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(54) Title: COMPOSITIONS AND METHODS TARGETING GLUTAMINASE

(57) Abstract: The invention provides compositions and methods featuring nucleic acid molecules that target glutaminase. The methods of the invention involve administering a nucleic acid to a cell of a subject diagnosed as having a neoplasia in order to inhibit the expression of glutaminase.



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COMPOSITIONS AND METHODS TARGETING GLUTAMINASE

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 61/ 098,092, filed on September 18, 2008, and U.S. Provisional Application No. 61/ 151,049, filed on February 9, 2009, the entire contents of each of which are incorporated herein by reference.

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

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BACKGROUND OF THE INVENTION

Altered glucose metabolism in cancer cells is termed the Warburg effect, which describes the propensity of most cancer cells to take up glucose avidly and convert it primarily to lactate, despite available oxygen (1, 2). Notwithstanding the renewed interest in the Warburg effect, cancer cells also depend on continued mitochondrial function for metabolism, specifically glutaminolysis that catabolizes glutamine to generate ATP and lactate (3). Glutamine, which is highly transported into proliferating cells (4, 5), is a major source of energy and nitrogen for biosynthesis, and a carbon substrate for anabolic processes in cancer cells, but the regulation of glutamine metabolism is not well understood (1, 6).

Oncogenes and tumor suppressors have been linked to the regulation of glucose metabolism, thereby connecting genetic alterations in cancers to their glucose metabolic phenotype (1,2). In particular, the MYC oncogene produces Myc protein that directly regulates glucose metabolic enzymes as well as genes involved in mitochondrial biogenesis (9, 12). Dysregulated expression or function of the Myc oncogenic transcription factor occurs frequently in human malignancies. Through the positive and negative regulation of an expansive network of target genes, Myc globally reprograms cells to drive proliferation and in some settings induce cell death. Myc utilizes distinct mechanisms for activating and repressing gene expression. When inducing transcription, Myc dimerizes with its binding partner Max and binds to genomic DNA directly upstream or within the first intron of target genes. When repressing transcription, Myc does not appear to contact DNA directly. Rather, Myc is recruited to core promoters via protein-protein interactions where it antagonizes the activity of positive regulators of transcription. For example, Myc can bind to and inhibit the

activity of the transcription factor Myc-interacting zinc finger protein 1 (Miz1), thus preventing Miz1 from activating transcription of the CDKN1A (p21WAF1/CIP1) and CDKN2B (p15INK4b) cell-cycle-inhibitory genes. Repression of other Myc targets is likely mediated through the ability of Myc to interact with and antagonize the activity of additional proteins including Sp1, Smad2, and NF-Y.

MicroRNAs (miRNAs) are a diverse family of ~18-24 nucleotide RNA molecules that have recently emerged as a novel class of Myc-regulated transcripts. miRNAs regulate the stability and translational efficiency of partially-complementary target messenger RNAs (mRNAs). miRNAs are initially transcribed by RNA polymerase II (pol II) as long primary transcripts (pri-microRNAs) that are capped, polyadenylated, and frequently spliced. The mature microRNA sequences are located in introns or exons of pri-microRNAs, within regions that fold into ~60-80 nucleotide hairpin structures. While the majority of pri-microRNAs are noncoding transcripts, a subset of microRNAs are located within introns of protein-coding genes. MicroRNA maturation requires a series of endonuclease reactions in which microRNA hairpins are excised from pre-miRNAs, the terminal loop of the hairpin is removed, and one strand of the resulting duplex is selectively loaded into the RNA-induced silencing complex (RISC). This microRNA-programmed RISC is the effector complex which carries out target mRNA regulation.

A large body of evidence has documented nearly ubiquitous dysregulation of miRNA expression in cancer cells. These miRNA expression changes are highly informative for cancer classification and prognosis. Moreover, altered expression of specific miRNAs has been demonstrated to promote tumorigenesis. Although select miRNAs are upregulated in cancer cells, global miRNA abundance appears to be generally reduced in tumors. miRNA downregulation likely contributes to neoplastic transformation by allowing the increased expression of proteins with oncogenic potential. Recent evidence suggests that a block in the first step of miRNA processing may contribute to the reduced abundance of select miRNAs in cancer cells. Cancer causes one in every four US deaths and is the second leading cause of death among Americans. Additional mechanisms of miRNA downregulation, including direct transcriptional repression, have not yet been investigated. Improved compositions and methods for the treatment or prevention of neoplasia are required.

SUMMARY OF THE INVENTION

As described in more detail below, the present invention has identified reductions in the expression of Myc regulated microRNAs (e.g., miR-23a or miR-23b) that are associated with greater expression of mitochondrial glutaminase. This leads to upregulated glutamine catabolism. The present invention provides compositions featuring microRNAs and methods of using them for the treatment of neoplasia. The present invention also provides compositions featuring microRNAs and methods of using them to protect against ischemic cell death, for example in the treatment or prevention of cardiac ischemia or stroke.

In one aspect, the invention generally provides an isolated oligonucleotide comprising a nucleobase sequence having at least 85% identity to the sequence of microRNA miR-23a or microRNA miR-23b or a fragment thereof, wherein expression of said oligonucleotide in a neoplastic cell reduces the survival of the cell or inhibits cell division.

In another aspect, the invention provides an isolated oligonucleotide comprising a nucleobase sequence having at least 85% identity to the sequence of microRNA miR-23a or microRNA miR-23b or a fragment thereof, wherein expression of said oligonucleotide in a cell decreases expression of mitochondrial glutaminase in the cell.

In still another aspect, the invention provides an inhibitory nucleic acid molecule that corresponds to or is complementary to at least a fragment of microRNA miR-23a or microRNA miR-23b, or a fragment thereof, and that increases expression of mitochondrial glutaminase in the cell.

In another further aspect, the invention provides an inhibitory nucleic acid molecule that corresponds to or is complementary to at least a fragment of microRNA miR-23a or microRNA miR-23b, or a fragment thereof, wherein expression of said inhibitory nucleic acid molecule in a cell protects the cell from ischemic cell death.

In one embodiment of the above aspects, the nucleic acid molecule is double-stranded.

In another embodiment of the above aspects, the nucleic acid molecule is single stranded.

In one embodiment, the nucleic acid molecule is an antisense nucleic acid molecule.

In one embodiment, the double-stranded nucleic acid molecule is an siRNA. In further embodiments, the double-stranded nucleic acid molecule is an shRNA. In still further related embodiments, each strand of the double-stranded nucleic acid molecule is about 19-21 nucleotides in length.

In another embodiment, the antisense nucleic acid molecule comprises a nucleic acid sequence that is at least 85% identical to microRNA miR-23a or microRNA miR-23b.

In another embodiment of the above aspects, the nucleotide sequence comprises at least one modified linkage. In still another embodiment of the above aspects, the inhibitory nucleic acid molecule comprises a modified backbone.

5 In still another embodiment of the above aspects, the oligonucleotide comprises the nucleobase sequence of said microRNA. In another related embodiment of the above aspects, the oligonucleotide consists essentially of the nucleobase sequence of said microRNA. In another embodiment of the above aspects, the microRNA sequence is a mature or hairpin form.

10 In another embodiment of the above aspects, the oligonucleotide comprises at least one modified linkage.

In still another embodiment of the above aspects, the oligonucleotide comprises at least one modified sugar moiety or one modified nucleobase.

15 The invention also features in another embodiment, an isolated nucleic acid molecule encoding the oligonucleotide of any of the above aspects, wherein expression of the oligonucleotide in a neoplastic cell reduces the survival of the cell or reduces cell division.

In one embodiments, the nucleic acid molecule consists essentially of the nucleotide sequence encoding a mature or hairpin form of microRNA miR-23a or microRNAmiR-23b, or a fragment thereof.

20 In another embodiment, the invention features an expression vector encoding an oligonucleotide of any one of the above aspects, wherein the nucleic acid molecule is positioned for expression in a mammalian cell.

In one embodiments, the vector encodes microRNA miR-23a or microRNAmiR-23b.

In one embodiments, the vector is a viral vector selected from the group consisting of a retroviral, adenoviral, lentiviral and adeno-associated viral vector.

25 In another embodiment, the invention features a host cell comprising the expression vector of any one of the above aspects or the oligonucleotide of any one of the above aspects.

30 In another aspect, the invention features a pharmaceutical composition for the decreasing the expression of glutaminase in a cell, the composition comprising an effective amount of an oligonucleotide having at least 85% identity to the sequence of microRNA miR-23a or microRNAmiR-23b, and a pharmaceutically acceptable excipient, wherein expression of said microRNA in a cell increases the expression of glutaminase.

In another aspect, the invention features a pharmaceutical composition for the treatment of a neoplasia, the composition comprising an effective amount of an oligonucleotide having at least 85% identity to the sequence of microRNA miR-23a or

microRNA miR-23b, and a pharmaceutically acceptable excipient, wherein expression of said microRNA in a neoplastic cell reduces the survival of the cell or reduces cell division.

In one embodiment, the amount of microRNA is sufficient to reduce cell survival, cell proliferation, or expression of Myc in a neoplastic cell by at least about 5% relative to an untreated control cell.

In one embodiment, the composition comprises at least one of miR-23a or miR-23b.

In another embodiment, the composition comprises an effective amount of an expression vector encoding microRNA miR-23a or microRNA miR-23b.

In one embodiment, the amount of microRNA is sufficient to reduce expression of Myc in a neoplastic cell by at least about 5% relative to an untreated control cell.

In another embodiment of the above aspects, the composition comprises at least one of miR-23a or miR-23b.

In yet another embodiment of the above aspects, the composition comprises microRNA miR-23a and microRNA miR-23b.

In another embodiment, the oligonucleotide comprises a modification.

In another aspect, the invention features a vector encoding an inhibitory nucleic acid molecule of any one of the above aspects.

In one embodiment, the vector is a retroviral, adenoviral, adeno-associated viral, or lentiviral vector.

In another embodiment, the vector comprises a promoter suitable for expression in a mammalian cell.

In another embodiment of the above aspects, the cell comprises the vector of the above aspects or an inhibitory nucleic acid molecule of the above aspects.

In another embodiment, the cell is an ischemic cell in vivo.

In another aspect, the invention features a pharmaceutical composition for increasing the expression of glutaminase in a subject comprising a therapeutically effective amount of an inhibitory nucleic acid molecule that corresponds to or is complementary to at least a fragment of a miR-23a or miR-23b, in a pharmaceutically acceptable excipient, wherein the fragment is capable of decreasing the expression of glutaminase.

In one embodiment, the inhibitory nucleic acid molecule is administered at a dosage of between about 100 to 300 mg/m²/day.

In another aspect, the invention features a method of decreasing glutaminase expression in a cell, the method comprising contacting the cell with an oligonucleotide

comprising a nucleobase sequence having at least 85% identity to microRNA miR-23a or microRNA miR-23b, thereby decreasing glutaminase expression in the cell relative to an untreated control cell.

In one embodiment, the cell is a neoplastic cell.

5 In another aspect, the invention features a method of reducing the growth, survival or proliferation of a neoplastic cell, the method comprising contacting the cell with an oligonucleotide comprising a nucleobase sequence having at least 85% identity to microRNA miR-23a or microRNA miR-23b, thereby reducing the growth, survival or proliferation of a neoplastic cell relative to an untreated control cell.

10 In another aspect, the invention features a method of reducing the growth, survival or proliferation of a neoplastic cell, the method comprising contacting the cell with an expression vector encoding microRNA miR-23a or microRNA miR-23b, thereby reducing the growth, survival or proliferation of a neoplastic cell relative to an untreated control cell.

15 In another aspect, the invention features a method of increasing glutaminase expression in a cell, the method comprising contacting the cell with an effective amount of an inhibitory nucleic acid molecule corresponding to or complementary to at least a portion of a miR-23a or miR-23b nucleic acid molecule.

In one embodiment, the cell is an ischemic cell.

In one embodiment, the cell is a mammalian cell.

20 In one embodiment, the cell is a human cell.

In one embodiment, the cell is a lymphoma cell or a prostate cell.

In one embodiment, the method induces apoptosis in the neoplastic cell.

25 In another aspect, the invention features a method of treating neoplasia in a subject, the method comprising administering to the subject an effective amount of an oligonucleotide comprising a nucleobase sequence having at least 85% identity to microRNA miR-23a or microRNA miR-23b, thereby treating a neoplasia in the subject.

30 In another aspect, the invention features a method of treating neoplasia in a subject, the method comprising administering to the subject an effective amount of an expression vector encoding a microRNA selected from miR-23a or miR-23b, thereby treating the neoplasia in the subject.

In one embodiment, the oligonucleotide comprises a modification that enhances nuclease resistance.

In another embodiment of any one of the above aspects, the subject is diagnosed as having prostate cancer, pancreatic cancer, or a lymphoma.

In another embodiment of any one of the above aspects, the method induces apoptosis in a neoplastic cell of the subject.

In another embodiment of any one of the above aspects, the effective amount is sufficient to reduce expression of glutaminase in a neoplastic cell by at least about 5%

5 relative to an untreated control cell.

In another aspect, the invention features a method of treating a subject suffering from an ischemic event, the method comprising administering to the subject an effective amount of an inhibitory nucleic acid molecule corresponding to or complementary to one or more of miR-23a and miR-23b, wherein the inhibitory nucleic acid molecule reduces miR-23a or
10 miR-23b expression thereby treating the ischemic event.

In one embodiment, the ischemic event is a cardiac ischemia.

In one embodiment, the ischemic event is a stroke.

In another aspect, the invention features a method comprising assaying the expression of microRNA miR-23a or microRNA miR-23b.

15 In one embodiment, the method comprises assaying the expression of a combination of microRNA miR-23a or microRNA miR-23b.

In another embodiment, the neoplasia is characterized as having Myc dysregulation.

In another aspect, the invention features a method of identifying an agent for the treatment of a neoplasia, the method comprising contacting a neoplastic cell with a candidate
20 agent; and assaying the expression of microRNA miR-23a or microRNA miR-23b, wherein an increase in said microRNA expression identifies the agent as useful for the treatment of a neoplasia.

In one embodiment, the method further comprises testing the agent in a functional assay. In a further embodiment, the functional assay analyses cell growth, proliferation, or
25 survival.

In another aspect, the invention features a method of identifying an agent for the protection of a cell against ischemic cell death, the method comprising exposing a cell to ischemic conditions; contacting a cell with a candidate agent; and assaying the expression of microRNA miR-23a or microRNA miR-23b, wherein an increase in said microRNA
30 expression identifies the agent as useful for the treatment of a neoplasia.

