAUGAUA-3′
	MGMT	5′-GCUGCUGAAGGUUGUGAAAUUCGGA-3′
Cocktail 5	VEGF	5′-CCCUGGUGGACAUCUUCCAGGAGUA-3′
	EGFR	5′-GAUCAUGGUCAAGUGCUGGAUGAUA-3′
	MGMT	5′-GCUGAAGGUUGUGAAAUUCGGAGAA-3′

Table 8. VEGF-EGFR-PDGF siRNA cocktails

siRNA Cocktail Combinations (siRNA sense sequences)		
VEGF EGFR PDGF		Human and Mouse homologues
Cocktail 1	VEGF	5′-CUGUAGACACACCCACCCACAUACA-3′
	EGFR	5′-CCAUCGAUGUCUACAUGAUCAUGGU-3′
	PDGF	5′-GCCUGCUGCUCCUCGGCUGCGGAUA-3′
Cocktail 2	VEGF	5′-CCCUGGUGGACAUCUUCCAGGAGUA-3′
	EGFR	5′-CCAUCGAUGUCUACAUGAUCAUGGU-3′
	PDGF	5′-GCCUGCUGCUCCUCGGCUGCGGAUA-3′
Cocktail 3	VEGF	5′-CUGUAGACACACCCACCCACAUACA-3′
	EGFR	5′-GAUCAUGGUCAAGUGCUGGAUGAUA-3′
	PDGF	5′-GCCUGCUGCUCCUCGGCUGCGGAUA-3′
Cocktail 4	VEGF	5′-CCCUGGUGGACAUCUUCCAGGAGUA-3′
	EGFR	5′-GAUCAUGGUCAAGUGCUGGAUGAUA-3′
	PDGF	5′-GCCUGCUGCUCCUCGGCUGCGGAUA-3′

Table 9. VEGF-EGFR-MMP-9 siRNA cocktails

siRNA Cocktail Combinations (siRNA sense sequences)		
VEGF EGFR MMP-9		Human and Mouse homologues
Cocktail 1	VEGF	5′-CUGUAGACACACCCACCCACAUACA-3′
	EGFR	5′-CCAUCGAUGUCUACAUGAUCAUGGU-3′
	MMP-9	5′-CCAGUUUGGUGUCGCGGAGCACGGA-3′
Cocktail 2	VEGF	5′-CCCUGGUGGACAUCUUCCAGGAGUA-3′
	EGFR	5′-CCAUCGAUGUCUACAUGAUCAUGGU-3′
	MMP-9	5′-CCAGUUUGGUGUCGCGGAGCACGGA-3′
Cocktail 3	VEGF	5′-CUGUAGACACACCCACCCACAUACA-3′
	EGFR	5′-GAUCAUGGUCAAGUGCUGGAUGAUA-3′
	MMP-9	5′-CCAGUUUGGUGUCGCGGAGCACGGA-3′
Cocktail 4	VEGF	5′-CCCUGGUGGACAUCUUCCAGGAGUA-3′
	EGFR	5′-GAUCAUGGUCAAGUGCUGGAUGAUA-3′
	MMP-9	5′-CCAGUUUGGUGUCGCGGAGCACGGA-3′

Table 10. VEGF-EGFR-MMP-2 siRNA cocktails

siRNA Cocktail Combinations (siRNA sense sequences)		
VEGF EGFR MMP-2		Human and Mouse homologues
Cocktail 1	VEGF	5′-CUGUAGACACACCCACCCACAUACA-3′
	EGFR	5′-CCAUCGAUGUCUACAUGAUCAUGGU-3′
	MMP-2	5′-CCCAAGUGGGACAAGAACCAGAUCA-3′
Cocktail 2	VEGF	5′-CCCUGGUGGACAUCUUCCAGGAGUA-3′
	EGFR	5′-CCAUCGAUGUCUACAUGAUCAUGGU-3′
	MMP-2	5′-CCCAAGUGGGACAAGAACCAGAUCA-3′
Cocktail 3	VEGF	5′-CUGUAGACACACCCACCCACAUACA-3′
	EGFR	5′-GAUCAUGGUCAAGUGCUGGAUGAUA-3′
	MMP-2	5′-CCCAAGUGGGACAAGAACCAGAUCA-3′
Cocktail 4	VEGF	5′-CCCUGGUGGACAUCUUCCAGGAGUA-3′
	EGFR	5′-GAUCAUGGUCAAGUGCUGGAUGAUA-3′
	MMP-2	5′-CCCAAGUGGGACAAGAACCAGAUCA-3′

Table 11. PDGF-EGFR-MGMT siRNA cocktails

siRNA Cocktail Combinations (siRNA sense sequences)		
PDGF EGFR MGMT		Human and Mouse homologues
Cocktail 1	PDGF	5′-GCCUGCUGCUCCUCGGCUGCGGAUA-3′
	EGFR	5′-CCAUCGAUGUCUACAUGAUCAUGGU-3′
	MGMT	5′-GCUGAAGGUUGUGAAAUUCGGAGAA-3′
Cocktail 2	PDGF	5′-GCCUGCUGCUCCUCGGCUGCGGAUA-3′
	EGFR	5′-CCAUCGAUGUCUACAUGAUCAUGGU-3′
	MGMT	5′-GCUGCUGAAGGUUGUGAAAUUCGGA-3′
Cocktail 3	PDGF	5′-GCCUGCUGCUCCUCGGCUGCGGAUA-3′
	EGFR	5′-GAUCAUGGUCAAGUGCUGGAUGAUA-3′
	MGMT	5′-GCUGCUGAAGGUUGUGAAAUUCGGA-3′
Cocktail 4	PDGF	5′-GCCUGCUGCUCCUCGGCUGCGGAUA-3′
	EGFR	5′-GAUCAUGGUCAAGUGCUGGAUGAUA-3′
	MGMT	5′-GCUGAAGGUUGUGAAAUUCGGAGAA-3′

Table 12. VEGF-EGFR-MGMT-other siRNA cocktails

siRNA Cocktail Combinations (siRNA sense sequences)		
VEGF EGFR MGMT	other	Human and Mouse homologues
Cocktail 1	VEGF	5′-CUGUAGACACACCCACCCACAUACA-3′
	EGFR	5′-CCAUCGAUGUCUACAUGAUCAUGGU-3′
	MGMT	5′-GCUGAAGGUUGUGAAAUUCGGAGAA-3′
	PDGF	5′-GCCUGCUGCUCCUCGGCUGCGGAUA-3′
Cocktail 2	VEGF	5′-CUGUAGACACACCCACCCACAUACA-3′
	EGFR	5′-CCAUCGAUGUCUACAUGAUCAUGGU-3′
	MGMT	5′-GCUGCUGAAGGUUGUGAAAUUCGGA-3′
	MMP-2	5′-CCCAAGUGGGACAAGAACCAGAUCA-3′
Cocktail 3	VEGF	5′-CUGUAGACACACCCACCCACAUACA-3′
	EGFR	5′-GAUCAUGGUCAAGUGCUGGAUGAUA-3′
	MGMT	5′-GCUGCUGAAGGUUGUGAAAUUCGGA-3′
	MMP-9	5′-CCAGUUUGGUGUCGCGGAGCACGGA-3′
Cocktail 4	VEGF	5′-CCCUGGUGGACAUCUUCCAGGAGUA-3′
	MMP-2	5′-CCCAAGUGGGACAAGAACCAGAUCA-3′
	MGMT	5′-GCUGCUGAAGGUUGUGAAAUUCGGA-3′
	MMP-9	5′-CCAGUUUGGUGUCGCGGAGCACGGA-3′
Cocktail 5	MMP-2	5′-CCCAAGUGGGACAAGAACCAGAUCA-3′
	EGFR	5′-GAUCAUGGUCAAGUGCUGGAUGAUA-3′
	MGMT	5′-GCUGAAGGUUGUGAAAUUCGGAGAA-3′
	PDGF	5′-GCCUGCUGCUCCUCGGCUGCGGAUA-3′

Experimental Design:

The design is summarized for three experiments that may be performed to evaluate the antitumorigenic effects of this method.

Experiment A. To evaluate the antitumorigenic effects of the selected siRNAs individually and collectively *in vitro* as well as *in vivo*. The most potent siRNA inhibitors for each gene are transfected into established cell lines. Measuring cellular proliferation, apoptosis, and signaling pathways downstream of these genes serves as parameters for drug-target validation and potential siRNA-mediated antitumorigenesis *in vivo*. Then, all three siRNAs are transfected simultaneously into these cell lines to validate the biological potential of a combinatorial approach. This method is applied *in vivo* to GBM xenograft nude mice

models. Evaluation of angiogenesis, proliferation, and apoptosis in these models is used to assess the efficacy of siRNA-mediated antitumorigenesis *in vivo*.

Experiment B. To evaluate, both *in vitro* and *in vivo*, the antitumorigenic effects of using individual siRNA and a combination of the three siRNAs in conjunction with clinically available agents, including Tarceva (anti-EGFR), Avastin (anti-VEGF), and Temozolomide (TMZ). Each candidate siRNA, coupled with its clinically available agent, is transfected into established cell lines *in vitro* to assess the antitumorigenic potential of combined administration. For example, the most potent siRNA for EGFR can be delivered, along with Tarceva, to evaluate the combined effect of these two agents. Comparing proliferation, apoptosis, and downstream signaling activity in these trials to those conducted with siRNA only provides a means to evaluate the effects of combined treatment, as well as the potential for enhanced therapeutic vulnerability. After each siRNA-therapeutic agent pair has been evaluated individually *in vitro*, all three siRNAs, along with their existing therapeutic agents, are transfected into cell lines simultaneously. These trials are used as a proof of concept for achieving synergistic antitumorigenesis. Corresponding *in vivo* experiments are conducted using nude mouse models.

Experiment C. To validate the efficacy of siRNA-mediated antitumorigenesis *in vivo* using a nanotechnology-based delivery system. Nanoparticle delivery of the siRNA and siRNA-therapeutic agent cocktails via local and systemic injection to the GBM mouse models is a means to validate multiple drug targets, as well as to indicate the potential therapeutic applications of siRNA. Moreover, the success of a systemic, nanoparticle delivery method in GBM animal models to effect antitumorigenesis serves to validate this approach for clinical trials.