In another aspect, the invention features a primer set comprising at least two pairs of oligonucleotides, each of which pair binds to microRNA miR-23a or microRNA miR-23b, or a fragment thereof.

In another aspect, the invention features a probe set comprising at least two oligonucleotides each of which binds to microRNA miR-23a or microRNA miR-23b, or a fragment thereof.

In another aspect, the invention features a microarray comprising a microRNA or nucleic acid molecule encoding microRNA miR-23a or microRNA miR-23b, or a fragment thereof.

Other features and advantages of the invention will be apparent from the detailed description, and from the claims.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton et al., Dictionary of Microbiology and Molecular Biology (2nd ed. 1994); The Cambridge Dictionary of Science and Technology (Walker ed., 1988); The Glossary of Genetics, 5th Ed., R. Rieger et al. (eds.), Springer Verlag (1991); and Hale & Marham, The Harper Collins Dictionary of Biology (1991). As used herein, the following terms have the meanings ascribed to them below, unless specified otherwise. The sequence of microRNAs referred to herein is known in the art. In particular, the sequence of microRNAs is publicly available via miRBase (<http://microrna.sanger.ac.uk/>), which provides microRNA data. Each entry in the miRBase Sequence database represents a predicted hairpin portion of a miRNA transcript, with information on the location and sequence of the mature miRNA sequence. Both hairpin and mature sequences are available for searching using BLAST and SSEARCH, and entries can also be retrieved by name, keyword, references and annotation.

By “miR-23a microRNA” is meant a nucleic acid molecule comprising a nucleobase sequence that is substantially identical to the sequence of hsa-mir-23a, or a fragment thereof. In certain embodiments, miR-23a or a fragment thereof, expression reduces the growth of a neoplasia.

By “miR-23a gene” is meant a polynucleotide that encodes a miR-23a microRNA or analog thereof.

By “miR-23b microRNA” is meant a nucleic acid molecule comprising a nucleobase sequence that is substantially identical to the sequence of hsa-mir-23b, or a fragment thereof.

In certain embodiments, miR-23b or a fragment thereof, expression reduces the growth of a neoplasia.

By "miR-23b gene" is meant a polynucleotide that encodes a miR-23b microRNA or analog thereof.

5 By "agent" is meant a polypeptide, polynucleotide, or fragment, or analog thereof, small molecule, or other biologically active molecule.

By "alteration" is meant a change (increase or decrease) in the expression levels of a gene or polypeptide as detected by standard art known methods such as those described above. As used herein, an alteration includes a 10% change in expression levels, preferably a
10 25% change, more preferably a 40% change, and most preferably a 50% or greater change in expression levels.

In this disclosure, "comprises," "comprising," "containing" and "having" and the like can have the meaning ascribed to them in U.S. Patent law and can mean "includes," "including," and the like; "consisting essentially of" or "consists essentially" likewise has the
15 meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

By "control" is meant a standard or reference condition.

20 By "an effective amount" is meant the amount of an agent required to ameliorate the symptoms of a disease relative to an untreated patient. The effective amount of active agent(s) used to practice the present invention for therapeutic treatment of a neoplasia varies depending upon the manner of administration, the age, body weight, and general health of the subject. Ultimately, the attending physician or veterinarian will decide the appropriate
25 amount and dosage regimen. Such amount is referred to as an "effective" amount.

By "fragment" is meant a portion (e.g., at least 10, 25, 50, 100, 125, 150, 200, 250, 300, 350, 400, or 500 amino acids or nucleic acids) of a protein or nucleic acid molecule that is substantially identical to a reference protein or nucleic acid and retains the biological activity of the reference protein or nucleic acid.

30 A "host cell" is any prokaryotic or eukaryotic cell that contains either a cloning vector or an expression vector. This term also includes those prokaryotic or eukaryotic cells that have been genetically engineered to contain the cloned gene(s) in the chromosome or genome of the host cell.

By "inhibitory nucleic acid" is meant a single or double-stranded RNA, siRNA (short interfering RNA), shRNA (short hairpin RNA), or antisense RNA, or a portion thereof, or a mimetic thereof, that when administered to a mammalian cell results in a decrease (e.g., by 10%, 25%, 50%, 75%, or even 90-100%) in the expression of a target gene. Typically, a nucleic acid inhibitor comprises or corresponds to at least a portion of a target nucleic acid molecule, or an ortholog thereof, or comprises at least a portion of the complementary strand of a target nucleic acid molecule.

By "antisense nucleic acid", it is meant a non-enzymatic nucleic acid molecule that binds to target RNA by means of RNA--RNA or RNA-DNA interactions and alters the activity of the target RNA (for a review, see Stein et al. 1993; Woolf et al., U.S. Pat. No.5, 849, 902). Typically, antisense molecules are complementary to a target sequence along a single contiguous sequence of the antisense molecule. However, in certain embodiments, an antisense molecule can bind to substrate such that the substrate molecule forms a loop, and/or an antisense molecule can bind such that the antisense molecule forms a loop. Thus, the antisense molecule can be complementary to two (or even more) non-contiguous substrate sequences or two (or even more) non-contiguous sequence portions of an antisense molecule can be complementary to a target sequence or both. For a review of current antisense strategies, see Schmajuk NA et al., 1999; Delihis N et al., 1997; Aboul-Fadl T, 2005.)

By "inhibits a neoplasia" is meant decreases the propensity of a cell to develop into a neoplasia or slows, decreases, or stabilizes the growth or proliferation of a neoplasia.

By "ischemia" is meant to refer to a condition in which the blood flow (and thus oxygen) is restricted to a part of the body. Cardiac ischemia is the name for lack of blood flow and oxygen to the heart muscle.

By "isolated nucleic acid molecule" is meant a nucleic acid (e.g., a DNA, RNA, microRNA or analog thereof) that is free of the genes which, in the naturally-occurring genome of the organism from which the nucleic acid molecule of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or that exists as a separate molecule (for example, a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. In addition, the term includes a microRNA or other RNA molecule which is transcribed from a DNA molecule, as well as a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

By “marker” is meant any protein or polynucleotide having an alteration in expression level or activity that is associated with a disease or disorder.

The term "microarray" is meant to include a collection of nucleic acid molecules or polypeptides from one or more organisms arranged on a solid support (for example, a chip, plate, or bead).

By “modification” is meant any biochemical or other synthetic alteration of a nucleotide, amino acid, or other agent relative to a naturally occurring reference agent.

By “neoplasia” is meant any disease that is caused by or results in inappropriately high levels of cell division, inappropriately low levels of apoptosis, or both. For example, cancer is a neoplasia. Examples of cancers include, without limitation, leukemias (e.g., acute leukemia, acute lymphocytic leukemia, acute myelocytic leukemia, acute myeloblastic leukemia, acute promyelocytic leukemia, acute myelomonocytic leukemia, acute monocytic leukemia, acute erythroleukemia, chronic leukemia, chronic myelocytic leukemia, chronic lymphocytic leukemia), polycythemia vera, lymphoma (Hodgkin's disease, non-Hodgkin's disease), Waldenstrom's macroglobulinemia, heavy chain disease, and solid tumors such as sarcomas and carcinomas (e.g., fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, uterine cancer, testicular cancer, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, schwannoma, meningioma, melanoma, neuroblastoma, and retinoblastoma). Lymphoproliferative disorders are also considered to be proliferative diseases. In certain preferred embodiments, the neoplasia is pancreatic cancer, prostate cancer or lymphoma.

By “mature form” is meant a microRNA that has, at least in part, been processed into a biologically active form that can participate in the regulation of a target mRNA.

By “hairpin form” is meant a microRNA that includes a double stranded portion.

By “microRNA” is meant a nucleobase sequence having biological activity that is independent of any polypeptide encoding activity. MicroRNAs may be synthetic or naturally occurring, and may include one or more modifications described herein. MicroRNAs include pri-microRNAs, hairpin microRNAs, and mature microRNAs.

5 By “Myc deregulation” is meant an alteration in the level of expression of one or more microRNAs usually repressed by Myc.

By “nucleic acid” is meant an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid, or analog thereof. This term includes oligomers consisting of naturally occurring bases, sugars, and intersugar (backbone) linkages as well as oligomers
10 having non-naturally occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example, enhanced stability in the presence of nucleases.

By “obtaining” as in “obtaining the inhibitory nucleic acid molecule” is meant synthesizing, purchasing, or otherwise acquiring the inhibitory nucleic acid molecule.

15 By “oligonucleotide” is meant any molecule comprising a nucleobase sequence. An oligonucleotide may, for example, include one or more modified bases, linkages, sugar moieties, or other modifications.

By “operably linked” is meant that a first polynucleotide is positioned adjacent to a second polynucleotide that directs transcription of the first polynucleotide when appropriate
20 molecules (e.g., transcriptional activator proteins) are bound to the second polynucleotide.

By “positioned for expression” is meant that the polynucleotide of the invention (e.g., a DNA molecule) is positioned adjacent to a DNA sequence that directs transcription and translation of the sequence (i.e., facilitates the production of, for example, a recombinant microRNA molecule described herein).

25 “Primer set” or “probe set” means a set of oligonucleotides. A primer set may be used, for example, for the amplification of a polynucleotide of interest. A probe set may be used, for example, to hybridize with a polynucleotide of interest. A primer set would consist of at least 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 30, 40, 50, 60, 80, 100, or more primers or probes.

By “fragment” is meant a portion of a polypeptide or nucleic acid molecule. This
30 portion contains, preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the entire length of the reference nucleic acid molecule or polypeptide. A fragment may contain 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 nucleotides.

By “reduces” is meant a negative alteration. A reduction includes, for example, a 5%, 10%, 25%, 50%, 75% or even 100% reduction.

By “reduces the survival” is meant increases the probability of cell death in a cell or population of cells relative to a reference. For example, a reduction in survival is measured in a cell treated with a microRNA of the invention relative to an untreated control cell. Cell death may be by any means, including apoptotic or necrotic cell death.

5 By “reduces cell division” is meant interferes with the cell cycle or otherwise reduces the growth or proliferation of a cell, tissue, or organ relative to a reference. For example, a reduction in cell division is measured in a cell treated with a microRNA of the invention relative to an untreated control cell.

By “reference” is meant a standard or control condition.

10 By “reporter gene” is meant a gene encoding a polypeptide whose expression may be assayed; such polypeptides include, without limitation, glucuronidase (GUS), luciferase, chloramphenicol transacetylase (CAT), and beta-galactosidase.

The term “siRNA” refers to small interfering RNA; a siRNA is a double stranded RNA that “corresponds” to or matches a reference or target gene sequence. This matching
15 need not be perfect so long as each strand of the siRNA is capable of binding to at least a portion of the target sequence. SiRNA can be used to inhibit gene expression, see for example Bass, 2001, Nature, 411, 428 429; Elbashir et al., 2001, Nature, 411, 494 498; and Zamore et al., Cell 101:25-33 (2000).

The term “subject” is intended to include vertebrates, preferably a mammal.

20 Mammals include, but are not limited to, humans.

The term “pharmaceutically-acceptable excipient” as used herein means one or more compatible solid or liquid filler, diluents or encapsulating substances that are suitable for administration into a human.

By “transformed cell” is meant a cell into which (or into an ancestor of which) has
25 been introduced, by means of recombinant DNA techniques, a polynucleotide molecule encoding (as used herein) a protein of the invention.

By “vector” is meant a nucleic acid molecule, for example, a plasmid, cosmid, or bacteriophage, that is capable of replication in a host cell. In one embodiment, a vector is an expression vector that is a nucleic acid construct, generated recombinantly or synthetically,
30 bearing a series of specified nucleic acid elements that enable transcription of a nucleic acid molecule in a host cell. Typically, expression is placed under the control of certain regulatory elements, including constitutive or inducible promoters, tissue-preferred regulatory elements, and enhancers.

In one embodiment, nucleic acid molecules useful in the methods of the invention include any nucleic acid molecule that encodes a polypeptide of the invention or a fragment thereof. In another embodiment, nucleic acid molecules useful in the methods of the invention include any nucleic acid molecule that encodes a polynucleotide (e.g., a
5 microRNA) that has biologic activity independent of providing a polypeptide sequence. Such nucleic acid molecules need not be 100% identical with an endogenous nucleic acid sequence, but will typically exhibit substantial identity. Polynucleotides having "substantial identity" to an endogenous sequence are typically capable of hybridizing with at least one strand of a double-stranded nucleic acid molecule. By "hybridize" is meant pair to form a
10 double-stranded molecule between complementary polynucleotide sequences (e.g., a gene described herein), or portions thereof, under various conditions of stringency. (See, e.g., Wahl, G. M. and S. L. Berger (1987) *Methods Enzymol.* 152:399; Kimmel, A. R. (1987) *Methods Enzymol.* 152:507).

For example, stringent salt concentration will ordinarily be less than about 750 mM
15 NaCl and 75 mM trisodium citrate, preferably less than about 500 mM NaCl and 50 mM trisodium citrate, and more preferably less than about 250 mM NaCl and 25 mM trisodium citrate. Low stringency hybridization can be obtained in the absence of organic solvent, e.g., formamide, while high stringency hybridization can be obtained in the presence of at least about 35% formamide, and more preferably at least about 50% formamide. Stringent
20 temperature conditions will ordinarily include temperatures of at least about 30° C, more preferably of at least about 37° C, and most preferably of at least about 42° C. Varying additional parameters, such as hybridization time, the concentration of detergent, e.g., sodium dodecyl sulfate (SDS), and the inclusion or exclusion of carrier DNA, are well known to those skilled in the art. Various levels of stringency are accomplished by combining these
25 various conditions as needed. In a preferred embodiment, hybridization will occur at 30° C in 750 mM NaCl, 75 mM trisodium citrate, and 1% SDS. In a more preferred embodiment, hybridization will occur at 37° C in 500 mM NaCl, 50 mM trisodium citrate, 1% SDS, 35% formamide, and 100 µg/ml denatured salmon sperm DNA (ssDNA). In a most preferred embodiment, hybridization will occur at 42° C in 250 mM NaCl, 25 mM trisodium citrate,
30 1% SDS, 50% formamide, and 200 µg/ml ssDNA. Useful variations on these conditions will be readily apparent to those skilled in the art.

For most applications, washing steps that follow hybridization will also vary in stringency. Wash stringency conditions can be defined by salt concentration and by temperature. As above, wash stringency can be increased by decreasing salt concentration or

by increasing temperature. For example, stringent salt concentration for the wash steps will preferably be less than about 30 mM NaCl and 3 mM trisodium citrate, and most preferably less than about 15 mM NaCl and 1.5 mM trisodium citrate. Stringent temperature conditions for the wash steps will ordinarily include a temperature of at least about 25° C, more preferably of at least about 42° C, and even more preferably of at least about 68° C. In a preferred embodiment, wash steps will occur at 25° C in 30 mM NaCl, 3 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 42.degree. C. in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 68° C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. Additional variations on these conditions will be readily apparent to those skilled in the art. Hybridization techniques are well known to those skilled in the art and are described, for example, in Benton and Davis (Science 196:180, 1977); Grunstein and Hogness (Proc. Natl. Acad. Sci., USA 72:3961, 1975); Ausubel et al. (Current Protocols in Molecular Biology, Wiley Interscience, New York, 2001); Berger and Kimmel (Guide to Molecular Cloning Techniques, 1987, Academic Press, New York); and Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.

By "substantially identical" is meant a polypeptide or nucleic acid molecule exhibiting at least 50% identity to a reference amino acid sequence (for example, any one of the amino acid sequences described herein) or nucleic acid sequence (for example, any one of the nucleic acid sequences described herein). Preferably, such a sequence is at least 60%, more preferably 80% or 85%, and more preferably 90%, 95% or even 99% identical at the amino acid level or nucleic acid to the sequence used for comparison.

Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, BLAST, BESTFIT, GAP, or PILEUP/PRETTYBOX programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. In an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between e^{-3} and e^{-100} indicating a closely related sequence.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 (a – d) shows Myc enhances the expression of mitochondrial protein glutaminase. a) shows the expanded insets of two-dimensional gels reveal the induction of glutaminase (GLS, highlighted by white circles) by Myc in P493-6 B cells. For each condition, 350 pg of mitochondrial protein lysate was resolved on 18 cm immobilized pH gradient strips as the first dimension followed by 10% Bis-Tris SDS-PAGE as the second dimension, which is marked by molecular mass markers. Protein spots were visualized by silver staining. Six independent biological experiments were performed for each condition, where Table 1 summarizes the identity of the spots with the same numbering system as depicted in the figure. b) shows that immunoblot with anti-GLS antibody of a one-dimensional SDS-PAGE gel of mitochondrial proteins (20 pg per lane) validates the induction of GLS by Myc discovered in a. TFAM represents a control mitochondrial protein. c) P493-6 cells were treated with tetracycline (Tet) for different lengths of time to inhibit Myc expression or were treated first with tetracycline for 48 h and then washed (Wash) to remove tetracycline, with the times after wash-out indicated. Cells were then harvested for immunoblot assay for GLS or c-Myc. Anti-tubulin antibody and anti-TFAM were used for loading controls. d) Human CB33 lymphoblastoid cells, CB33 -Myc cells and PC3 cells transfected with siRNA against c-Myc (MYC siRNA) or control siRNA (Ctrl siRNA) were used for immunoblot assays. Experiments were replicated with similar results.

Figure 2 (a – e) shows glutamine and glutaminase are necessary for Myc-mediated cancer cell proliferation and survival. a), top panel is immunoblots showing that GLS protein level is diminished by transfecting cells with siRNA for GLS1 (GLS siRNA,) as compared with non transfection (No tx) or control siRNA, (Ctrl siRNA). The bottom panel shows growth inhibition of P493 and PC3 cells by GLS siRNA.. The results shown are mean $\pm$ s.d, n = 3. b) shows growth inhibition of P493 and PC3 cells cultured under control, glucose- or glutamine-deprived conditions. The results shown are mean $\pm$ s.d., n = 3. c) shows cells were cultured with normal medium or medium without glucose ((---)Gluc) or glutamine ((—)Q) for 48h and harvested for ATP assay as described in Methods. The results shown (mean $\pm$ s.d., n = 2) are relative ATP levels per microgram total protein normalized to the control (Ctrl) normal medium group. d) shows ATP levels in control cells or cells transfected with GUS siRNA or control siRNA. Seventy-two hours after transfection, cells were harvested for ATP assay. The results shown (mean $\pm$ n = 2) are relative ATP levels per microgram total protein normalized to the non-transfected control group. e) shows cells were transfected with GLS siRNA or control siRNA and cultured with 10 mM N-acetylcysteine (NAC), or 5 mM

oxaloacetate (OAA), as indicated. The left panel shows cell counts (relative units (RU); mean $\pm$ s.d., $n = 5$) of different groups at 72 h after transfection (see Fig. 8 for the complete cell growth curve). The right panel shows the percentage cell death at 72h after transfection.

Percentage cell death indicates annexin-positive plus annexin V and 7-AAD-positive cells.

- 5 Primary data are shown in Fig. 7. All experiments in Fig. 2 were repeated at least twice. All experiments with P493 cells were in the absence of tetracycline. Asterisk denotes mean ($\pm$ s.d.) that is significantly different ($P < 0.05$, t test).

Figure 3 shows Myc increases GLS protein by transcriptionally repressing miR-23a/b that target the GLS 3' UTR. a) shows GLS1 mRNA levels were determined by real-time PCR after treatment of P493 cells with tetracycline or on removal of tetracycline (after 10 48 h of tetracycline pre-treatment). Data are mean $\pm$ s.d., $n = 3$ PCR reactions. b, Northern blot analysis of miR-23a/b expression in P493 cells treated with or without tetracycline for 24h and then transfected with miR-23a/b TN As or scrambled control LNA and cultured for 48 h. c) shows chromatin immunoprecipitation assay with P493 cells showing Myc binding to the 15 promoter region of *C9orf3*, whose transcript is processed to miR-23b. The positions of the amplicons are depicted in the cartoon of the *C9orf3* gene below the bar graphs (mean $\pm$ s.d., $n = 3$). Anti I-IGF serves as a nonspecific antibody control. d) shows inhibition of GLS 3' UTR luciferase reporter by miR-23a/b. Top: glutaminase reporter (wild-type GLS 3' UTR or mutant Mut-GLS 3' UTR.) or control (PGL3) luciferase constructs were co-transfected with 20 pSV-Renilla into MCP-7 cells, or further co-transfected with miR-23a/b LNAs or control LNA. After 24h, luciferase activities (relative light units, RLU) were measured. Data shown are RLUs normalized to the control group (mean $\pm$ s.d., $n = 4$). Bottom: miR-23a, miR-23b, GLS 3' UTR and Mut-GLS 3' UTR sequences. e) shows analysis of GLS protein levels in P493 and PC3 cells treated with control or antisense miR-23a/b LNAs. Left: P493 cells were 25 treated with or -without tetracycline for 24 h and then transfected with antisense miR-23a/b LNAs or scrambled control LNA. After 72 h, cells were harvested for immunoblot assay. Right: PC3 cells were transfected with MYC siRNA or control siRNA. After 24h, cells were transfected with miR-23a/b LNAs or scrambled control. Cells were cultured for 72 h and then were harvested for immunoblot assay.

- 30 Figure 4 is a graph showing GLS1, not GLS2, is predominantly expressed in P493 and PC3 cells as determined by real-time quantitative PCR. HT-29 cells, a colorectal cancer cell line known to express GLS1 and GLS2, was used as a control. Data shown are mean $\pm$ SD, $n=3$ PCR reaction.

Figure 5 (a – f) shows ectopic GLS1 overexpression in PC3 cells did not rescue the diminished growth rate with siRNA-mediated reduction of c-Myc. a) shows GLS1 overexpressing cell line was established by transfection of retroviral vector PQCXIN-GLS1 in PC3 cells. PQCXIN-GFP vector was used as a control. Note that the cloned GLS1 sequence includes the coding sequence and partial 3'UTR but does not include the predicted binding sites, or the seed sequence, for miR-23. Immunoblot shows the ectopic expression of GLS1, with tubulin as a loading control. b) ATP levels in parental PC3, PC3-GFP control and PC-GLS1 cells were detected as described in Materials and Methods section. c) There is no significant difference in the growth of parental PC3, PC3-GFP control and PC-GLS1 cells. Data shown are mean $\pm$ SD, n=2 d) PC3 cells (parental, PQCXIN-GFP and PGCXIN-GLS) were untreated (No tx) or transfected with siRNA against c-Myc (siMyc) or control siRNA (siCont). After 4 days, cells were collected for immunoblot for c-Myc and GLS. Tubulin was also blotted as a loading control. e) ATP levels in PC3-GFP control and PC-GLS1 cells when cells were transfected with control siRNA (siCont) or siRNA against c-Myc. Relative ATP levels per microgram total protein (mean $\pm$ SD) normalized to the siCont transfected control PC3-GFP cells are shown. S2f. The growth curves of PC3 cells, parental (PC3), PQCXIN-GFP (GFP) and PGCXIN-GLS (GLS), when c-Myc was suppressed by siRNA (siMyc) transfection as compared with untreated control (No Tx) or control siRNA (siCont). c-Myc and GLS expression was confirmed in d) The results indicated that ectopic GLS1 overexpression in PC3 cells did not have significant effect on cell growth (mean $\pm$ SD, n=2) when c-Myc expression was suppressed by siRNA.

Figure 6 shows depletion of GLS or glutamine decreases oxygen (O₂) consumption. The curves in a) show the decrease of O₂ concentration under different conditions, while the bar graph in b) is derived from a), representing the normalized rate of O₂ consumption. Data shown are means $\pm$ SD, n=3 measurements. (*) denotes mean ($\pm$ SD) that is significantly different ($P < 0.05$ by t test). c) shows flow cytometry data document that reactive oxygen species (ROS) production as measured with DCF fluorescence was increased in P493 cells when cells were transfected with siGLS or cultured under glutamine-deprived conditions. The increased ROS was attenuated by N-acetylcysteine (NAC). The experiments were repeated twice with similar results.

Figure 7 shows depletion of GLS or glutamine decreases glutathione. Flow cytometry data generated by bromobimane staining of glutathione showed that glutathione production was decreased in P493 and PC3 cells when cells were transfected with siGLS or cultured under glutamine-deprived conditions. Glucose deprivation or control siRNA (siCont)

serves as controls. Experiments were repeated twice with similar results. Refer to the companion Table 3 for statistical analysis.

Figure 8 shows the results of partial rescue of diminished GLS or glutamine-deprivation induced cell death by oxaloacetate (OAA) or N-acetylcysteine (NAC). Primary flow cytometry data for P493-6 cell death as detected by using a Annexin V-7AAD staining kit. FL2 and FL3 represent Annexin V and 7-AAD fluorescence, respectively in the dot plot figures. Control cells (siCont or Control) were compared with those treated with siRNA against GLS (siGLS) or deprived of glutamine ((-)Q)). Experiments were replicated with similar results.

Figure 9 (a – c) shows rescue of inhibition of cell proliferation by diminished GLS or glutamine-deprivation inhibition with oxaloacetate (OAA) or N-acetylcysteine (NAC). a) P493-6 cells were transfected with siRNA for GLS (siGLS) or control siRNA (siCont) and cultured with 10mM N-acetylcysteine (NAC), or 5mM oxaloacetate (OAA), or no addition control. Cell counts were performed every 24h and presented as means $\pm$ SD, n = 3. b) and c) cells cultured with (Control) or without glutamine ((-)Q)) were treated with OAA or NAC for 4 days and cell counts were performed every 24h and presented as means $\pm$ SD, n = 4.

Figure 10 shows the results of Taqman MicroRNA assay of miR-23a and miR-23b expression in P493 cells. P493 cells were treated with or without tetracycline (Tet) for different time points, or treated with tetracycline (Tet) for 48h followed by removal of Tet (Wash) and continued to culture for different time points. Cells were collected for RNA purification using a QIAGEN miRNeasy mini kit. Real-time PCR was performed using a TaqMan MicroRNA Assay kit. The results (mean $\pm$ SD, n = 3 PCR reactions) were normalized using U6 snRNA as control.

Figure 11 shows the results of Taqman MicroRNA assay of miR-23a and miR-23b expression in P493-6, CB33 and PC3 cells. Tetracycline (tet); siRNA against Myc treated sample (siMYC); siRNA against GLS treated sample (siGLS). The data shown are mean $\pm$ SD, n = 3 PCR reactions. (*) denotes mean ($\pm$ SD,) that is significantly different from the control (P < 0.05 by t test).

Figure 12 shows c-Myc knockdown by siRNA in PC3 cells suppresses GLS-3'UTR luciferase reporter activity. a) shows Western blot showing c-Myc knockdown by siRNA in PC3 cells. Tubulin was blotted as a loading control. S9b. Luciferase activity (RLU = relative light unit) was measured as described in Figure 3d. PC3 cells were transfected with control siRNA (siCont) or siRNA against c-Myc (siMyc). After 48h, cells were seeded in 48-well

plates and incubated overnight to allow cells to attach. Then cells were co-transfected with wild-type or mutant GLS-3'UTR or control PGL3 vectors, and pSV Renilla vector. After 24h, cells were collected for luciferase activity measurement. The results shown are mean $\pm$ SD, n=3 measurements. * denotes mean ($\pm$ SD) that is significantly different ($P < 0.05$ by t test).

Figure 13 shows the correlation between Myc and GLS protein levels in primary human prostate cancer (T) and paired normal prostate (N) samples (each patient sample is shown numbered at the top). Immunoblots were performed after polypeptide separation in a 10% polyacrylamide SDS gel. Tubulin serves as a sample loading control.

DETAILED DESCRIPTION OF THE INVENTION

The present invention reports that the c-Myc oncogenic transcription factor, which is known to regulate microRNAs (7,8) and stimulate cell proliferation (9), transcriptionally represses miR-23a and miR-23b, resulting in greater expression of their target protein, mitochondrial glutaminase, in human P-493 B lymphoma cells and PC3 prostate cancer cells. This leads to upregulation of glutamine catabolism (10). Glutaminase converts glutamine to glutamate, which is further catabolized through the tricarboxylic acid cycle for the production of ATP or serves as substrate for glutathione synthesis (11). The present invention reports a unique means by which Myc regulates glutaminase and uncovers a previously unsuspected link between Myc regulation of miRNAs, glutamine metabolism, and energy and reactive oxygen species homeostasis.