Polymers Enhance siRNA Cocktail Delivery

As shown in a study using siRNA cocktails to inhibit ocular neovascularization induced by a herpes simplex viral sequence, the histidine-lysine (HK) polymer-siRNA nanoparticle-mediated local delivery has achieved potent anti-angiogenesis activity. In a separate study using HK polymer to enhance siRNA delivery intratumorally, the tumor growth curves showed significant anti-tumor efficacy with clear downregulation of the target gene expression. At 10 days after the injection of MDA-MB-435 cells into the mammary fat pad, mice with visible tumors were separated into treatment groups. Each group had four mice with eight tumors, and tumor size was assessed in two dimensions and calculated. Mice received 4 μg/tumor of siRNA with each intratumoral injection every 5 days. To confirm the

antitumor efficacy of siRNA Raf-1 with the optimal polymer in greater detail, mice with tumors were divided into these groups: untreated, b-galactosidase siRNA, and Raf-1 siRNA. Clearly, HK polymer has been validated as an effective local siRNA delivery carrier.

Over the past few decades, biodegradable polyesters, such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and poly(lactic-co-glycolic acid) (PLGA), have been extensively studied for a wide variety of pharmaceutical and biomedical applications. The biodegradable polyester family has been regarded as one of the few synthetic biodegradable polymers with controllable biodegradability, excellent biocompatibility, and high safety. The need for a variety of drug formulations for different drugs and delivery pathways resulted in development of various types of block copolymers (e.g., diblock, triblock, multiblock, and star-shaped block) consisting of the biodegradable polyesters and PEG.

PAMAM dendrimers represent an exciting new class of macromolecular architecture called "dense star" polymers. Unlike classical polymers, dendrimers have a high degree of molecular uniformity, narrow molecular weight distribution, specific size and shape characteristics, and a highly functionalized terminal surface. The manufacturing process is a series of repetitive steps starting with a central initiator core. Each subsequent growth step represents a new "generation" of polymer with a larger molecular diameter, twice the number of reactive surface sites, and approximately double the molecular weight of the preceding generation. PAMAM dendrimers are the most common class of dendrimers suitable for many materials science and biotechnology applications. PAMAM dendrimers consist of alkyl-diamine core and tertiary amine branches.

Cationic polymers have gained prominence in nonviral DNA delivery, although their use in siRNA delivery is much more recent. Of these polymers, PEI, which contains primary, secondary, and tertiary amines, has the unique abilities to complex nucleic acid and serve as a low pH (4-5) buffer. Owing to this buffering capacity or "proton sponge" effect, PEI has shown higher transfection efficiency than other cationic polymers. Recently, several groups have reported the use of PEI-siRNA complexes for the treatment of influenza in mice and systemic delivery for treatment of breast cancer in a mouse model, etc. However, in vivo toxicity has been noted for PEI upon intravenous administration. To reduce PEI toxicity, PEG was grafted onto PEI, greatly reducing toxicity for the resulting gene delivery complexes. The PEI component of the copolymer allows for complexation with polynucleotides, and it increases endosomal release of the nanocomplexes into the cytoplasm. The PEG component not only reduces toxicity of the PEI component, but also stabilizes the resulting nanocomplex. This modified vector was

recently used to deliver siRNA targeting VEGF for treatment of angiogenic tumors. A copolymer composed of 10 PEG grafts (2 kDa each) per PEI polymer (2k 10 copolymer) gave the highest binding affinity to siRNA by ethidium bromide exclusion assays, and it had the smallest nanocomplex size (115 ± 13 nm diameter). When we tested this PEG-PEI-siRNA nanoparticle in mouse for pulmonary delivery, the anti-influenza A activity was very much enhanced, which allows us to achieve potent therapeutic effect. Therefore, this PEG-PEI could be used in our multi-targeted siRNA regimen for treatment of glioblastoma.

DOTAP Chloride has been used as DNA transfection reagent. Racemic DOTAP Chloride is a cationic lipid which forms liposomes in aqueous media, alone or in combination with other lipids. These liposomes can carry polar and non-polar molecules (APIs or diagnostics). They can enter cells carrying their load through the cell membrane. We will use either R DOTAP Chloride or S DOTAP Chloride for siRNA cocktail delivery into the tumor tissue.

To test HK polymer, PLGA, and PAMAM for their role in enhancement of siRNA delivery in vivo, we need to define the formulations for each of these carrier-siRNA nanoparticles.

Ligands Useful for Targeted siRNA Delivery

The targeting ligands may also improve intracerebral or intratumoral siRNA delivery. The EGF receptor ligands, IL13 ligand, hepatocyte growth factor ligand, and other single chain monoclonal antibodies may be used for this type of targeting design.

RGD (arginine-glycine-aspartic acid) peptide ligands, such as the 'cyclic' 10mer RGD peptide with the sequence H-ACRGDMFGCA-OH, and -(D)CR(D)WKTCT-(ol) have been used for neovasculature targeted nucleic acid delivery.

A short peptide derived from rabies virus glycoprotein (RVG) recently has been identified for its ability to enable the transvascular delivery of small interfering RNA (siRNA) (27). This 29-amino-acid peptide specifically binds to the acetylcholine receptor expressed by neuronal cells. To enable siRNA binding, a chimaeric peptide was synthesized by adding nonamer arginine residues at the carboxy terminus of RVG. This RVG-9R peptide was able to bind and transduce siRNA to neuronal cells in vitro and in vivo, resulting in efficient gene silencing. Peptides: RVG (YTIWMPENPRPGTPCDIFTNSRGKRASNG), RVG-9R (YTIWMPENPRPGTPCDIFTNS-RGKRASNGGGGRRRRRRRRR).

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All publications, including issued patents and published applications, and all database entries identified by url addresses or accession numbers are incorporated herein by reference in their entirety.

Although this invention has been described in relation to certain embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

WHAT IS CLAIMED IS:

1. An isolated siRNA molecule that binds to a single-stranded RNA molecule, wherein said single-stranded RNA molecule comprises an mRNA that encodes at least part of a peptide or protein whose activity promotes tumorigenesis, angiogenesis, or cell proliferation in the brain or spinal cord of a mammal, or wherein said single stranded RNA molecule comprises an miRNA whose activity promotes tumorigenesis, angiogenesis, or cell proliferation in the brain or spinal cord of a mammal.

2. The siRNA molecule of claim 1 wherein said molecule comprises an oligonucleotide with a length of 19-35 base pairs.

3. The siRNA molecule of claim 1 wherein said molecule comprises an oligonucleotide with a length of 19-27 base pairs.

4. The siRNA molecule of claim 1 wherein said molecule comprises an oligonucleotide with a length of 21-25 base pairs.

5. The siRNA molecule of any of claims 1-4 wherein said molecule comprises an oligonucleotide with blunt ends at both ends, or sticky ends at both ends, or one of each.

6. The siRNA molecule of any of claims 1-5 wherein said molecule comprises a chemical modification in one or more of its individual nucleotides or in its oligonucleotide backbone.

7. The siRNA molecule of any of claims 1-6 wherein the mammal is a human, a mouse, or a non-human primate.

8. The siRNA molecule of any of claims 1-7 wherein said peptide or protein causes or promotes the growth of a glioma.

9. The siRNA molecule of claim 8 wherein said glioma is an astrocytoma.

10. The siRNA molecule of claim 9 wherein said astrocytoma is a glioblastoma multiforme.

11. The siRNA molecule of any of claims 1-10 wherein said mRNA molecule encodes a protein selected from the group consisting of pro-tumorigenic pathway proteins, pro-angiogenesis pathway proteins, pro-cell proliferation pathway proteins, and anti-apoptotic pathway proteins.

12. The siRNA molecule of claim 11 wherein said mRNA molecule encodes a protein selected from the group consisting of VEGF pathway proteins, EGFR pathway proteins, MGMT pathway proteins, Raf1 pathway proteins, MMP pathway proteins, mTOR pathway proteins, TGFβ pathway proteins, and Cox-2 pathway proteins.

13. The siRNA molecule of claim 11 wherein said protein is selected from the group consisting of VEGF, EGFR, PI3K, AKT, AGT, RAF, RAS, MAPK, ERK, MGMT, MMP-2, MMP-9, PDGF, PDGFR, IGF-1, HGF, mTOR, Cox-2 and TGFβ1.

14. The siRNA molecule of claim 11 wherein said protein is VEGF, EGFR, MGMT, MMP-2, MMP-9, RAF1, mTOR, Cox-2, or PDGF.

15. The siRNA molecule of claim 11 wherein said protein is VEGF, EGFR, MGMT, MMP-2, MMP-9, or PDGF.

16. The siRNA molecule of claim 11 wherein said protein is RAF1, mTOR, or Cox-2.

17. The siRNA molecule of any of claims 11-16 wherein said siRNA molecule binds to both a human mRNA molecule and a homologous mouse mRNA molecule.

18. A composition comprising the siRNA molecule of any of claims 1-17 and a pharmaceutically acceptable carrier.

19. A composition comprising at least three of the siRNA molecules of any of claims 1-17.

20. The composition of claim 19 comprising three different siRNA molecules.

21. The composition of claim 19 wherein each siRNA molecule binds to at least one mRNA molecule that encodes at least one protein.

22. The composition of claim 19 comprising siRNA molecules that bind to at least two or more different mRNA molecules.

23. The composition of claim 19 wherein each siRNA molecules binds to a different mRNA molecule that encodes a different protein.

24. The composition of claim 23 wherein each of the proteins is in a different pathway.

25. The composition of any of claims 19-24 wherein the siRNA molecules target one or more of the mRNA molecules that are transcribed from one or more of the gene sequences listed in Tables 1-6.

26. The composition of any of claims 19-24 wherein the siRNA molecules are selected from those listed in Tables 7-12.

27. The composition of claim 26, wherein at least one siRNA (sense: 5'-CUGUAGACACACCCACCCACAUACA-3', antisense: 5'-UGUAUGUGGGUGGGUGUGUCUACAG-3') or (sense 5'-CCCUGGUGGACAUCUUCCAGGAGUA-3', antisense 5'-UACUCCUGGAAGAUGUCCACCAGGG-3') binds to an mRNA molecule that encodes both a human and a mouse VEGF protein, at least one siRNA molecule (sense: 5'-

CCAUCGAUGUCUACAUGAUCAUGGU-3', antisense: 5'-ACCAUGAUCAUGUAGACAUCGAUGG-3') or (sense 5'-GAUCAUGGUCAAGUGCUGGAUGAUA-3', antisense 5'-UAUCAUCCAGCACUUGACCAUGAUC-3') binds to an mRNA molecule that encodes both a human and mouse EGFR protein, and at least one siRNA molecule (sense: 5'-GCUGAAGGUUGUGAAAUUCGGAGAA-3', antisense: 5'-UUCUCCGAAUUUCACAACCUUCAGC-3') or (sense 5'-GCUGCUGAAGGUUGUGAAAUUCGGA-3', antisense 5'-UCCGAAUUUCACAACCUUCAGCAGC-3') binds to an mRNA molecule that encodes both a human and mouse MGMT protein.