As reported in more detail below the present inventors describe a pathway by which Myc suppression of miR-23a/b, which target GLS, enhances glutamine catabolism through increased mitochondrial glutaminase expression. Therefore, the expression of one or more of these Myc-repressed microRNAs or a fragment thereof, is expected to be useful for the treatment or prevention of a neoplasia. Further, the repression of one or more of these Myc-repressed microRNAs or a fragment thereof, is expected to be useful for the treatment or prevention of ischemic cell death, such as cardiac ischemia or stroke. Taken together, these observations provide a regulatory mechanism involving Myc and miRNAs for elevated expression of glutaminase and glutamine metabolism in human cancers.

MicroRNAs

MicroRNAs are small noncoding RNA molecules that are capable of causing post-transcriptional silencing of specific genes in cells by the inhibition of translation or through

degradation of the targeted mRNA. A microRNA can be completely complementary or can have a region of noncomplementarity with a target nucleic acid, consequently resulting in a "bulge" at the region of non-complementarity. A microRNA can inhibit gene expression by repressing translation, such as when the microRNA is not completely complementary to the target nucleic acid, or by causing target RNA degradation, which is believed to occur only when the microRNA binds its target with perfect complementarity. The invention also can include double-stranded precursors of microRNA.

A microRNA or pre- microRNA can be 18-100 nucleotides in length, and more preferably from 18-80 nucleotides in length. Mature miRNAs can have a length of 19-30 nucleotides, preferably 21-25 nucleotides, particularly 21, 22, 23, 24, or 25 nucleotides. MicroRNA precursors typically have a length of about 70-100 nucleotides and have a hairpin conformation. MicroRNAs are generated *in vivo* from pre-miRNAs by the enzymes Dicer and Drosha, which specifically process long pre-miRNA into functional miRNA. The hairpin or mature microRNAs, or pre- microRNA agents featured in the invention can be synthesized *in vivo* by a cell-based system or *in vitro* by chemical synthesis.

The invention provides isolated microRNAs and polynucleotides encoding such sequences. A recombinant microRNA of the invention (e.g., miR-23a or miR-23b) or a polynucleotide encoding such a microRNA may be administered to reduce the growth, survival, or proliferation of a neoplastic cell in a subject in need thereof. In one approach, the microRNA is administered as a naked RNA molecule. In another approach, it is administered in an expression vector suitable for expression in a mammalian cell.

Alternatively, an antisense microRNA, (e.g., miR-23a or miR-23b) or a polynucleotide encoding such a microRNA may be administered to protect against ischemic cell death in a subject in need of treatment. In one approach, the antisense microRNA is administered as a naked RNA molecule. In another approach, it is administered in an expression vector suitable for expression in a mammalian cell.

One exemplary approach provided by the invention involves administration of a recombinant therapeutic, such as a recombinant microRNA molecule, variant, or fragment thereof, either directly to the site of a potential or actual disease-affected tissue or systemically (for example, by any conventional recombinant administration technique). The dosage of the administered microRNA depends on a number of factors, including the size and health of the individual patient. For any particular subject, the specific dosage regimes should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions.

For example, a microRNA of the invention (e.g., miR-23a or miR-23b) may be administered in dosages between about 1 and 100 mg/kg (e.g., 1, 5, 10, 20, 25, 50, 75, and 100 mg/kg). In other embodiments, the dosage ranges from between about 25 and 500 mg/m²/day. Desirably, a human patient having a neoplasia receives a dosage between about 50 and 300 mg/m²/day (e.g., 50, 75, 100, 125, 150, 175, 200, 250, 275, and 300).

MicroRNAs can be synthesized to include a modification that imparts a desired characteristic. For example, the modification can improve stability, hybridization thermodynamics with a target nucleic acid, targeting to a particular tissue or cell-type, or cell permeability, e.g., by an endocytosis-dependent or -independent mechanism. Modifications can also increase sequence specificity, and consequently decrease off-site targeting. Methods of synthesis and chemical modifications are described in greater detail below.

The invention further provides solid supports, including microarrays, comprising one, two, three, four, five, six or more microRNAs, oligonucleotides comprising such microRNAs, or nucleic acid sequences encoding or binding to such microRNAs. In addition, the invention provides probes that hybridize to and/or that may be used to amplify a microRNA of the invention. In particular embodiments, the invention provides collections of such probes that include one, two, three, four, or more microRNAs or probes described herein.

MicroRNA Analogs

If desired, microRNA molecules may be modified to stabilize the microRNAs against degradation, to enhance half-life, or to otherwise improve efficacy. Desirable modifications are described, for example, in U.S. Patent Publication Nos. 20070213292, 20060287260, 20060035254, 20060008822, and 20050288244, each of which is hereby incorporated by reference in its entirety.

For increased nuclease resistance and/or binding affinity to the target, the single-stranded oligonucleotide agents featured in the invention can include 2'-O-methyl, 2'-fluorine, 2'-O-methoxyethyl, 2'-O-aminopropyl, 2'-amino, and/or phosphorothioate linkages. Inclusion of locked nucleic acids (LNA), ethylene nucleic acids (ENA), e.g., 2'-4'-ethylene-bridged nucleic acids, and certain nucleobase modifications can also increase binding affinity to the target. The inclusion of pyranose sugars in the oligonucleotide backbone can also decrease endonucleolytic cleavage. An antagomir can be further modified by including a 3' cationic group, or by inverting the nucleoside at the 3'-terminus with a 3'-3' linkage. In another alternative, the 3'-terminus can be blocked with an aminoalkyl group. Other 3' conjugates

can inhibit 3'-5' exonucleolytic cleavage. While not being bound by theory, a 3' may inhibit exonucleolytic cleavage by sterically blocking the exonuclease from binding to the 3' end of the oligonucleotide. Even small alkyl chains, aryl groups, or heterocyclic conjugates or modified sugars (D-ribose, deoxyribose, glucose etc.) can block 3'-5'-exonucleases.

5 In one embodiment, the microRNA includes a 2'-modified oligonucleotide containing oligodeoxynucleotide gaps with some or all internucleotide linkages modified to phosphorothioates for nuclease resistance. The presence of methylphosphonate modifications increases the affinity of the oligonucleotide for its target RNA and thus reduces the IC₅₀. This modification also increases the nuclease resistance of the modified oligonucleotide. It is
10 understood that the methods and reagents of the present invention may be used in conjunction with any technologies that may be developed to enhance the stability or efficacy of an inhibitory nucleic acid molecule.

MicroRNA molecules include nucleobase oligomers containing modified backbones or non-natural internucleoside linkages. Oligomers having modified backbones include those
15 that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone are also considered to be nucleobase oligomers. Nucleobase oligomers that have modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates,
20 phosphotriesters, aminoalkyl-phosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates. Various salts, mixed salts and free acid forms are also included. Representative United States patents that teach the preparation of
25 the above phosphorus-containing linkages include, but are not limited to, U.S. Pat. Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050, each of which is herein incorporated by reference.

30 Nucleobase oligomers having modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a

nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed

5 N, O, S and CH₂ component parts. Representative United States patents that teach the preparation of the above oligonucleotides include, but are not limited to, U.S. Pat. Nos.

5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312;

10 5,633,360; 5,677,437; and 5,677,439, each of which is herein incorporated by reference.

Nucleobase oligomers may also contain one or more substituted sugar moieties. Such modifications include 2'-O-methyl and 2'-methoxyethoxy modifications. Another desirable modification is 2'-dimethylaminoethoxy, 2'-aminopropoxy and 2'-fluoro. Similar modifications may also be made at other positions on an oligonucleotide or other nucleobase

15 oligomer, particularly the 3' position of the sugar on the 3' terminal nucleotide. Nucleobase oligomers may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 20 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, each of which is herein incorporated by reference in its entirety.

In other nucleobase oligomers, both the sugar and the internucleoside linkage, i.e., the backbone, are replaced with novel groups. The nucleobase units are maintained for hybridization with a nucleic acid molecule of the miR-17-92 cluster. Methods for making and using these nucleobase oligomers are described, for example, in "Peptide Nucleic Acids (PNA): Protocols and Applications" Ed. P. E. Nielsen, Horizon Press, Norfolk, United Kingdom, 1999. Representative United States patents that teach the preparation of PNAs include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found 30 in Nielsen et al., Science, 1991, 254, 1497-1500.

In other embodiments, a single stranded modified nucleic acid molecule (e.g., a nucleic acid molecule comprising a phosphorothioate backbone and 2'-O-Me sugar modifications is conjugated to cholesterol.

RNA interference (RNAi)

RNAi is a method for decreasing the cellular expression of specific proteins of interest (reviewed in Tuschl, *ChemBiochem* 2:239-245, 2001; Sharp, *Genes & Devel.* 15:485-490, 2000; Hutvagner and Zamore, *Curr. Opin. Genet. Devel.* 12:225-232, 2002; and Hannon, *Nature* 418:244-251, 2002). In RNAi, gene silencing is typically triggered post-transcriptionally by the presence of double-stranded RNA (dsRNA) in a cell. This dsRNA is processed intracellularly into shorter pieces called small interfering RNAs (siRNAs). The introduction of siRNAs into cells either by transfection of dsRNAs or through expression of shRNAs using a plasmid-based expression system is currently being used to create loss-of-function phenotypes in mammalian cells. The present invention contemplates siRNAs that target miR-23a/b and that block miR-23a/b. Accordingly, by blocking miR-23a/b the result is a greater expression of mitochondrial Glutaminase (GLS) which results in an upregulation of glutamine catabolism, and glutamine is transported into proliferating cells.

Inhibitory Nucleic Acid Molecules

Inhibitory nucleic acid molecules are essentially nucleobase oligomers that may be employed as single-stranded or double-stranded nucleic acid molecule to decrease miR-23a/b expression. In certain embodiments, an inhibitory nucleic acid may increase GLS expression.

In certain preferred embodiments, the present invention features an inhibitory nucleic acid molecule that corresponds to or is complementary to at least a fragment of a microRNA selected from miR-23a or miR-23b, or a fragment thereof, and that increases expression of mitochondrial glutaminase in the cell.

The present invention also features an inhibitory nucleic acid molecule that corresponds to or is complementary to at least a fragment of a microRNA selected from miR-23a or miR-23b, or a fragment thereof, wherein expression of said inhibitory nucleic acid molecule in a cell protects the cell from ischemic cell death.

The nucleic acid molecule can be single stranded or double stranded.

In one approach, for example, the inhibitory nucleic acid molecule is a double-stranded RNA used for RNA interference (RNAi)-mediated knock-down of gene expression.

In one embodiment, a double-stranded RNA (dsRNA) molecule is made that includes between eight and twenty-five (e.g., 8, 10, 12, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25) consecutive nucleobases of a nucleobase oligomer of the invention. The dsRNA can be two complementary strands of RNA that have duplexed, or a single RNA strand that has self-duplexed (small hairpin (sh)RNA). Typically, dsRNAs are about 21 or 22 base pairs, but

may be shorter or longer (up to about 29 nucleobases) if desired. Double stranded RNA can be made using standard techniques (e.g., chemical synthesis or in vitro transcription). Kits are available, for example, from Ambion (Austin, Tex.) and Epicentre (Madison, Wis.).

Methods for expressing dsRNA in mammalian cells are described in Brummelkamp et al.

5 Science 296:550-553, 2002; Paddison et al. Genes & Devel. 16:948-958, 2002. Paul et al. Nature Biotechnol. 20:505-508, 2002; Sui et al. Proc. Natl. Acad. Sci. USA 99:5515-5520, 2002; Yu et al. Proc. Natl. Acad. Sci. USA 99:6047-6052, 2002; Miyagishi et al. Nature Biotechnol. 20:497-500, 2002; and Lee et al. Nature Biotechnol. 20:500-505 2002, each of which is hereby incorporated by reference. An inhibitory nucleic acid molecule that
10 “corresponds” to an gene comprises at least a fragment of the double-stranded gene, such that each strand of the double-stranded inhibitory nucleic acid molecule is capable of binding to the complementary strand of the target gene. The inhibitory nucleic acid molecule need not have perfect correspondence to the reference sequence. In one embodiment, an siRNA has at least about 85%, 90%, 95%, 96%, 97%, 98%, or even 99% sequence identity with the target
15 nucleic acid. For example, a 19 base pair duplex having 1-2 base pair mismatch is considered useful in the methods of the invention. In other embodiments, the nucleobase sequence of the inhibitory nucleic acid molecule exhibits 1, 2, 3, 4, 5 or more mismatches.

In certain exemplary embodiments, the single stranded inhibitory nucleic acid is an antisense nucleic acid molecule. In other exemplary embodiments, the double-stranded
20 nucleic acid molecule is an siRNA. In other certain exemplary embodiments, the double-stranded nucleic acid molecule is an shRNA. Preferably, each strand of the double-stranded nucleic acid molecule is about 19-21 nucleotides in length.

In certain preferred embodiments of the invention, siRNAs target human *GLS1* or *MYC*. Exemplary target sequences are (human *GLS1*) CCUGAAGCAGUUCGAAAUA,
25 CUGAAUAUGUGCAUCGAUA, AGAAAGUGGAGAUCGAAAU and GCACAGACAUGGUUGGUAU, and (*MYC*) ACGGAACUCUUGUGCGUAAUU, GAACACACAACGUCUUGGAUU, AACGUUAGCUUCACCAACAUAU and CGAUGUUGUUUCUGUGGAAUU.

In other preferred embodiments of the invention miR-23a/b is knowcked down with
30 anti-sense locked nucleic acid (LNA) oligomers. Exemplary LNA knockdown probes for miR-23a (miRCURY knockdown, 118119-00 target sequence) is AUCACAUUGCCAGGGAUUACC) and miR-23b (miRCURY knockdown, 138120-00, target sequence) is AUCACAUUGCCAGGGAUUACC).

The inhibitory nucleic acid molecules provided by the invention are not limited to siRNAs, but include any nucleic acid molecule sufficient to decrease the expression of target nucleic acid molecule or polypeptide. Each of the DNA sequences provided herein may be used, for example, in the discovery and development of therapeutic antisense nucleic acid molecule to decrease the expression of miR-23a/b or GLS. The invention further provides catalytic RNA molecules or ribozymes. Such catalytic RNA molecules can be used to inhibit expression of a nucleic acid molecule in vivo. The inclusion of ribozyme sequences within an antisense RNA confers RNA-cleaving activity upon the molecule, thereby increasing the activity of the constructs. The design and use of target RNA-specific ribozymes is described in Haseloff et al., *Nature* 334:585-591. 1988, and U.S. Patent Application Publication No. 2003/0003469 A1, each of which is incorporated by reference. In various embodiments of this invention, the catalytic nucleic acid molecule is formed in a hammerhead or hairpin motif. Examples of such hammerhead motifs are described by Rossi et al., *Aids Research and Human Retroviruses*, 8:183, 1992. Example of hairpin motifs are described by Hampel et al., "RNA Catalyst for Cleaving Specific RNA Sequences," filed Sep. 20, 1989, which is a continuation-in-part of U.S. Ser. No. 07/247,100 filed Sep. 20, 1988, Hampel and Tritz, *Biochemistry*, 28:4929, 1989, and Hampel et al., *Nucleic Acids Research*, 18: 299, 1990. These specific motifs are not limiting in the invention and those skilled in the art will recognize that all that is important in an enzymatic nucleic acid molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule.

Modified Inhibitory Nucleic Acid Molecules

A desirable inhibitory nucleic acid molecule is one based on 2'-modified oligonucleotides containing oligodeoxynucleotide gaps with some or all internucleotide linkages modified to phosphorothioates for nuclease resistance. The presence of methylphosphonate modifications increases the affinity of the oligonucleotide for its target RNA and thus reduces the IC₅₀. This modification also increases the nuclease resistance of the modified oligonucleotide. It is understood that the methods and reagents of the present invention may be used in conjunction with any technologies that may be developed to enhance the stability or efficacy of an inhibitory nucleic acid molecule.

Inhibitory nucleic acid molecules include nucleobase oligomers containing modified backbones or non-natural internucleoside linkages. Oligomers having modified

backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone are also considered to be nucleobase oligomers. Nucleobase oligomers that have modified

5 oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkyl-phosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates. Various
10 salts, mixed salts and free acid forms are also included. Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Pat. Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253;
15 5,571,799; 5,587,361; and 5,625,050, each of which is herein incorporated by reference.

Nucleobase oligomers having modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These
20 include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed
25 N, O, S and CH₂ component parts. Representative United States patents that teach the preparation of the above oligonucleotides include, but are not limited to, U.S. Pat. Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312;
30 5,633,360; 5,677,437; and 5,677,439, each of which is herein incorporated by reference.

Nucleobase oligomers may also contain one or more substituted sugar moieties. Such modifications include 2'-O-methyl and 2'-methoxyethoxy modifications. Another desirable modification is 2'-dimethylaminoethoxy, 2'-aminopropoxy and 2'-fluoro. Similar modifications may also be made at other positions on an oligonucleotide or other nucleobase

oligomer, particularly the 3' position of the sugar on the 3' terminal nucleotide. Nucleobase oligomers may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, each of which is herein incorporated by reference in its entirety. In other nucleobase oligomers, both the sugar and the internucleoside linkage, i.e., the backbone, are replaced with novel groups. The nucleobase units are maintained for hybridization with a desired nucleic acid molecule (e.g. miR-23 or GLS). Methods for making and using these nucleobase oligomers are described, for example, in "Peptide Nucleic Acids (PNA): Protocols and Applications" Ed. P. E. Nielsen, Horizon Press, Norfolk, United Kingdom, 1999. Representative United States patents that teach the preparation of PNAs include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., Science, 1991, 254, 1497-1500.

Delivery of Nucleobase Oligomers

A microRNA of the invention, which may be in the mature or hairpin form, may be provided as a naked oligonucleotide that is capable of entering a tumor cell. In some cases, it may be desirable to utilize a formulation that aids in the delivery of a microRNA or other nucleobase oligomer to cells (see, e.g., U.S. Pat. Nos. 5,656,611, 5,753,613, 5,785,992, 6,120,798, 6,221,959, 6,346,613, and 6,353,055, each of which is hereby incorporated by reference).

In some examples, the microRNA composition is at least partially crystalline, uniformly crystalline, and/or anhydrous (e.g., less than 80, 50, 30, 20, or 10% water). In another example, the microRNA composition is in an aqueous phase, e.g., in a solution that includes water. The aqueous phase or the crystalline compositions can be incorporated into a delivery vehicle, e.g., a liposome (particularly for the aqueous phase), or a particle (e.g., a microparticle as can be appropriate for a crystalline composition). Generally, the microRNA composition is formulated in a manner that is compatible with the intended method of administration.

A microRNA composition can be formulated in combination with another agent, e.g., another therapeutic agent or an agent that stabilizes an oligonucleotide agent, e.g., a protein

that complexes with the oligonucleotide agent. Still other agents include chelators, e.g., EDTA (e.g., to remove divalent cations such as Mg^{2+}), salts, and RNase inhibitors (e.g., a broad specificity RNase inhibitor, such as RNasin).

In one embodiment, the microRNA composition includes another microRNA, e.g., a second microRNA composition (e.g., a microRNA that is distinct from the first). Still other preparations can include at least three, five, ten, twenty, fifty, or a hundred or more different oligonucleotide species.

Polynucleotide Therapy

The present invention features polynucleotide therapy. For example, polynucleotide therapy featuring a polynucleotide encoding a microRNA is another therapeutic approach for inhibiting neoplasia in a subject. Expression vectors encoding the microRNAs can be delivered to cells, e.g., prostate cancer or lymphoma cells, of a subject for the treatment or prevention of a neoplasia. The nucleic acid molecules must be delivered to the cells of a subject in a form in which they can be taken up and are advantageously expressed so that therapeutically effective levels can be achieved.

Polynucleotide therapy featuring a polynucleotide encoding an inhibitory nucleic acid molecule is another therapeutic approach that can be used. For example, inhibitory nucleic acids can be used to restore cardiac function following any event or procedure involving the heart in which ischemia has occurred or is likely to occur. An exemplary use is in subjects who have recently experienced myocardial infarction, or cardiac patients who have undergone heart surgery and are at risk of reperfusion damage; e.g., subjects who have undergone cardiac bypass procedures, valve repairs or replacements, heart transplantation, or balloon angioplasty.

Other ischemic conditions that can be treated or prevented according to the invention include ischemic events involving other internal organs such as the lungs, liver, and kidneys; regions containing skeletal muscle, e.g., limbs and trunk muscles; and ischemic conditions involving smooth muscle, e.g. surgeries involving the smooth muscle of the gastrointestinal tract, e.g., surgeries to treat lesions of the organs of the gastrointestinal tract, and surgeries to correct blockages, e.g., intestinal blockages.

Methods for delivery of the polynucleotides to the cell according to the invention include using a delivery system, such as liposomes, polymers, microspheres, gene therapy vectors, and naked DNA vectors.

Transducing viral (e.g., retroviral, adenoviral, lentiviral and adeno-associated viral) vectors can be used for somatic cell gene therapy, especially because of their high efficiency of infection and stable integration and expression (see, e.g., Cayouette et al., Human Gene Therapy 8:423-430, 1997; Kido et al., Current Eye Research 15:833-844, 1996; Bloomer et al., Journal of Virology 71:6641-6649, 1997; Naldini et al., Science 272:263-267, 1996; and Miyoshi et al., Proc. Natl. Acad. Sci. U.S.A. 94:10319, 1997). For example, a polynucleotide encoding a microRNA molecule can be cloned into a retroviral vector and expression can be driven from its endogenous promoter, from the retroviral long terminal repeat, or from a promoter specific for a target cell type of interest. Other viral vectors that can be used include, for example, a vaccinia virus, a bovine papilloma virus, or a herpes virus, such as Epstein-Barr Virus (also see, for example, the vectors of Miller, Human Gene Therapy 15-14, 1990; Friedman, Science 244:1275-1281, 1989; Eglitis et al., BioTechniques 6:608-614, 1988; Tolstoshev et al., Current Opinion in Biotechnology 1:55-61, 1990; Sharp, The Lancet 337:1277-1278, 1991; Cornetta et al., Nucleic Acid Research and Molecular Biology 36:311-322, 1987; Anderson, Science 226:401-409, 1984; Moen, Blood Cells 17:407-416, 1991; Miller et al., Biotechnology 7:980-990, 1989; Le Gal La Salle et al., Science 259:988-990, 1993; and Johnson, Chest 107:77S-83S, 1995). Retroviral vectors are particularly well developed and have been used in clinical settings (Rosenberg et al., N. Engl. J. Med 323:370, 1990; Anderson et al., U.S. Pat. No.5,399,346).

Non-viral approaches can also be employed for the introduction of a microRNA therapeutic to a cell of a patient diagnosed as having a neoplasia. For example, a microRNA can be introduced into a cell by administering the nucleic acid in the presence of lipofection (Feigner et al., Proc. Natl. Acad. Sci. U.S.A. 84:7413, 1987; Ono et al., Neuroscience Letters 17:259, 1990; Brigham et al., Am. J. Med. Sci. 298:278, 1989; Staubinger et al., Methods in Enzymology 101:512, 1983), asialoorosomucoid-polylysine conjugation (Wu et al., Journal of Biological Chemistry 263:14621, 1988; Wu et al., Journal of Biological Chemistry 264:16985, 1989), or by micro-injection under surgical conditions (Wolff et al., Science 247:1465, 1990). Preferably the microRNA molecules are administered in combination with a liposome and protamine.

Gene transfer can also be achieved using non-viral means involving transfection *in vitro*. Such methods include the use of calcium phosphate, DEAE dextran, electroporation, and protoplast fusion. Liposomes can also be potentially beneficial for delivery of DNA into a cell.

MicroRNA expression for use in polynucleotide therapy methods can be directed from any suitable promoter (e.g., the human cytomegalovirus (CMV), simian virus 40 (SV40), or metallothionein promoters), and regulated by any appropriate mammalian regulatory element. For example, if desired, enhancers known to preferentially direct gene expression in specific cell types can be used to direct the expression of a nucleic acid. The enhancers used can include, without limitation, those that are characterized as tissue- or cell-specific enhancers.

For any particular subject, the specific dosage regimes should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions.

Pharmaceutical Compositions

The present invention provides pharmaceutical compositions. In preferred embodiments, the invention provides pharmaceutical compositions that increase the expression of glutaminase in a cell. In other preferred embodiments, the invention provides pharmaceutical compositions that decrease the expression of glutaminase in a cell.

In certain embodiments, the present invention features a pharmaceutical composition for the decreasing the expression of glutaminase in a cell, the composition comprising an effective amount of an oligonucleotide having at least 85% identity to the sequence of a microRNA selected from miR-23a or miR-23b, and a pharmaceutically acceptable excipient, wherein expression of said microRNA in a cell increases the expression of glutaminase.

In other embodiments, the invention features a pharmaceutical composition for increasing the expression of glutaminase in a subject comprising a therapeutically effective amount of an inhibitory nucleic acid molecule that corresponds to or is complementary to at least a fragment of a miR-23a or miR-23b, in a pharmaceutically acceptable excipient, wherein the fragment is capable of decreasing the expression of glutaminase.

As reported herein, a reduction in the expression of specific microRNAs regulated by Myc is associated with neoplasia or tumorigenesis. Accordingly, the invention provides therapeutic compositions that increase the expression of a microRNAs described herein for the treatment or prevention of a neoplasm. In one embodiment, the present invention provides a pharmaceutical composition comprising a microRNA of the invention or a nucleic acid molecule encoding a microRNA of the invention. If desired, the nucleic acid molecule is administered in combination with a chemotherapeutic agent. In another embodiment, a recombinant microRNA or a polynucleotide encoding such a microRNA, is administered to reduce the growth, survival or proliferation of a neoplastic cell or to increase apoptosis of a

neoplastic cell. Polynucleotides of the invention may be administered as part of a pharmaceutical composition. The compositions should be sterile and contain a therapeutically effective amount of a microRNA or nucleic acid molecule encoding a microRNA in a unit of weight or volume suitable for administration to a subject.

5 A recombinant microRNA or a nucleic acid molecule encoding a microRNA described herein may be administered within a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer the compounds to patients suffering from a neoplasia. Administration may begin before the patient is symptomatic.

10 Any appropriate route of administration may be employed, for example, administration may be parenteral, intravenous, intraarterial, subcutaneous, intratumoral, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intrahepatic, intracapsular, intrathecal, intracisternal, intraperitoneal, intranasal, aerosol, suppository, or oral administration. For example, therapeutic formulations may be in the form of liquid solutions or suspensions; for
15 oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found, for example, in "Remington: The Science and Practice of Pharmacy" Ed. A. R. Gennaro, Lippincott Williams & Wilkins, Philadelphia, Pa., 2000. Formulations for parenteral administration may,
20 for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for inhibitory nucleic acid molecules include
25 ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

30 The formulations can be administered to human patients in therapeutically effective amounts (e.g., amounts which prevent, eliminate, or reduce a pathological condition) to provide therapy for a neoplastic disease or condition. The preferred dosage of a nucleobase oligomer of the invention is likely to depend on such variables as the type and extent of the

disorder, the overall health status of the particular patient, the formulation of the compound excipients, and its route of administration.

With respect to a subject having a neoplastic disease or disorder, an effective amount is sufficient to stabilize, slow, or reduce the proliferation of the neoplasm. Generally, doses of active polynucleotide compositions of the present invention would be from about 0.01 mg/kg per day to about 1000 mg/kg per day. It is expected that doses ranging from about 50 to about 2000 mg/kg will be suitable. Lower doses will result from certain forms of administration, such as intravenous administration. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits. Multiple doses per day are contemplated to achieve appropriate systemic levels a microRNA of the invention or of a polynucleotide encoding such a microRNA.

Accordingly, the present invention provides methods of treating disease and/or disorders or symptoms thereof which comprise administering a therapeutically effective amount of a composition comprising a microRNA described herein to a subject (e.g., a mammal, such as a human). Thus, one embodiment is a method of treating a subject suffering from or susceptible to a neoplastic disease or disorder or symptom thereof. The method includes the step of administering to the mammal a therapeutic amount of a microRNA or nucleic acid encoding such a microRNA herein sufficient to treat the neoplastic disease or disorder or symptom thereof, under conditions such that the disease or disorder is treated.

The methods herein include administering to the subject (including a subject identified as in need of such treatment) an effective amount of a compound described herein, or a composition described herein to prevent, treat, stabilize, or reduce the growth or survival of a neoplasia in a subject in need thereof. Identifying a subject in need of such treatment can be in the judgment of a subject or a health care professional and can be subjective (e.g. opinion) or objective (e.g. measurable by a test or diagnostic method).

As used herein, the terms “treat,” “treating,” “treatment,” and the like refer to reducing or ameliorating a disorder and/or symptoms associated therewith. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition or symptoms associated therewith be completely eliminated.

As used herein, the terms “prevent,” “preventing,” “prevention,” “prophylactic treatment” and the like refer to reducing the probability of developing a disorder or condition

in a subject, who does not have, but is at risk of or susceptible to developing a disorder or condition.