28. The composition of claim 26, wherein at least one siRNA (sense, 5'-GCCUGCUGCUCCUCGGCUGCGGAUA-3' antisense, 5'-UAUCCGCAGCCGAGGAGCAGCAGGC-3') binds to RAF-1 mRNA, at least one siRNA (sense,: 5'-GGUCUGGUGCCUGGUCUGAUGAUGU-3', antisense, ACAUCAUCAGACCAGGCACCAGACC-3',) binds to mTOR mRNA, at least one siRNA (sense 5'-CCCAAGGGCUACCAUGCCAACUUCU-3', antisense,5'-AGAAGUUGGCAUGGUAGCCCUUGGG-3'" binds to hmTGFβ1 mRNA, and at least one siRNA (sense, 5'-GGUCUGGUGCCUGGUCUGAUGAUGU-3', antisense, 5'-ACAUCAUCAGACCAGGCACCAGACC-3'" binds to hmCox-2 mRNA.

29. The composition of any of claims 19-28 further comprising a pharmaceutically acceptable carrier.

30. The composition claim 29 wherein said carrier is at least one of the following: saline, a sugar, a polypeptide, a polymer, a lipid, a cream, a gel, a micelle material, and a metal nanoparticle.

31. The composition of claim 29 wherein said carrier comprises at least one of the following: a glucose solution, a polycationic binding agent, a cationic lipid, a cationic micelle, a cationic polypeptide, a hydrophilic polymer grafted polymer, a non-natural cationic polymer, a cationic polyacetal, a hydrophilic polymer grafted polyacetal, a ligand functionalized cationic polymer, a ligand functionalized-hydrophilic polymer grafted polymer, and a ligand functionalized liposome.

32. The composition of claim 31 wherein said ligand comprises one or more of an RGD peptide, an RVG peptide, or a FROP peptide.

33. The composition of claim 32 wherein said RGD peptide is H-ACRGDMFGCA-OH, said RVG peptide is H- YTIWMPENPRPGTPCDIFTNSRGKRASNG-OH, and said FROP peptide is H-EDYELMDLLAYL-OH.

34. The composition of claim 31 wherein said polymers comprise a biodegradable histidine-lysine polymer, a biodegradable polyester, a polyamidoamine (PAMAM) dendrimer, a cationic lipid, optionally DOTAP, and a PEGylated PEI.

35. The composition of claim 34 wherein said biodegradable polyester comprises poly(lactic acid) (PLA), poly(glycolic acid) (PGA), or poly(lactic-co-glycolic acid) (PLGA).

36. The composition of claim 29 wherein said carrier comprises a histidine-lysine copolymer that forms a nanoparticle with the siRNA molecule or molecules.

37. The composition of claim 36 wherein the diameter of said nanoparticle is about 100nm to about 500 nm.

38. The composition of any of claims 18-37 further comprising a therapeutic agent that impedes or blocks tumorigenesis, angiogenesis, or cell proliferation in the brain or spinal cord of a mammal.

39. The composition of claim 38 wherein said agent impedes or blocks the activity of a peptide or protein whose activity promotes tumorigenesis, angiogenesis, or cell proliferation in the brain or spinal cord of a mammal.

40. The composition of claim 39 wherein said protein is selected from the group consisting of pro-tumorigenic pathway proteins, pro-angiogenesis pathway proteins, pro-cell proliferation pathway proteins, and anti-apoptotic pathway proteins.

41. The composition of claim 39 wherein said protein is selected from the group consisting of VEGF pathway proteins, EGFR pathway proteins, MGMT pathway proteins, RAF pathway proteins, MMP pathway proteins, mTOR pathway proteins, TGFβ pathway proteins, and Cox-2 pathway proteins.

42. The composition of claim 39 wherein said protein is selected from the group consisting of VEGF, EGFR, PI3K, AKT, AGT, RAF1, RAS, MAPK, ERK, MGMT, MMP-2, MMP-9, PDGF, PDGFR, IGF-1, HGF, mTOR, Cox-2, and TGFβ.

43. The composition of claim 42 wherein said protein is VEGF, EGFR, MGMT, MMP-2, MMP-9, PDGF, RAF1, mTOR, or Cox-2.

44. The composition of claim 42 wherein said protein is VEGF, EGFR, MGMT, MMP-2, MMP-9, or PDGF.

45. The composition of claim 42 wherein said protein is RAF1, mTOR, or Cox-2.

46. The composition of any of claims 39-44 wherein said peptide or protein causes or promotes the growth of a glioma.

47. The composition of claim 46 wherein said glioma is an astrocytoma.

48. The composition of claim 47 wherein said astrocytoma is a glioblastoma multiforme.

49. The composition of any of claims 38-48 wherein said mammal is a human.

50. The composition of any of claims 38-49 wherein said therapeutic agent is selected from the group consisting of bevacizumab, sunitinib, sorafenib, temsirolimus, and temozolomide.

51. The composition of claim 50 wherein said compound is temozolomide and wherein said siRNA molecules inhibit the expression of MGMT and two of the following: EGFR, VEGF, PDFG, MMP-2, and MMP-9.

52. The composition of claim 50 wherein said compound is bevacizumab and wherein said siRNA molecules inhibit the expression of two of the following: EGFR, PDFG, MMP-2, and MMP-9.

53. The composition of claim 50 wherein said compound is temozolomide and wherein said siRNA molecules inhibit the expression of EGFR, VEGF, and MGMT.

54. A method for treating a subject with a glioma comprising administering to said subject an effective amount of the siRNA molecule of any of claims 1-17.

55. A method for treating a subject with a glioma comprising administering to said subject an effective amount of the composition of any of claims 18-53.

56. The method of claims 54 or 55 wherein said glioma is characterized at least in part by neovascularization and inflammation in the brain or spinal cord of said subject.

57. The method of any of claims 54-56 wherein said glioma is an astrocytoma.

58. The method of claim 57 wherein said astrocytoma is a glioblastome multiforme.

59. The method of any of claims 48-58 wherein said subject is a human.

60. The method of any of claims 54-59 wherein said composition comprises at least three siRNA molecules at a ratio determined by the potency of each siRNA molecule and the therapeutic needs of the subject.

61. The method of claim 60 comprising three siRNA molecules at a ratio of 1:1:1, 1:1.5:0.5, or 0.5:0.5:2.

62. A nanoparticle comprising the siRNA molecule of any of claims 1-17, a carrier, and a targeting ligand.

63. The nanoparticle of claim 62 wherein said carrier is a polymer or a lipid.

64. The nanoparticle of claims 62 or 63 wherein said carrier is a histidine-lysine copolymer.

65. The nanoparticle of claim 64 wherein the diameter of said nanoparticle is about 100nm to about 500nm.

66. The nanoparticle of any of claims 62-65 wherein said targeting ligand is selected from the group consisting of EGF receptor ligands, IL13 ligand, hepatocyte growth factor ligand, single chain monoclonal antibodies, RGD peptide ligands, and RVG peptide ligands.

67. The nanoparticle of claim 66 wherein said ligand is an RGD peptide ligand or an RVG peptide ligand.

68. The nanoparticle of claim 67 wherein said RGD peptide ligand is 'cyclic' 10mer RGD peptide with the sequence H-ACRGDMFGCA-OH, and -(D)CR(D)WKTCT-(ol).

69. The nanoparticle of claim 67 wherein said RVG peptide ligand is YTIWMPENPRPGTPCDIFTNSRGKRASNG or YTIWMPENPRPGTPCDIFTNS-RGKRASNGGGGRRRRRRRRR.

70. A composition comprising 3 or more of the nanoparticles of claims 62-69.

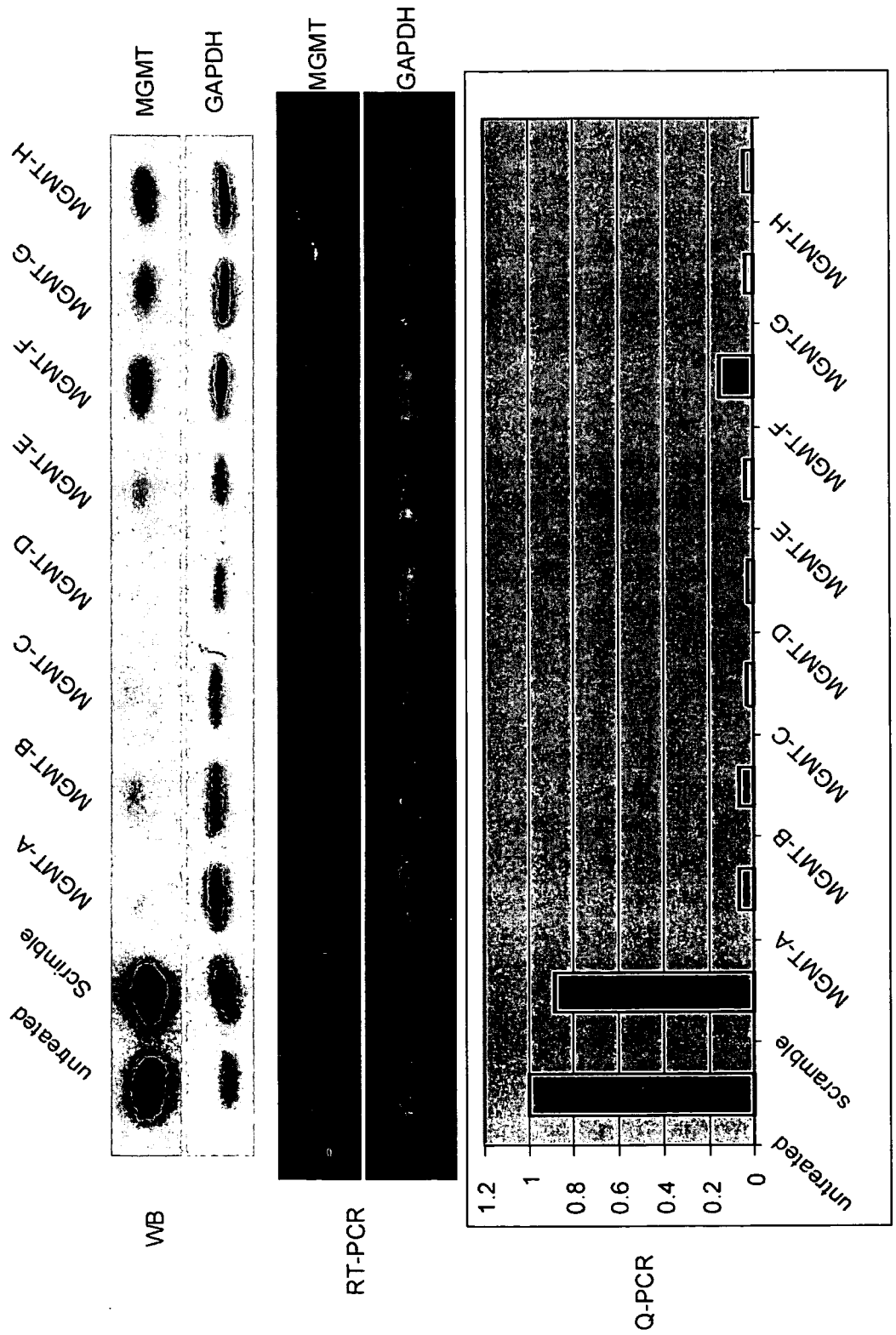

WB
MGMT
GAPDH
untreated
Scrimble
MGMT-A
MGMT-B
MGMT-C
MGMT-D
MGMT-E
MGMT-F
MGMT-G
MGMT-H
RT-PCR
MGMT
GAPDH
Q-PCR
1.2
1
0.8
0.6
0.4
0.2
0
untreated
scramble
MGMT-A
MGMT-B
MGMT-C
MGMT-D
MGMT-E
MGMT-F
MGMT-G
MGMT-H
siRNA knocking down MGMT (H80TR)

Figure 1.