The therapeutic methods of the invention (which include prophylactic treatment) in general comprise administration of a therapeutically effective amount of the agents herein, such as a microRNA or a nucleic acid encoding such a microRNA herein to a subject (e.g., animal, human) in need thereof, including a mammal, particularly a human. Such treatment will be suitably administered to subjects, particularly humans, suffering from, having, susceptible to, or at risk for a disease, disorder, or symptom thereof. Determination of those subjects "at risk" can be made by any objective or subjective determination by a diagnostic test or opinion of a subject or health care provider (e.g., genetic test, enzyme or protein marker, Marker (e.g., increased Myc expression or a neoplasia associated with an alteration in Myc regulation, or as defined herein), family history, and the like). The compounds herein may be also used in the treatment of any other disorders in which Myc dysregulation may be implicated.

In one embodiment, the invention provides a method of monitoring treatment progress. The method includes the step of determining a level of diagnostic marker (Marker) (e.g., any target delineated herein modulated by a compound herein, a protein or indicator thereof, etc.) or diagnostic measurement (e.g., screen, assay) in a subject suffering from or susceptible to a disorder or symptoms thereof associated with glutaminase dysregulation, in which the subject has been administered a therapeutic amount of a compound herein sufficient to treat the disease or symptoms thereof. The level of Marker determined in the method can be compared to known levels of Marker in either healthy normal controls or in other afflicted patients to establish the subject's disease status. In preferred embodiments, a second level of Marker in the subject is determined at a time point later than the determination of the first level, and the two levels are compared to monitor the course of disease or the efficacy of the therapy. In certain preferred embodiments, a pre-treatment level of Marker in the subject is determined prior to beginning treatment according to this invention; this pre-treatment level of Marker can then be compared to the level of Marker in the subject after the treatment commences, to determine the efficacy of the treatment.

Therapy

Therapy may be provided wherever cancer therapy is performed: at home, the doctor's office, a clinic, a hospital's outpatient department, or a hospital. Therapy may also be performed where heart surgery or bypass procedures are performed. For cancer, for example,

treatment generally begins at a hospital so that the doctor can observe the therapy's effects closely and make any adjustments that are needed. The duration of the therapy depends on the kind of neoplasia being treated, the age and condition of the patient, the stage and type of the patient's disease, and how the patient's body responds to the treatment. Drug
5 administration may be performed at different intervals (e.g., daily, weekly, or monthly). Therapy may be given in on-and-off cycles that include rest periods so that the patient's body has a chance to build healthy new cells and regain its strength.

Depending on the type of cancer and its stage of development, the therapy can be used to slow the spreading of the cancer, to slow the cancer's growth, to kill or arrest cancer cells
10 that may have spread to other parts of the body from the original tumor, to relieve symptoms caused by the cancer, or to prevent cancer in the first place. As described above, if desired, treatment with a microRNA or a polynucleotide encoding such a microRNA may be combined with therapies for the treatment of proliferative disease (e.g., radiotherapy, surgery, or chemotherapy). For any of the methods of application described above, microRNA of the
15 invention is desirably administered intravenously or is applied to the site of neoplasia (e.g., by injection).

Diagnostics

As described in more detail below, the present invention has identified reductions in
20 the expression of Myc regulated microRNAs (e.g., miR-23a or miR-23b) that are associated with greater expression of mitochondrial glutaminase. This leads to upregulated glutamine catabolism. Accordingly, the present invention has identified that reductions in the expression of Myc regulated microRNAs (e.g., miR-23a or miR-23b) are associated with neoplasia. The present invention has also identified that antisense directed to miR-23a or
25 miR-23b can be used to provide protection against ischemic cell death. Reductions in the expression level of one or more of these markers is used to diagnose a subject as having a neoplasia associated with Myc dysregulation. In one embodiment, the method identifies a neoplasia as amenable to treatment using a method of the invention by assaying a decrease in the level of any one or more of the following markers: miR-23a, miR-23b.

30 In one embodiment, a subject is diagnosed as having or having a propensity to develop a neoplasia, the method comprising measuring markers in a biological sample from a patient, and detecting an alteration in the expression of one or more marker molecules relative to the sequence or expression of a reference molecule. The markers typically include a microRNA.

Certain exemplary neoplasias include, but are not limited to, pancreatic cancer, prostate cancer or lymphoma.

Reduced expression of a microRNA of the invention (e.g., miR-23a or miR-23b) is used to identify a neoplasia that is amenable to treatment using a composition or method described herein. Accordingly, the invention provides compositions and methods for identifying such neoplasias in a subject. Alterations in gene expression are detected using methods known to the skilled artisan and described herein. Such information can be used to diagnose a neoplasia or to identify a neoplasia as being amenable to a therapeutic method of the invention.

In one approach, diagnostic methods of the invention are used to assay the expression of a microRNA (e.g., miR-23a or miR-23b) in a biological sample relative to a reference (e.g., the level of microRNA present in a corresponding control tissue, such as a healthy tissue). Exemplary nucleic acid probes that specifically bind a microRNA of the invention are described herein. By “nucleic acid probe” is meant any nucleic acid molecule, or fragment thereof, that binds or amplifies a microRNA of the invention. Such nucleic acid probes are useful for the diagnosis of a neoplasia.

In one approach, quantitative PCR methods are used to identify a reduction in the expression of a microRNA of the invention. In another approach, a probe that hybridizes to a microRNA of the invention is used. The specificity of the probe determines whether the probe hybridizes to a naturally occurring sequence, allelic variants, or other related sequences. Hybridization techniques may be used to identify mutations indicative of a neoplasia or may be used to monitor expression levels of these genes (for example, by Northern analysis (Ausubel et al., *supra*).

In general, the measurement of a nucleic acid molecule or a protein in a subject sample is compared with a diagnostic amount present in a reference. A diagnostic amount distinguishes between a neoplastic tissue and a control tissue. The skilled artisan appreciates that the particular diagnostic amount used can be adjusted to increase sensitivity or specificity of the diagnostic assay depending on the preference of the diagnostician. In general, any significant increase or decrease (e.g., at least about 10%, 15%, 30%, 50%, 60%, 75%, 80%, or 90%) in the level of test nucleic acid molecule or polypeptide in the subject sample relative to a reference may be used to diagnose or characterize a neoplasia. Test molecules include any one or more of miR-23a or miR-23b. In one embodiment, the reference is the level of test polypeptide or nucleic acid molecule present in a control sample obtained from a patient that does not have a neoplasia. In another embodiment, the reference is a baseline level of

test molecule present in a biologic sample derived from a patient prior to, during, or after treatment for a neoplasia. In yet another embodiment, the reference can be a standardized curve.

5 Types of Biological Samples

The level of markers in a biological sample from a patient having or at risk for developing a neoplasia can be measured, and an alteration in the expression of marker molecule relative to the sequence or expression of a reference molecule, can be determined in different types of biologic samples. Test markers include any one or all of the following:

- 10 miR-23a or miR-23b. The biological samples are generally derived from a patient, preferably as a bodily fluid (such as blood, cerebrospinal fluid, phlegm, saliva, or urine) or tissue sample (e.g. a tissue sample obtained by biopsy).

Kits

- 15 The invention provides kits for the prevention, treatment, diagnosis or monitoring of a neoplasia. In one embodiment, the kit provides a microRNA molecule for administration to a subject. In another embodiment, the kit detects an alteration in the sequence or expression of miR-23a or miR-23b derived from a subject relative to a reference sequence or reference level of expression. In related embodiments, the kit includes reagents for monitoring the
20 expression of a miR-23a or miR-23b nucleic acid molecule, such as primers or probes that hybridize to a miR-23a or miR-23b nucleic acid molecule.

- Optionally, the kit includes directions for monitoring the nucleic acid molecule levels of a Marker in a biological sample derived from a subject. In other embodiments, the kit comprises a sterile container which contains the primer, probe, antibody, or other detection
25 reagents; such containers can be boxes, ampoules, bottles, vials, tubes, bags, pouches, blister-packs, or other suitable container form known in the art. Such containers can be made of plastic, glass, laminated paper, metal foil, or other materials suitable for holding nucleic acids. The instructions will generally include information about the use of the primers or probes described herein and their use in diagnosing a neoplasia. Preferably, the kit further
30 comprises any one or more of the reagents described in the diagnostic assays described herein. In other embodiments, the instructions include at least one of the following: description of the primer or probe; methods for using the enclosed materials for the diagnosis of a neoplasia; precautions; warnings; indications; clinical or research studies; and/or references. The instructions may be printed directly on the container (when present), or as a

label applied to the container, or as a separate sheet, pamphlet, card, or folder supplied in or with the container.

Screening Assays

5 One embodiment of the invention encompasses a method of identifying an agent that increases the expression or activity of a miR-23a or miR-23b microRNA. Accordingly, compounds that increase the expression or activity of a microRNA of the invention or a variant, or portion thereof are useful in the methods of the invention for the treatment or prevention of a neoplasm. The method of the invention may measure an increase in
10 transcription of one or more microRNAs of the invention. Any number of methods are available for carrying out screening assays to identify such compounds. In one approach, the method comprises contacting a cell that expresses a microRNA of the invention (e.g., miR-23a or miR-23b) with an agent and comparing the level of expression in the cell contacted by the agent with the level of expression in a control cell, wherein an agent that increases the
15 expression of a microRNA of the invention thereby inhibits a neoplasia.

 In other embodiments, the agent acts as a microRNA mimetic, which substantially fulfills the function of an microRNA of the invention. Candidate mimetics include organic molecules, peptides, polypeptides, nucleic acid molecules. Small molecules of the invention preferably have a molecular weight below 2,000 daltons, more preferably between 300 and
20 1,000 daltons, and still more preferably between 400 and 700 daltons. It is preferred that these small molecules are organic molecules. Compounds isolated by any approach described herein may be used as therapeutics to treat a neoplasia in a human patient.

 In addition, compounds that increase the expression of a microRNA of the invention are also useful in the methods of the invention. Any number of methods are available for
25 carrying out screening assays to identify new candidate compounds that increase the expression of miR-23a or miR-23b. The invention also includes novel compounds identified by the above-described screening assays. Optionally, such compounds are characterized in one or more appropriate animal models to determine the efficacy of the compound for the treatment of a neoplasia. Desirably, characterization in an animal model can also be used to
30 determine the toxicity, side effects, or mechanism of action of treatment with such a compound. Furthermore, novel compounds identified in any of the above-described screening assays may be used for the treatment of a neoplasia in a subject. Such compounds are useful alone or in combination with other conventional therapies known in the art.

Test Compounds and Extracts

In general, compounds capable of inhibiting the growth or proliferation of a neoplasia by increasing the expression or biological activity of a microRNA (e.g., miR-23a or miR-23b) are identified from large libraries of either natural product or synthetic (or semi-synthetic) extracts or chemical libraries according to methods known in the art. Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds. Synthetic compound libraries are commercially available from Brandon Associates (Merrimack, N.H.) and Aldrich Chemical (Milwaukee, Wis.). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceanographics Institute (Ft. Pierce, Fla.), and PharmaMar, U.S.A. (Cambridge, Mass.).

In one embodiment, test compounds of the invention are present in any combinatorial library known in the art, including: biological libraries; peptide libraries (libraries of molecules having the functionalities of peptides, but with a novel, non-peptide backbone which are resistant to enzymatic degradation but which nevertheless remain bioactive; see, e.g., Zuckermann, R.N. *et al.*, *J. Med. Chem.* 37:2678-85, 1994); spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library and peptoid library approaches are limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, *Anticancer Drug Des.* 12:145, 1997).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 90:6909, 1993; Erb *et al.*, *Proc. Natl. Acad. Sci. USA* 91:11422, 1994; Zuckermann *et al.*, *J. Med. Chem.* 37:2678, 1994; Cho *et al.*, *Science* 261:1303, 1993; Carrell *et al.*, *Angew. Chem. Int. Ed. Engl.* 33:2059, 1994; Carrell *et al.*, *Angew. Chem. Int. Ed. Engl.* 33:2061, 1994; and Gallop *et al.*, *J. Med. Chem.* 37:1233, 1994.

Libraries of compounds may be presented in solution (e.g., Houghten, *Biotechniques* 13:412-421, 1992), or on beads (Lam, *Nature* 354:82-84, 1991), chips (Fodor, *Nature* 364:555-556, 1993), bacteria (Ladner, U.S. Patent No. 5,223,409), spores (Ladner U.S. Patent No. 5,223,409), plasmids (Cull *et al.*, *Proc Natl Acad Sci USA* 89:1865-1869, 1992) or on

phage (Scott and Smith, *Science* 249:386-390, 1990; Devlin, *Science* 249:404-406, 1990; Cwirla *et al. Proc. Natl. Acad. Sci.* 87:6378-6382, 1990; Felici, *J. Mol. Biol.* 222:301-310, 1991; Ladner *supra.*).

In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (e.g., taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their anti-neoplastic activity should be employed whenever possible.

In an embodiment of the invention, a high throughput approach can be used to screen different chemicals for their potency to enhance the activity of miR-23a or miR-23b.

Those skilled in the field of drug discovery and development will understand that the precise source of a compound or test extract is not critical to the screening procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using the methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as modification of existing compounds.

When a crude extract is found to enhance the biological activity of a miR-23a or miR-23b, variant, or fragment thereof, further fractionation of the positive lead extract is necessary to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract having anti-neoplastic activity. Methods of fractionation and purification of such heterogeneous extracts are known in the art. If desired, compounds shown to be useful agents for the treatment of a neoplasm are chemically modified according to methods known in the art.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are well within the purview of the skilled artisan. Such techniques are explained fully in the literature, such as, “Molecular Cloning: A Laboratory Manual”, second edition (Sambrook, 1989); “Oligonucleotide Synthesis” (Gait, 1984); “Animal Cell Culture” (Freshney, 1987); “Methods in Enzymology” “Handbook of Experimental Immunology” (Weir, 1996); “Gene Transfer Vectors for Mammalian Cells” (Miller and Calos, 1987); “Current Protocols in Molecular Biology” (Ausubel, 1987); “PCR: The Polymerase Chain Reaction”, (Mullis,

1994); “Current Protocols in Immunology” (Coligan, 1991). These techniques are applicable to the production of the polynucleotides and polypeptides of the invention, and, as such, may be considered in making and practicing the invention. Particularly useful techniques for particular embodiments will be discussed in the sections that follow.

5 The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the assay, screening, and therapeutic methods of the invention, and are not intended to limit the scope of what the inventors regard as their invention.