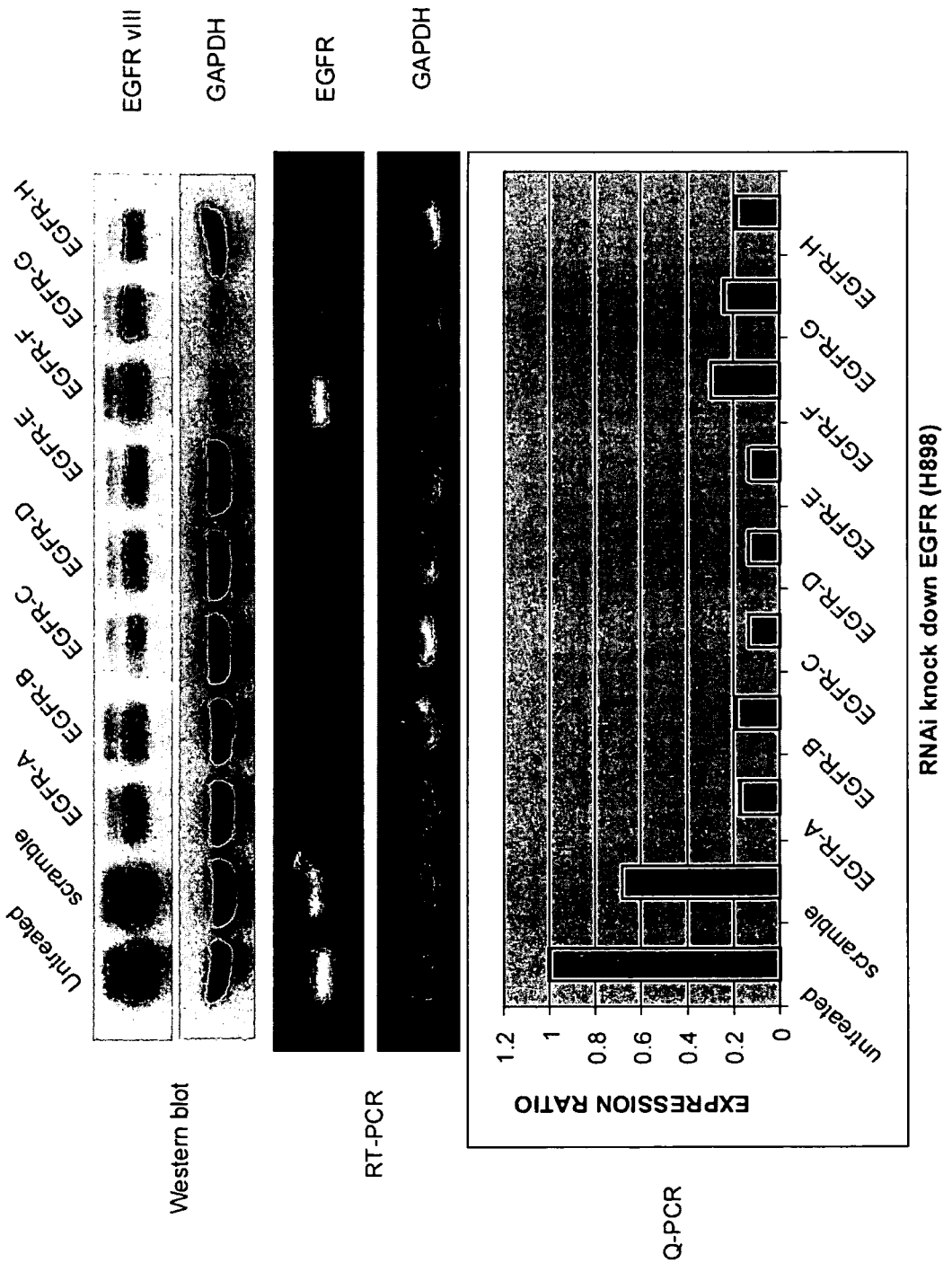
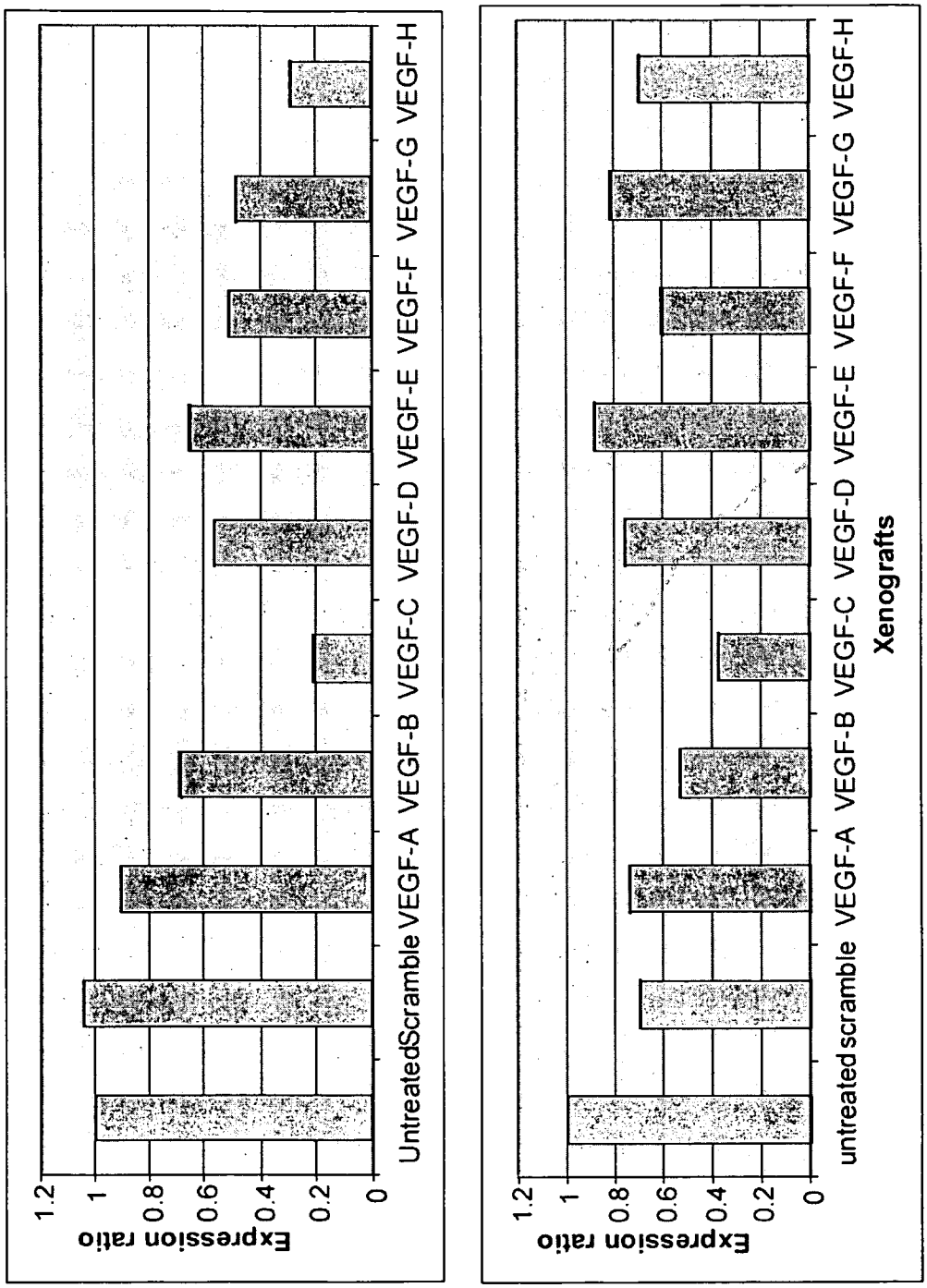
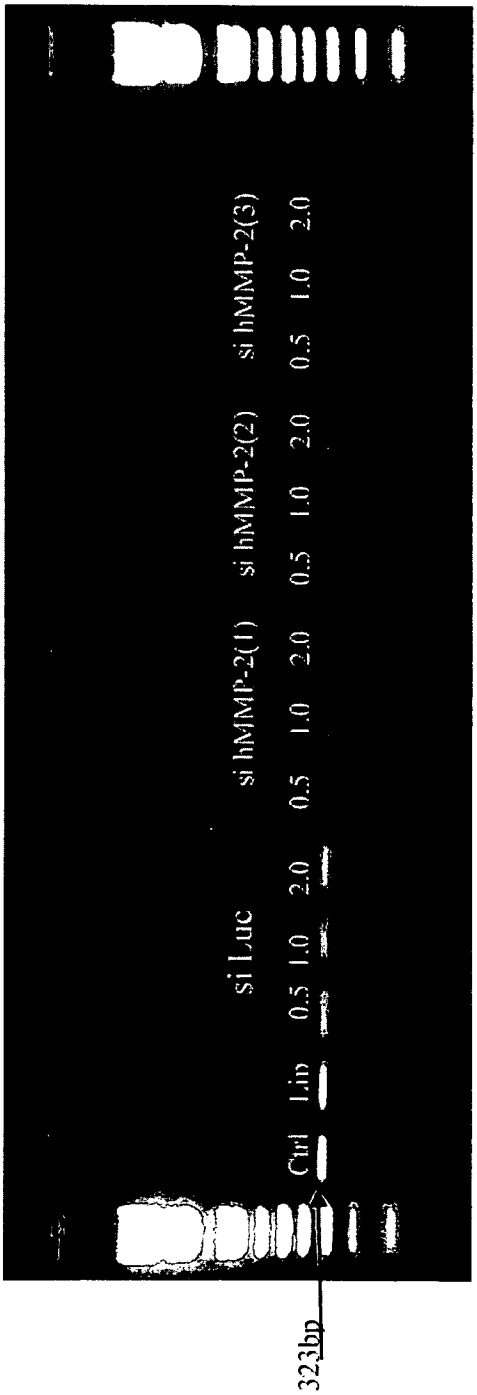