10 **EXAMPLES**

Example 1: Myc enhances the expression of mitochondrial protein glutaminase.

Oncogenes and tumor suppressors have been linked to the regulation of glucose metabolism, thereby connecting genetic alterations in cancers to their glucose metabolic phenotype, and in particular, the MYC oncogene produces Myc protein that directly regulates
15 glucose metabolic enzymes as well as genes involved in mitochondrial biogenesis (9, 12).

The experiments described herein make use of human P-493 B cells that bear a tetracycline-repressible MYC construct, such that tetracycline withdrawal results in rapid induction of Myc and mitochondrial biogenesis, followed by cell proliferation. By
20 comparing the mitochondrial proteomes of tetracycline-treated and untreated cells with high Myc expression, eight mitochondrial proteins were identified that are distinctly differentially expressed in response to Myc (Fig. 1a, b and Table 1, below).

25

30
Table 1

Spot No.	Name	NCBI Accession Number	Cellular Function	Cellular Localization	Fold-Change*
1	No hit				>10
2	Glutaminase C	AAD47056	Glutamine catabolism	Mitochondrial matrix	>10
3	Ornithine aminotransferase	AAB35211	Ornithine and proline metabolism	Mitochondrial matrix	>10
4	RPS22	AAH09296	Protein biosynthesis	Mitochondrial ribosomal small subunit	>10
6	Mitochondrial RPL7L12	CAA56249	Protein biosynthesis	Mitochondrial ribosomal large subunit	>10
11	proteasome activator PA28 beta chain	I53518	proteasome activator	Proteasome complex	>10
12	proteasome activator subunit 1 isoform 1	NP_006254	proteasome activator	Proteasome complex	>10
15	CDC 42 homolog	1AN0E			>10

*Fold-change was determined on the basis of equal total mitochondrial protein loading

Mitochondrial glutaminase expression (GLS, molecular mass of 58 kDa) was increased 10-fold in response to Myc. As such, the response of glutaminase to Myc induction was then determined in a time-course study using anti-GLS antibody (Fig. 1c) and it was found that GLS levels diminish with decreased Myc expression and recover on Myc re-induction. However, the level of the mitochondrial protein TFAM remained virtually unaltered. GLS levels also correlate with Myc levels in another human B cell line (CB33) and one (CB33-Myc) with constitutive Myc expression (14). Because human prostate cancer is linked to Myc expression (15), it was next determined whether reduction of Myc expression by short interfering RNA (siRNA) in the human PC3 prostate cancer cell line is also associated with reduction of GLS expression (Fig. 1d). Similar to the human lymphoid cells, the PC3 cells also displayed a correlation between Myc and GLS levels. Table 2 below shows Glutaminase peptides identified.

Table 2

Glutaminase Peptides Identified	MM+	z	MW Sp	Peptide (bits) prior	Count
K LFLNEDDKPHNFMVNAQAVYTSUK Q	2835.50733	1	642.0	23.75	1
R HSGPLDYESLQDELAK E	2075.04440	1	410.5	18.51	1
K MAGNEYVGFSNATEQSER E	2007.86052	1	349.3	19.51	1
K FRYVMQFLRK M	1304.83437	1	543.7	14.37	1
R LTLQTTSDGVMLQKDLFK K	2025.05737	1	55.9	14.51	1
R NEMISYYLK E	1008.57251	1	367.7	10.24	1
K GETHFQGVSPFAAPAAGPK Q	1920.89264	1	712.7	23.05	2
K QGLIPSELDLFTAEQDEK I	2364.23332	1	234.5	18.05	2
K DQSGETDAFQNSEQK E	1480.81868	1	310.2	17.42	2
K VADYIPQAK F	1117.82519	1	444.6	15.37	1
K YAIANDLQTEYVRR Y	1720.86531	1	173.2	12.42	1

Example 2. Glutamine and glutaminase are necessary for Myc-mediated cancer cell proliferation and survival.

5 In a next set of experiments, it was determined whether the marked alteration of GLS levels in response to Myc is functionally linked to Myc-induced cell proliferation. Although there are two major known tissue-specific GLS isoforms, GLS1 and GLS2 (16, 17), results indicate show that only GLS1 is predominantly expressed in P493-6 or PC3 cells (Fig. 4). It was first determined whether gain of GLS1 function through overexpression in PC3 cells

10 would rescue the diminished growth rate associated with siRNA-mediated reduction of Myc (Fig. 5) and it was found that ectopic GLS1 expression alone is insufficient to stimulate growth. In light of the observation that no single gene could substitute for Myc (18, 19) and that Myc is a pleiotropic transcription factor, this outcome was not particularly surprising. As such, the expression of GLS1 was reduced (hereafter referred to as GLS) by RNA

15 interference (GLSSiRNA) and it was found that P-493-6 cell proliferation is markedly attenuated by GLS siRNA but not by control siRNA (Fig. 2a). Likewise, proliferation of the human PC3 prostate cancer cell line was diminished by GLS siRNA (Fig. 2a), indicating that GLS is necessary for cell proliferation.

Because glutamine is converted by GLS to glutamate for further catabolism by the

20 tricarboxylic acid (TCA) cycle, and previous studies indicate that overexpression of Myc sensitizes human cells to glutamine-withdrawal-induced apoptosis (11), the metabolic responses of P493-6 or PC3 cells to glutamine deprivation was then determined (Fig. 2b). The growth of both cell lines was diminished significantly by glutamine withdrawal and moderately with glucose withdrawal. Glutamine withdrawal also resulted in a decrease in

ATP levels (Fig. 2c) associated with a diminished cellular oxygen consumption rate (Fig. 6a, b). Reduction of GLS by RNA interference (RNAi) also reduced ATP levels (Fig. 2d).

Because glutamine is a precursor for glutathione (20), glutathione levels were measured by flow cytometry and were found to be diminished with glutamine withdrawal or RNAi-

- 5 mediated reduction of GLS (Fig. 7 and Table 3, below) that is also associated with an increase in reactive oxygen species (ROS) levels (Supplementary Fig. 3c) and cell death in the P493-6 cells (Fig. 2e and Fig. 8).

Table 3

P493 cells	Events	Median	Mean	SD	P-value (vs. Control)
No stain	11.769	191	200	89	
control	11.819	102.966	105.425	37.368	
(-) glutamine	13.165	43.917	44.384	23.450	< 0.001
(-)glucose	12.807	83.388	85.516	30.911	< 0.001

PC3 cells	Events	Median	Mean	SD	P-value (vs. Control)
No stain	10.926	86	95	199	
control	11.651	86.024	92.153	35.467	
(-) glutamine	11.693	66.207	72.169	30.838	< 0.001
(-)glucose	11.654	86.970	92.591	37.355	0.359975398

P493 cells	Events	Median	Mean	SD	P-value (vs. Control)
No stain	10.081	96	101	50	
si-control	10.222	77.339	80.433	29.985	
siGLS	10.460	51.410	53.845	20.508	< 0.001

PC3 cells	Events	Median	Mean	SD	P-value (vs. Control)
No stain	10.926	86	95	199	
si-control	11.651	86.024	92.153	35.467	
siGLS	11.693	66.207	72.169	30.838	< 0.001

10

Shortly after the MYC proto-oncogene was discovered, the MC29 retrovirus which bears the v-myc oncogene was found to enhance glutamine catabolism and mitochondrial respiration in transplantable avian liver tumor cells (21). Thus, these findings functionally link historical observations with Myc, glutaminase and glutamine metabolism.

Because GLS catabolizes glutamine for ATP and glutathione synthesis, its reduction affects proliferation and cell death presumably through depletion of ATP and augmentation of ROS, respectively. Hence, the next experiments were aimed to rescue the P493-6 cells with the TCA cycle metabolite oxaloacetate (OAA) and the oxygen radical scavenger N-acetylcysteine (NAC) (11). Both OAA and NAC partially rescued the decreased proliferation and death of P493-6 cells deprived of GLS (Fig. 2e and Fig. 9). Similarly, OAA and NAC both partially rescued glutamine-deprived P493-6 cells (Figs 8 and 9). These findings support the notion that glutamine catabolism through GLS is critical for cell proliferation induced by Myc and protection against ROS generated by enhanced mitochondrial function in response to Myc (11, 20).

Example 3. Myc increases GLS protein by transcriptionally repressing miR-23a/b that target the GLS 3' UTR.

Given that GLS is critical for cell proliferation and is induced by Myc, the mechanism by which Myc regulates GLS was next determined. Because Myc is a transcription factor (9), it was hypothesized that myc transactivates GLS directly as a target gene. Despite the presence of a canonical Myc binding site (5'-CACGTG-3') in the *GLS* gene intron 1, GLS messenger RNA levels do not respond to alterations in Myc levels in the P493-6 cells, suggesting that GLS is regulated at the post-transcriptional level (Fig. 3a). As such, it was hypothesized that GLS could be regulated by miRNAs that are in turn directly regulated by Myc. The TargetScan algorithm predicts that miR-23a and miR-23b could target the GLS 3' untranslated region (UTR) seed sequence. Notably, the earlier studies uncovered that both miR-23a and miR-23b are suppressed by Myc in P493-6 cells (7), and both miR-23a and miR-23b are decreased in human prostate cancers (22), which are associated with elevated Myc expression (15).

To verify that miR-23a and miR-23b (hereafter referred to as miR-23a/b) are suppressed by Myc and can be diminished by antisense miR-23a/b locked nucleic acid (LNA) oligomers, a northern blot analysis was performed; the results show that miR-23a/b are indeed suppressed by Myc and profoundly diminished by antisense miR-23a/b LNAs (Fig. 3b). Quantitative real-time polymerase chain reaction (PCR) assays show (Fig. 10) that miR-23a/b levels increase with diminished Myc expression and then decrease on Myc re-induction in a manner that is compatible with the GLS protein levels seen in Fig. 1c. The results also showed an inverse relationship between Myc and the levels of miR-23a/b in the CB33 human lymphoid cells and PC3 prostate cancer cell line (Fig. 11). Furthermore, a chromatin

immunoprecipitation assay (Fig. 3c) shows that Myc directly binds the transcriptional unit, *C9orf3*, encompassing miR-23b, as demonstrated for other Myc miRNA targets (7). Because the transcriptional unit involving miR-23a has not been mapped, miR-23a was not studied in this context. These observations indicate that Myc represses miR-23a and miR-23b, which
5 seem to be directly regulated by Myc.

It was next determined whether miR-23a/b target and inhibit the expression of GLS through the 3' UTR. In this regard, the 3' UTR sequence of GLS including the predicted binding site for miR-23a/b was cloned to the pGL3 luciferase reporter vector and transfected into MCF-7 cells, which are known to express miR-23a/b (23). The GLS 3' UTR inhibited
10 luciferase activity in a fashion that was blocked by co-transfection with the antisense miR-23a/b LNAs, but not with control LNAs (Fig. 3d). Next, the predicted binding was mutated by site by a site-directed mutagenesis strategy (8) and it was observed that mutant 3' UTR does not inhibit luciferase activity as the wild-type sequence does. Using these reporters in PC3 cells, it was observed that diminished expression of Myc via siRNA results in decreased
15 luciferase activity with wild type but not with the mutant 3' LTR reporter (Fig. 12). Notably, reduced GLS protein level, a result of decreased Myc expression (Fig. 1c), was rescued by antisense miR-23a/b LNAs (Fig. 3e). The antisense miR-23a/b LNAs also partially rescued the diminished GLS level associated with RNAi-mediated reduction of Myc expression in PC3 cells (Fig. 3e).

Events upstream and downstream of GLS (24) were examined, and it was found that the glutamine transporter SLC7A5 is induced by Myc in P493-6 cells at the transcriptional level (five-fold by nuclear run-on), with a >7-fold induction of its mRNA level. The glutamine transporter ASCT2 (also called SLC1A5) shows a twofold induction by Myc at the mRNA level, whereas glutamate dehydrogenase mRNA levels appear unaltered.

Furthermore, it was found that elevated levels of Myc protein in human prostate cancer correspond to levels of GLS, which are not increased in the accompanying normal tissue from the same patients (Fig. 13). Intriguingly, miR-23a and miR-23b are significantly decreased in human prostate cancer as compared with normal prostate tissue (22). It is notable that loss of GLS function by antisense suppression significantly inhibits the
25 tumorigenesis of Ehrlich ascites tumour cells in vivo. The results presented herein uncover a pathway by which Myc suppression of miR-23a/b, which target GLS, enhances glutamine catabolism through increased mitochondrial glutaminase expression. Taken together, these observations provide a regulatory mechanism involving Myc and miRNAs for elevated
30 expression of glutaminase and glutamine metabolism in human cancers.

Methods

The foregoing experiments were performed using, but not limited to, the following methods.