Western blot
EGFR vIII
GAPDH
Untreated
Scramble
EGFR-A
EGFR-B
EGFR-C
EGFR-D
EGFR-E
EGFR-F
EGFR-G
EGFR-H
RT-PCR
EGFR
GAPDH
Q-PCR
EXPRESSION RATIO
1.2
1
0.8
0.6
0.4
0.2
0
RNAi knock down EGFR (H898)

Figure 2



SiRNA Knockdown of VEGF in 293 cells and F3 cells

Figure 3


Ctrl
Lip
si Luc
0.5 1.0 2.0
si hMMP-2(1)
0.5 1.0 2.0
si hMMP-2(2)
0.5 1.0 2.0
si hMMP-2(3)
0.5 1.0 2.0
323bp

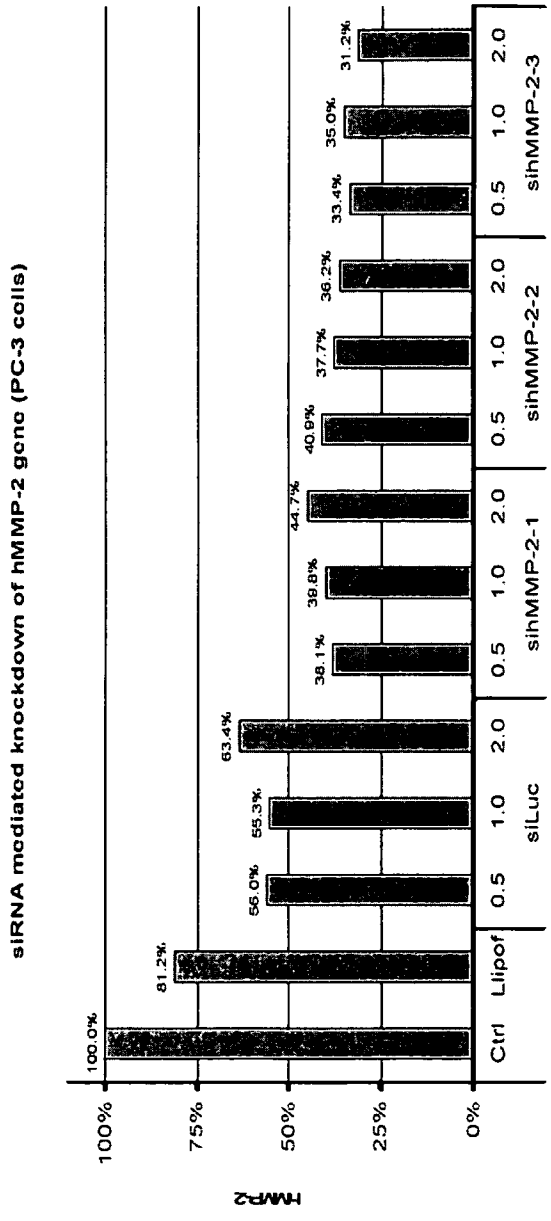
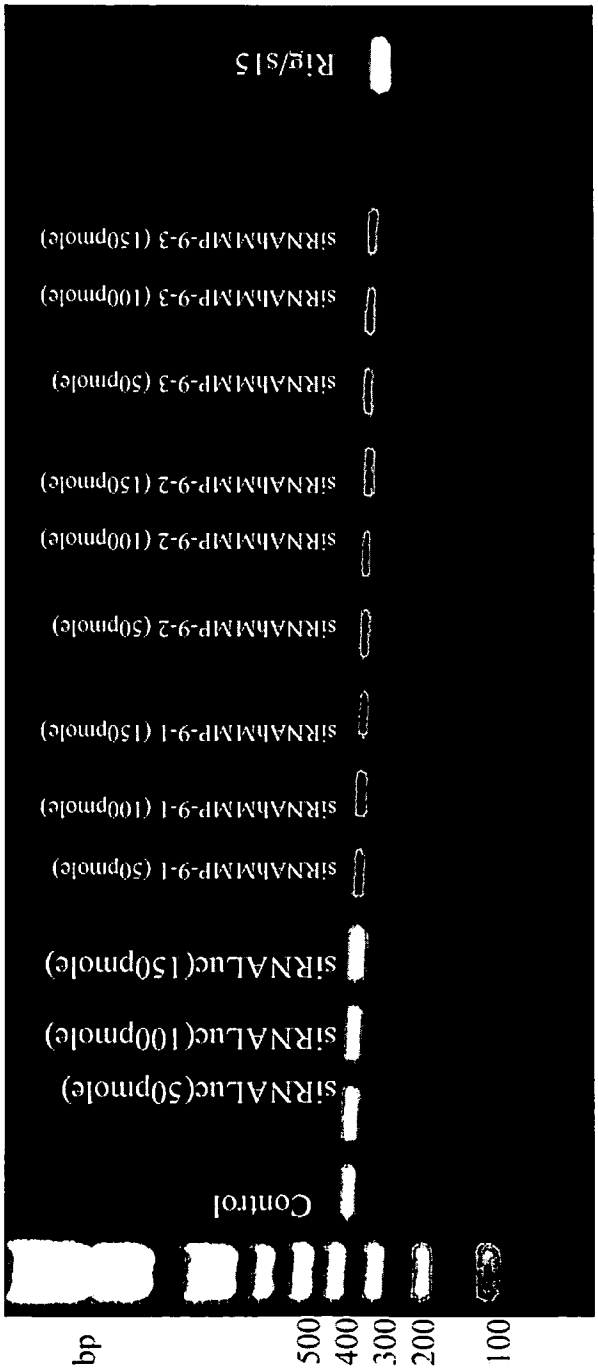

siRNA mediated knockdown of hMMP-2 gene (PC-3 cells)
MMP2
100%
75%
50%
25%
0%
100.0%
81.2%
55.0%
55.3%
63.4%
38.1%
38.8%
44.7%
40.9%
37.7%
36.2%
33.4%
35.0%
31.2%
Ctrl
Lipof
siLuc
sihMMP-2-1
sihMMP-2-2
sihMMP-2-3
0.5
1.0
2.0

Figure 4


bp
500
400
300
200
100
Control
siRNALuc(50pmole)
siRNALuc(100pmole)
siRNALuc(150pmole)
siRNAhMMP-9-1 (50pmole)
siRNAhMMP-9-1 (100pmole)
siRNAhMMP-9-1 (150pmole)
siRNAhMMP-9-2 (50pmole)
siRNAhMMP-9-2 (100pmole)
siRNAhMMP-9-2 (150pmole)
siRNAhMMP-9-3 (50pmole)
siRNAhMMP-9-3 (100pmole)
siRNAhMMP-9-3 (150pmole)
Rig/S15

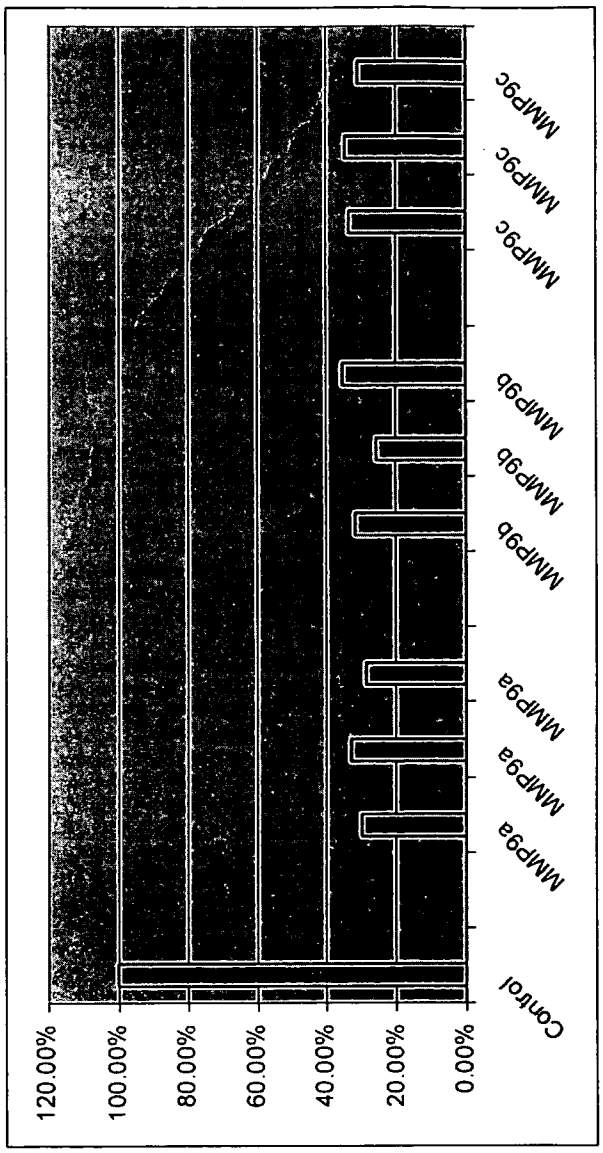

120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
Control
MMP9a
MMP9a
MMP9a
MMP9b
MMP9b
MMP9b
MMP9c
MMP9c
MMP9c

Figure 5

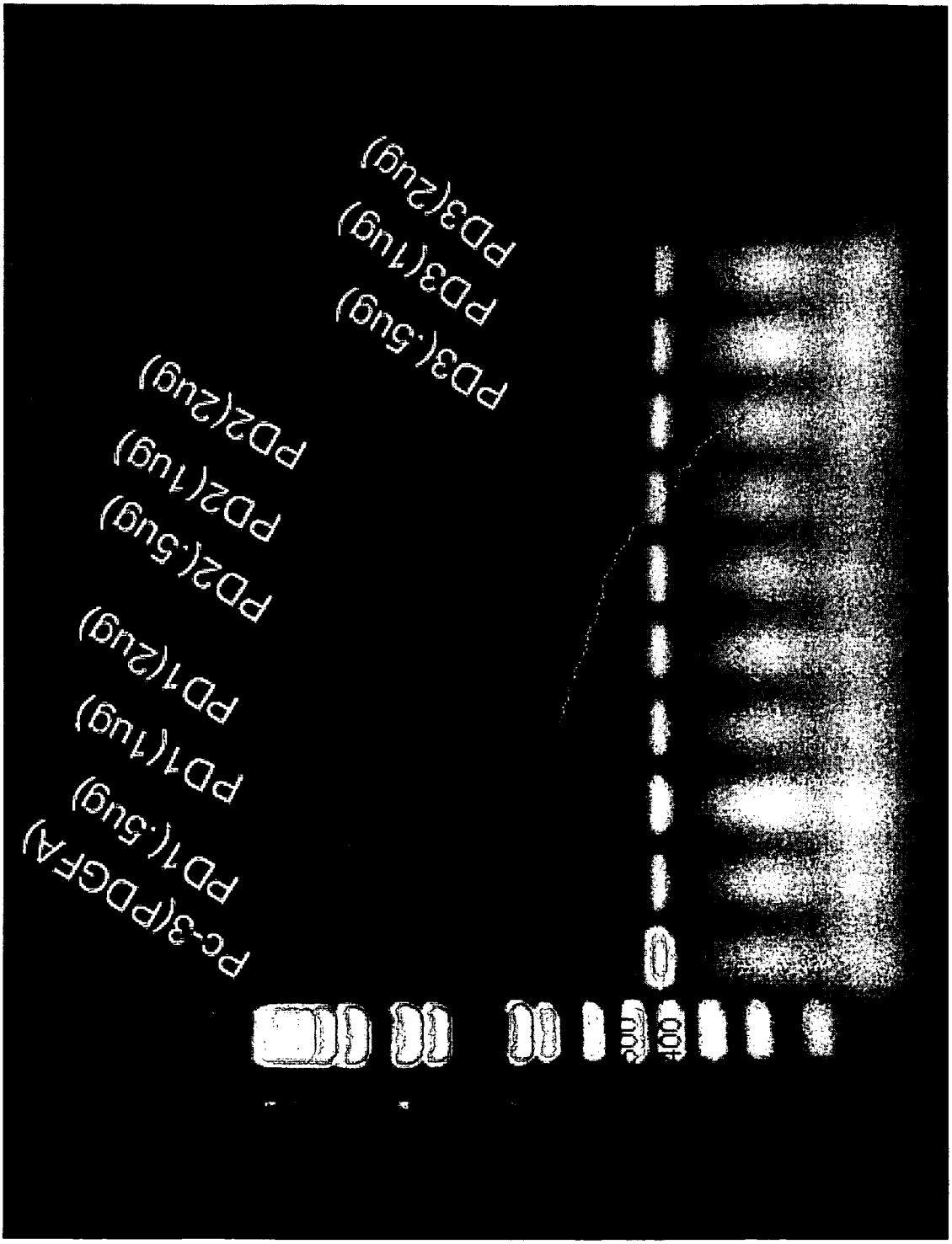

PC-3(PDGFA)
PD1(.5ug)
PD1(1ug)
PD1(2ug)
PD2(.5ug)
PD2(1ug)
PD2(2ug)
PD3(.5ug)
PD3(1ug)
PD3(2ug)
100
50

Figure 6

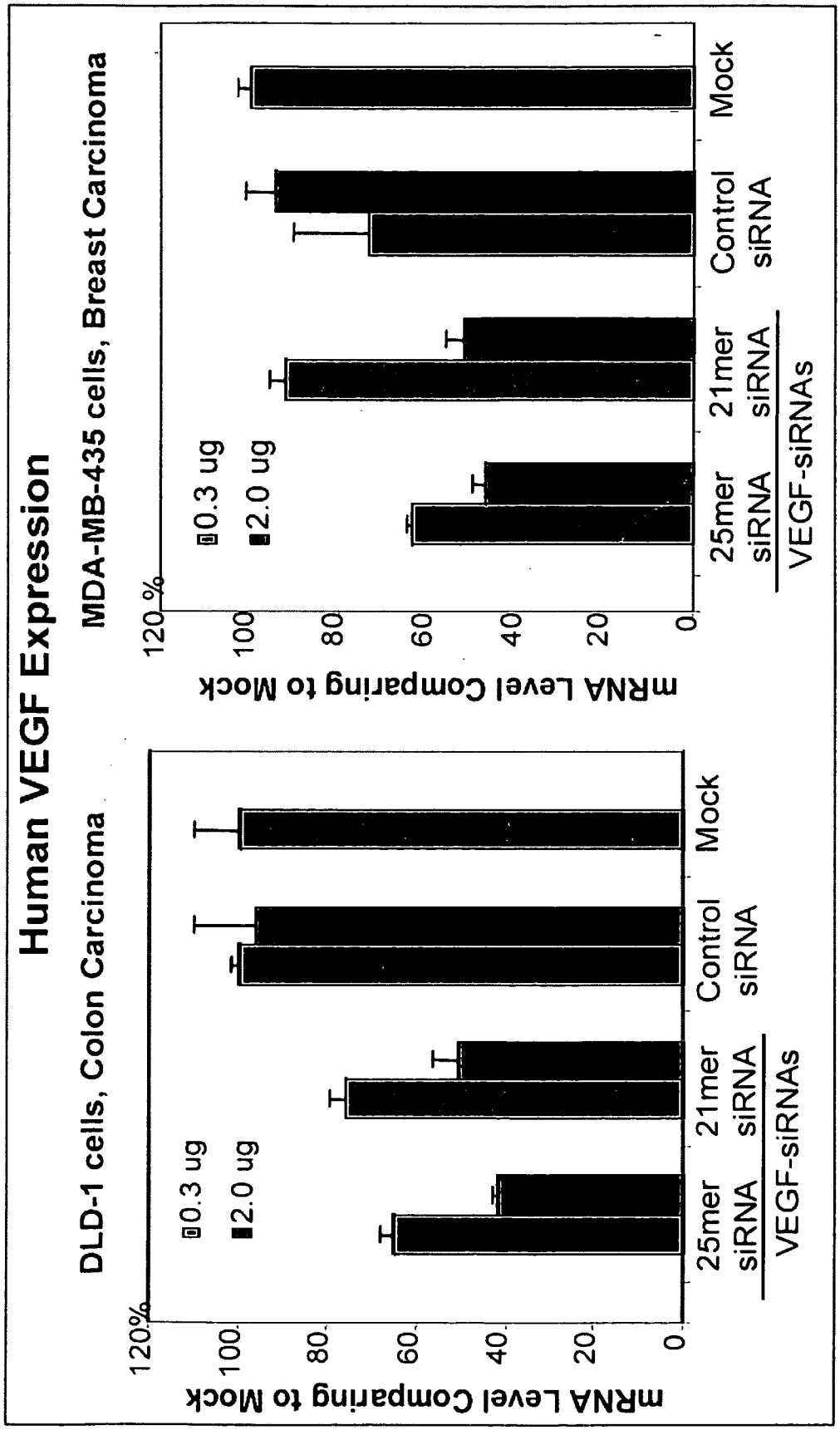

Human VEGF Expression
DLD-1 cells, Colon Carcinoma
MDA-MB-435 cells, Breast Carcinoma
mRNA Level Comparing to Mock
120%
100
80
60
40
20
0
0.3 ug
2.0 ug
25mer siRNA
21mer siRNA
VEGF-siRNAs
Control siRNA
Mock

Figure 7

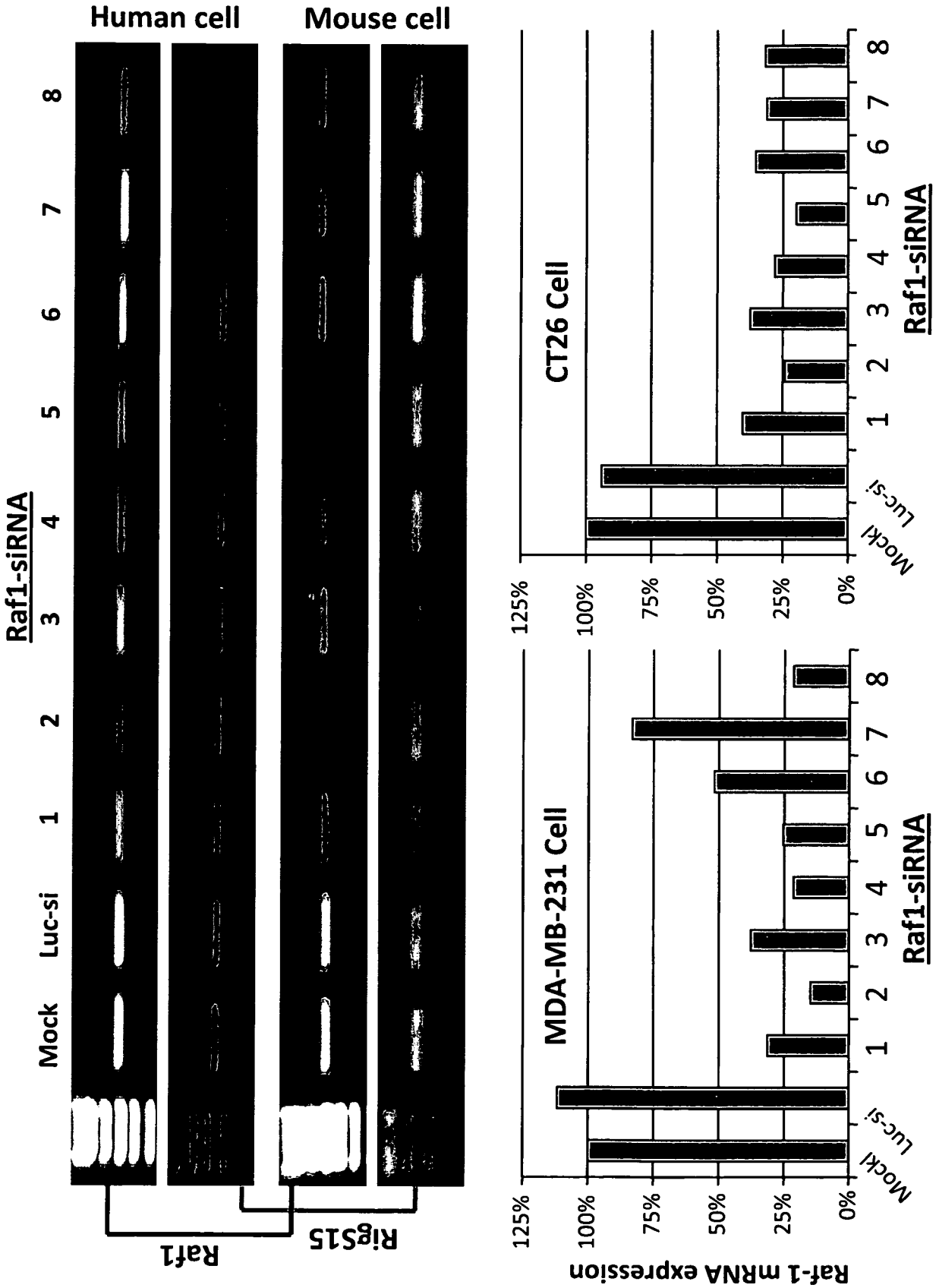

Human cell
Mouse cell
Raf1-siRNA
Mock
Luc-si
1
2
3
4
5
6
7
8
Raf1
RigS15
Raf-1 mRNA expression
125%
100%
75%
50%
25%
0%
MDA-MB-231 Cell
CT26 Cell

Figure 8

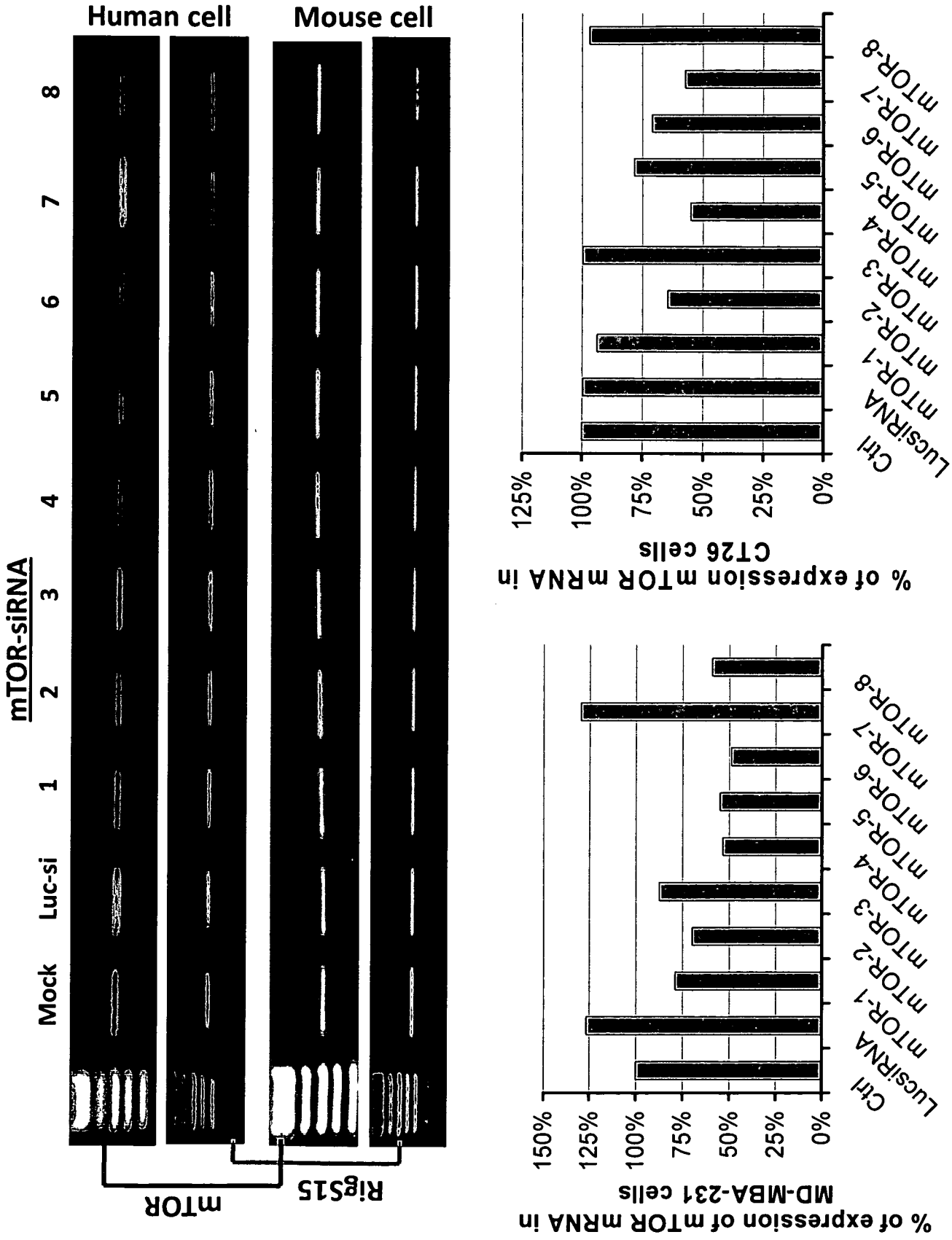

Human cell
Mouse cell
mTOR-siRNA
Mock
Luc-si
1
2
3
4
5
6
7
8
mTOR
RigS15
% of expression of mTOR mRNA in MD-MBA-231 cells
150%
125%
100%
75%
50%
25%
0%
Ctrl
LucsiRNA
mTOR-1
mTOR-2
mTOR-3
mTOR-4
mTOR-5
mTOR-6
mTOR-7
mTOR-8
% of expression mTOR mRNA in CT26 cells
125%
100%
75%
50%
25%
0%
Ctrl
LucsiRNA
mTOR-1
mTOR-2
mTOR-3
mTOR-4
mTOR-5
mTOR-6
mTOR-7
mTOR-8

Figure 9

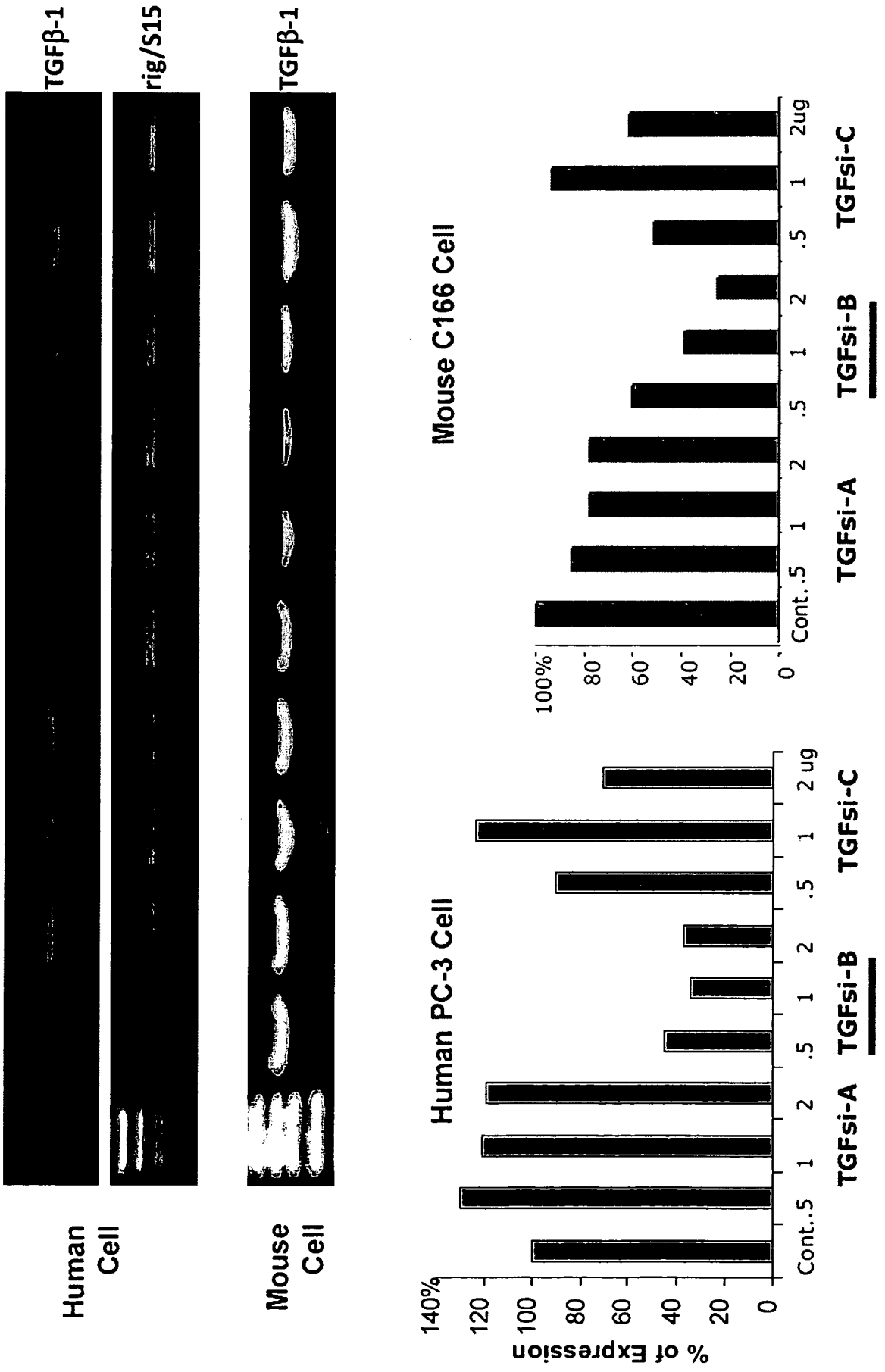
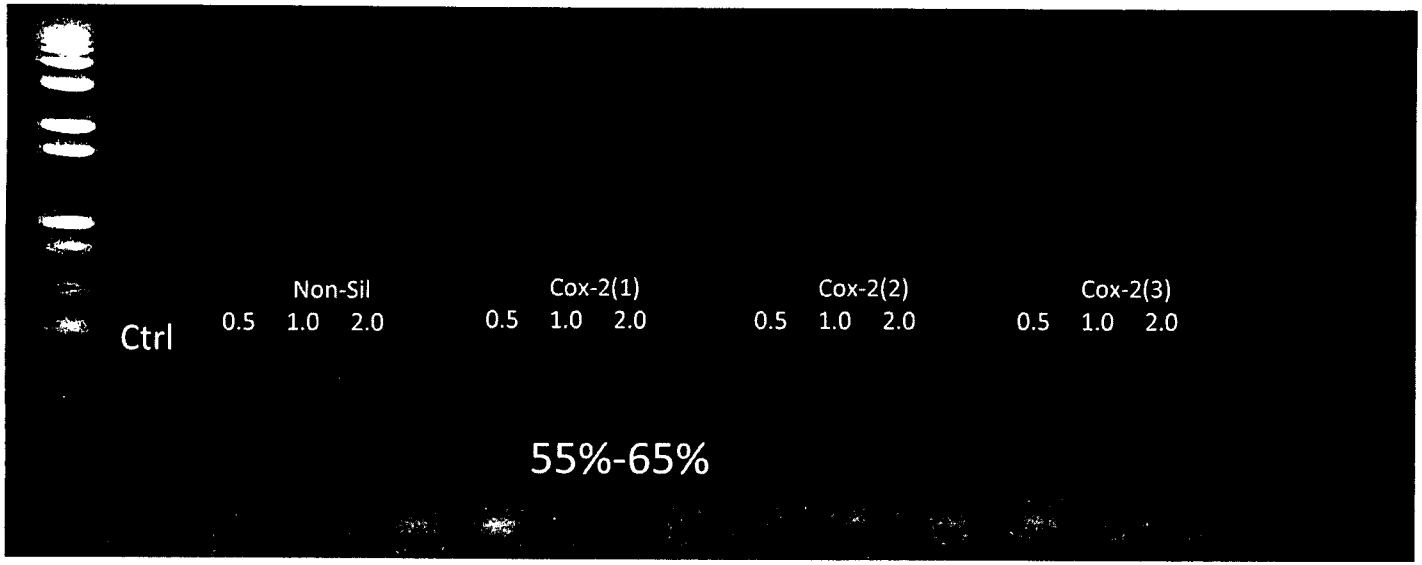

Ctrl
TGFβsi-A
0.5
1.0
2.0
TGFβsi-B
0.5
1.0
2.0
TGFβsi-C
0.5
1.0
2.0
TGFβ-1
rig/S15
TGFβ-1
Human Cell
Mouse Cell
Human PC-3 Cell
% of Expression
140%
120
100
80
60
40
20
0
Cont.
.5
1
2
.5
1
2
2 ug
TGFsi-A
TGFsi-B
TGFsi-C
Mouse C166 Cell
100%
80
60
40
20
0
Cont.
.5
1
2
.5
1
2
2ug
TGFsi-A
TGFsi-B
TGFsi-C

Figure 10

Cox-2(1) siRNA is able to knockdown target gene expression in PC3 cell



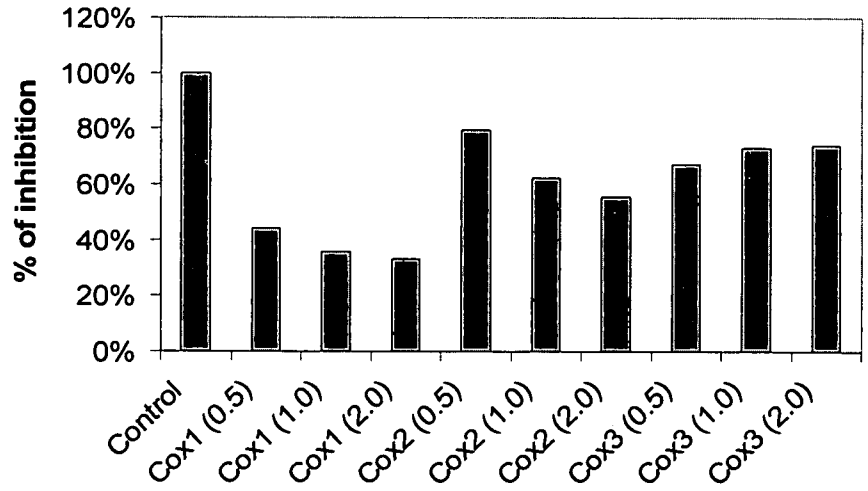


Figure 11

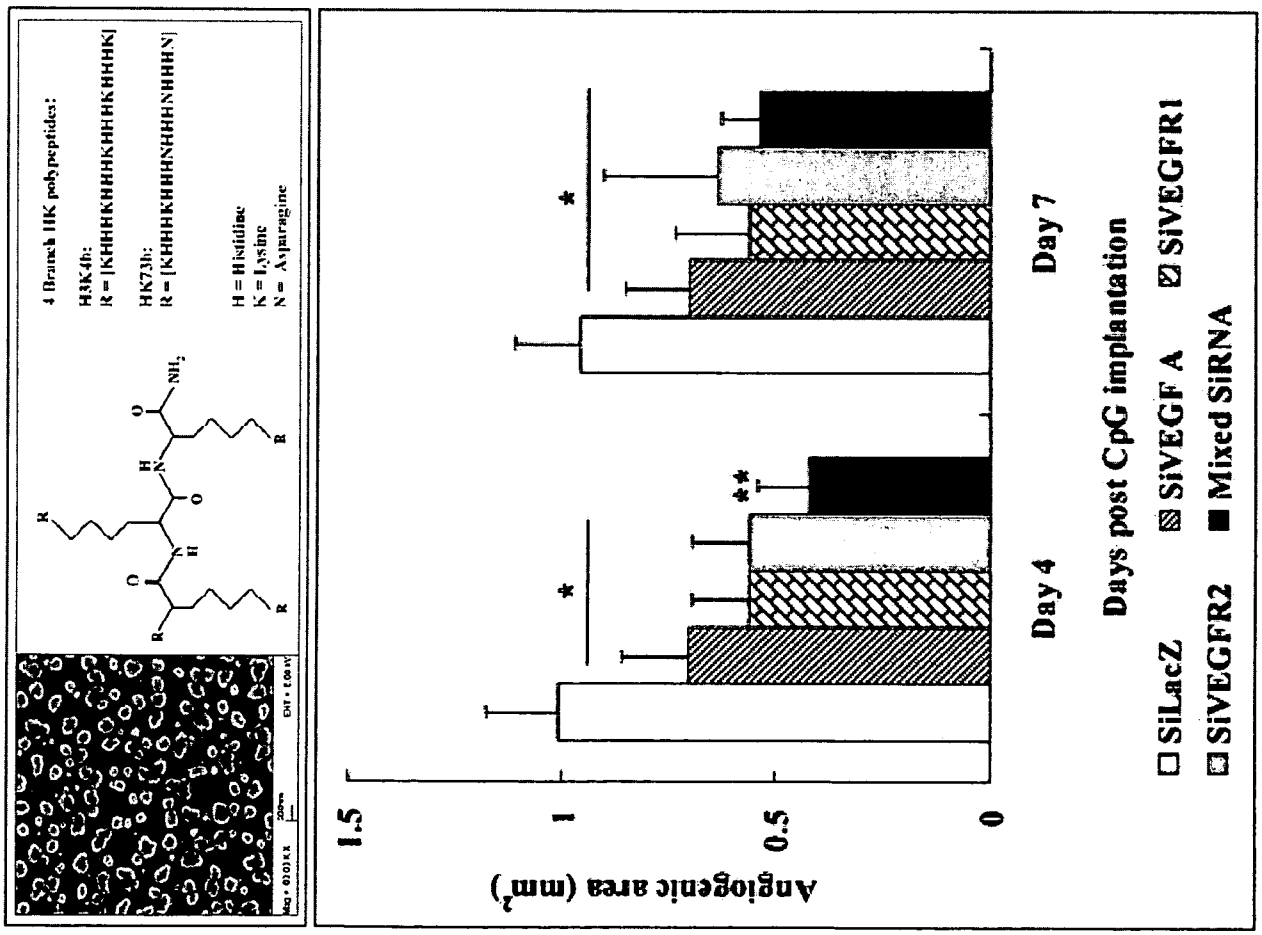
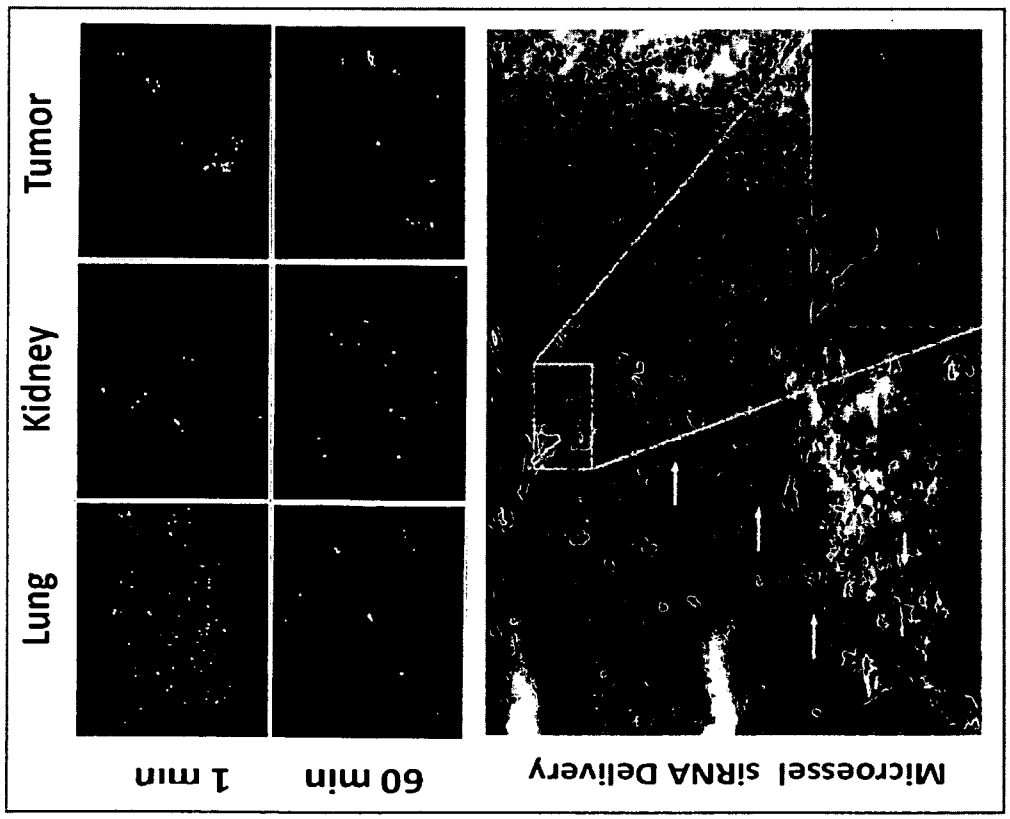

A
4 Branch HK polypeptides:
H3K4b:
R = [KHHHKHHHKHHHKHHHK]
HK73b:
R = [KHHHKHHHNHHNHHHN]
H = Histidine
K = Lysine
N = Asparagine
B
Angiogenic area (mm²)
1.5
1
0.5
0
Day 4
Day 7
Days post CpG implantation
SiLacZ
SiVEGF A
SiVEGFR1
SiVEGFR2
Mixed SiRNA

Figure 12

Figure 13



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/03196

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07H 21/02, A61K 31/7088 (2009.01)
USPC - 536/24.5, 536/23.1, 514/44
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 -- 536/24.5, 536/23.1, 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST -- PGPB,USPT,USOC,EPAB,JPAB; Dialog Classic Files ? 654, 65, 351, 349, 315, 6, 35, 65, 155; Google Scholar; USPTO Web Page; PCT Patentscope; Search terms -- isolated siRNA, mRNA target, glioblastoma, glioma, brain/spinal cord cell proliferation, 21-25 bps, blunt ends

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2007/0003519 A1 (LU et al.) 04 January 2007 (04.01.2007) para [0030], [0055], [0056], [0083], [0108], [0131], [0154], [0171], [0191], [0194], Fig 7, 35	1-4 ---- 5
Y	US 2006/0134787 A1 (ZAMORE et al.) 22 June 2006 (22.06.2006) para [0008], [0009], [0021], [0031]	5

☐ 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
23 September 2009 (23.09.2009)	05 OCT 2009
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 09/03196

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6-70
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.