- 5 **Cell culture.** P493-6 human B lymphoma cells, PC3 human prostate cancer cells, CB33 lymphoblastoid cells, CB33-Myc cells and MCF7 human breast cancer cells were maintained in RPMI 1640 with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. HT-29 cells were maintained in McCoy's 5A medium with 10% FBS and 1% penicillin-streptomycin. PC3-GLS1 and control PC3-GFP cells were established by infecting
- 10 PC3 cells with retroviral supernatants from PQCXIN-GLS1 or PQCXIN-GIT vector-transfected phoenix cells. The cells were selected by and maintained with RPMI 1640 medium containing $500 \mu\text{g ml}^{-1}$ G418. Experiments under deprived glutamine or glucose culture conditions were performed by using RPMI 1640 without L-glutamine (GIBCO 21870) or RPMI 1640 without D-glucose (GIBCO 11879).
- 15 **Mitochondrial protein enrichment.** To enrich for mitochondrial protein, protocols were adapted as described (26). 1×10^9 cells were used for high Myc no tetracycline treatment) and 1.5×10^9 for tetracycline treated cells. Cells were harvested, washed with cold PBS extensively then washed with cold homogenization buffer (220 mM mannitol, 70 mM sucrose, 2 mM HEPES, pH 7.4 with KOH) to remove PBS. Harvested cell pellets were re-
- 20 suspended in 8 ml of homogenization buffer with protease inhibitors (Roche), phosphatase inhibitors (EMD Chemicals) and homogenized with 15 strokes by a tight fitting dounce homogenizer. Homogenate was diluted with 60 ml of cold homogenization buffer and centrifuged at 800g for 10 min at 4 °C. The supernatant was collected and centrifuged at 7,000g for 15 min. The resulting pellet was homogenized in 8 ml homogenization buffer,
- 25 diluted in 60 ml of the same buffer and centrifuged at 800g for 10 min. Supernatant was further centrifuged at 12,000g for 15 min. The mitochondrial pellet after this step was washed once more by re-suspending in 60 ml homogenization buffer and centrifugation for 15 min at 12,000g. Finally, the pellet was re-suspended in 1.5 ml of homogenization buffer, transferred to microcentrifuge tube, centrifuged at 16,000g for 20 min and solubilized in 40 μl of 5%
- 30 ASB-14 (w/v) and then diluted in an appropriate amount of IEF buffer (8 M urea, 2 M thiourea, 4% w/v CHAPS, 1% w/v dithiothreitol, 0.5% v/v carrier ampholytes pH 4 - 7, and a trace amount of bromophenol blue) to make 5 mg ml^{-1} protein solution

Two-dimensional gel electrophoresis and proteomics. Two-dimensional gel electrophoresis and mass spectrometry identification of proteins were performed as described

with modifications (27). A vMALDI linear ion trap mass spectrometer (vMALDI-LTQ, ThermoElectron) with XCalibur 1.4 SRI software package was used to perform protein identification. Protein digests were re-suspended in 50% AcCN/ 0.1% TFA and mixed with an equal volume of 2,5- dihydroxybenzoic acid (2,5-DHEI; Laser BioLab) 50 mg ml⁻¹ in 50% acetonitrile/ 0.1% TFA. 0.5 µl of this mixture was spotted on a vMALDI plate. A survey scan from *m/z* 750 to *m/z* 4,000 (full MS) was followed by data-dependent MS/ MS scans on 30 most intense ions with normalized collision energy value of 40, activation Q value of 0.25, and activation time of 30 ms. Raw data files were searched with BioworkBrowser 3.3 (ThermoElectron) against the IPI human protein sequence database, using search algorithm SEQUEST (28, 29).

RNAi experiments. siRNAs targeting human *GLS1* (ON-TARGETplus SMARTpool, L-004548-01, target sequences are CCUGAAGCAGUUCGAAAUA, CUGAAUAUGUGCAUCGAUA, AGAAAGUGGAGAUCGAAAUA and GCACAGACAUGGUUGGUAU, *MYC* (siGENOME SMART pool, J-003282- 23, target sequences are ACGGAACUCUUGUGCGUAAUU, GAACACACAACGUCUUGGAUU, AACGUUAGCUUCACCAACAUAU and CGAUGUUGUUUCUGUGGAAUU, or control siRN A (Sicontrol, D-001210-02, sequence is UAAGGCUAUGAAGAGAUAC) were purchased from Dharmacon Research Inc. Transfection of the siRNAs into P493 or PC3 cells was performed as described previously (30).

Knocking-down miR-23a/b with anti-sense LNA oligomers. miRCURY LNA knockdown probes for miR-23a (miRCURY knockdown, 118119-00, target sequence is AUCACAUUGCCAGGGAUUUCC) and miR-23b (miRCURY knockdown, 138120-00, target sequence is AUCACAUUGCCAGGGAUUACC), and for scramble miRNA (miRCURY knockdown, 199002-04, scramble-miR) LNA probes as negative control, were purchased from EXIQON, Inc. The transfection of LNA probes into cells was performed using the same protocol for siRNA transfection as described above.

3'UTR luciferase assays and site-directed mutagenesis. The 3' UTR sequence of human GLS was generated by PCR with the following primers: 5' - GCTCTAGACATGTGTATTTCTATCTGGTAGTG-3' and 5' - GCTCTAGAGCATATCAGCAGATCATCACCATA-3'. The PCR products were digested with XbaI and then inserted into the PGL3 reporter vector downstream of the luciferase gene. The correct clones were confirmed by sequencing analysis. The mutagenesis of predicted miR-23a/b binding sites (seed sequences) was performed using a QuikChange site: directed mutagenesis kit (Stratagene, catalogue number 200519-5) and the following primers: 51-

CAATCTCCCTCCATGACGAGAGCAATATTACCTCG-3' and 5' -
 GTTAGAGGGAGGTACTGCTCTCGTTATAATGGAGC-3'. For luciferase assay, cells
 were seeded in 48-well plates. After overnight incubation, cells were co-transfected either
 with 100 ng reporter vectors and 4 ng Or further co-transfected with 10 mM LNA anti-sense
 for miR -23-a/b or control LNA. After 24 h, luciferase activities were measured using the
 Dual-Luciferase Reporter Assay System (Promega).

Chromatin immunoprecipitation and real-time PCR. Chromatin
 immunoprecipitation assay was carried out as described (7). Chromatin immunoprecipitation
 primers for *C9orf3* were: amplicon A 5'-ATTCTTCTCTTGGCTGTTCTTTCC-3', 5'-
 GAAGCAGCCAACTCTGTGGAG-3'; amplicon B 5'-
 GGAATACTAGGGTACCAGGGCA-3', 5'-GCAGCTTGGCTGGCTAGG-3';
 amplicon C 5'-ACTTAGGATCCAATCCACTGTTGAG-3', 5'-
 CTCAACAGTGGATTGGATCCTAAGT-3'.

For real-time PCR, total RNA was extracted using the RNeasy kit (QIAGEN)
 followed by DNAase (Ambion) treatment according to the manufacturer's instructions.
 Primers were designed using Beacon Designer software, and cDNA teas prepared using
 TaqA/lan Reverse. Transcription Reagents (Roche, Applied Biosystems). The primers used
 were: GLS1-F, 5'-TGGTGGCCTCAGGTGAAAAT-3'; GLS1-R, 5' -
 CCAAGCTAGGTAACAGACCCTGTTT 3'; GLS2-F, --
 AACGAATCCCTATCCACAAGTTCA-3'; GLS2-R, 5'-GCAGTCCAGTGGCCTTTAGTG-
 3'; 18s-F, 5' -CGGCGACGACCCATTCGAAC-3'; 18s--R, 5' -
 GAATCGAACCCTGATTCCCCGTC-3'. Quantitative real-time PCR for GLS1, GLS2 and
 18S was performed using the ABI 7500 sequence detection system. All PCRs were
 performed in triplicate.

Immunoblot analysis. Rabbit antibody for GLS for immunoblots was described
 previously (10). Rabbit anti-TFAM antibody seas a gift from D. Rang. Monoclonal anti c-
 Myc antibody from Santa Cruz (9E10) was used, and mouse antibody for tubulin from
 CalBiochem (catalogue number CP06) was used, and immunoblot assays were performed
 according to the manufacturer's instructions.

Northern blot analysis. Northern blotting for miR-23a and miR-23b was performed
 as described (7) using Ultrahyb-Oligo (Ambion) and oligonucleotide probes perfectly
 complementary to the mature miRNA sequences.

Intracellular ATP. ATP levels were measured using a Somatic Cell ATP assay kit
 (Sigma) according to the manufacturer's instructions. Luminescence was measured using a

Wallace microplate luminescence reader (Perkin Elmer) and normalized to the protein concentration.

Flow cytometric measurement of glutathione, ROS and cell death. The measurement of glutathione levels in cells was performed using monobromobimane (Sigma Aldrich) as described previously (19). Intracellular ROS production was measured by staining cells with dichlorodihydrofluorescein diacetate (Molecular Probes). Cell apoptosis was detected using an Annexin V -PE Apoptosis Detection Kit (BD Pharmingen, catalogue number 559763). Stained cells were analysed in FACScan flow cytometers (BDBioscience).

Measurement of cellular O₂ consumption. Cells were harvested and re-suspended at 1×10^7 per ml in RPMI 1640 medium with 10% FBS and 25 mM HEPES buffer. For each experiment, equal numbers of cells suspended in 0.5 ml were pipetted into the chamber of an Oxytherm electrode unit (Hansatech Instrument Ltd), which uses a Clark-type electrode to monitor the dissolved oxygen concentration in the sealed chamber over time. The data were exported to a computerized chart recorder (Oxygraph, Hansatech Instrument Ltd), which calculated the rate of O₂ consumption. The temperature was maintained at 37 °C during the measurement. The O₂ concentration in 0.5 ml of RPMI 1640 medium without cells was also measured over time to provide background values. Relative O₂ consumption rate was calculated after correcting for background.

TaqMan microRNA assays. TaqMan microRNA assay kits were purchased for hsa-miR-23a (catalogue number 4373074) and has-miR-23b (catalogue number 4373073) and control probes from Applied Biosystems, and performed real-time PCR assays according to the manufacturer's instructions.

Human prostate cancer samples. Human samples were acquired with the approval of the Johns Hopkins University School of Medicine Institutional Review Board

Other Embodiments

From the foregoing description, it will be apparent that variations and modifications may be made to the invention described herein to adopt it to various usages and conditions. Such embodiments are also within the scope of the following claims.

The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or subcombination) of listed elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

All patents and publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent patent and publication was specifically and individually indicated to be incorporated by reference.

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What is claimed is:

1. An isolated oligonucleotide comprising a nucleobase sequence having at least 85% identity to the sequence of microRNA miR-23a or microRNA miR-23b or a fragment thereof,
5 wherein expression of said oligonucleotide in a neoplastic cell reduces the survival of the cell or inhibits cell division.
2. An isolated oligonucleotide comprising a nucleobase sequence having at least 85% identity to the sequence of microRNA miR-23a or microRNA miR-23b or a fragment thereof,
10 wherein expression of said oligonucleotide in a cell decreases expression of mitochondrial glutaminase in the cell.
3. An inhibitory nucleic acid molecule that corresponds to or is complementary to at least a fragment of microRNA miR-23a or microRNA miR-23b, or a fragment thereof, and
15 that increases expression of mitochondrial glutaminase in the cell.
4. An inhibitory nucleic acid molecule that corresponds to or is complementary to at least a fragment of microRNA miR-23a or microRNA miR-23b, or a fragment thereof,
wherein expression of said inhibitory nucleic acid molecule in a cell protects the cell from
20 ischemic cell death.
5. The inhibitory nucleic acid molecule of claim 3 or 4, wherein the nucleic acid molecule is double-stranded.
- 25 6. The inhibitory nucleic acid molecule of claim 3 or 4, wherein the nucleic acid molecule is single stranded.
7. The inhibitory nucleic acid molecule of claim 6, wherein the nucleic acid molecule is an antisense nucleic acid molecule.
30
8. The inhibitory nucleic acid molecule of claim 5, wherein the double-stranded nucleic acid molecule is an siRNA.

9. The inhibitory nucleic acid molecule of claim 8, wherein the double-stranded nucleic acid molecule is an shRNA.

10. The inhibitory nucleic acid molecule of claim 8, wherein each strand of the double-stranded nucleic acid molecule is about 19-21 nucleotides in length.

11. The inhibitory nucleic acid molecule of claim 7, wherein the antisense nucleic acid molecule comprises a nucleic acid sequence that is at least 85% identical to microRNA miR-23a or microRNAmiR-23b.

12. The inhibitory nucleic acid molecule of claim 3 or claim 4, wherein the nucleotide sequence comprises at least one modified linkage.

13. The inhibitory nucleic acid molecule of claim 3 or claim 4, wherein the inhibitory nucleic acid molecule comprises a modified backbone.

14. The isolated oligonucleotide of claim 1 or claim 2, wherein said oligonucleotide comprises the nucleobase sequence of said microRNA.

15. The isolated oligonucleotide of claim 1 or claim 2, wherein said oligonucleotide consists essentially of the nucleobase sequence of said microRNA.

16. The isolated oligonucleotide of claim 1 or claim 2, wherein said microRNA sequence is a mature or hairpin form.

17. The isolated oligonucleotide of claim 1 or claim 2, wherein said oligonucleotide comprises at least one modified linkage.

18. The isolated oligonucleotide of claim 1 or claim 2, wherein said oligonucleotide comprises at least one modified sugar moiety or one modified nucleobase.

19. An isolated nucleic acid molecule encoding the oligonucleotide of any of claims 1 or 2, wherein expression of the oligonucleotide in a neoplastic cell reduces the survival of the cell or reduces cell division.

20. The isolated nucleic acid molecule of claim 19, said nucleic acid molecule consisting essentially of the nucleotide sequence encoding a mature or hairpin form of microRNA miR-23a or microRNA miR-23b, or a fragment thereof.

5

21. An expression vector encoding an oligonucleotide of any one of claims 1-20, wherein the nucleic acid molecule is positioned for expression in a mammalian cell.

22. The expression vector of claim 16, wherein the vector encodes microRNA miR-23a or microRNA miR-23b.

10

23. The expression vector of claim 21, wherein the vector is a viral vector selected from the group consisting of a retroviral, adenoviral, lentiviral and adeno-associated viral vector.

24. A host cell comprising the expression vector of claim 19 or the oligonucleotide of any one of claims 1 or 2.

15

25. A pharmaceutical composition for the decreasing the expression of glutaminase in a cell, the composition comprising an effective amount of an oligonucleotide having at least 85% identity to the sequence of microRNA miR-23a or microRNA miR-23b, and a pharmaceutically acceptable excipient, wherein expression of said microRNA in a cell increases the expression of glutaminase.

20

26. A pharmaceutical composition for the treatment of a neoplasia, the composition comprising an effective amount of an oligonucleotide having at least 85% identity to the sequence of microRNA miR-23a or microRNA miR-23b, and a pharmaceutically acceptable excipient, wherein expression of said microRNA in a neoplastic cell reduces the survival of the cell or reduces cell division.

25

27. The pharmaceutical composition of claim 25, wherein the amount of microRNA is sufficient to reduce cell survival, cell proliferation, or expression of Myc in a neoplastic cell by at least about 5% relative to an untreated control cell.

30

28. The pharmaceutical composition of claim 25, wherein the composition comprises at least one of miR-23a or miR-23b.

29. A pharmaceutical composition for the treatment of a neoplasia, the composition comprising an effective amount of an expression vector encoding microRNA miR-23a or microRNA miR-23b.

30. The pharmaceutical composition of claim 29, wherein the amount of microRNA is sufficient to reduce expression of Myc in a neoplastic cell by at least about 5% relative to an untreated control cell.

31. The pharmaceutical composition of claim 25 or 29, wherein the composition comprises at least one of miR-23a or miR-23b.

32. The pharmaceutical composition of claim 25 or 29, wherein the composition comprises microRNA miR-23a and microRNA miR-23b.

33. The pharmaceutical composition of claim 25, wherein the oligonucleotide comprises a modification.

34. A vector encoding an inhibitory nucleic acid molecule of claim 3 or claim 4.

35. The vector of claim 34, wherein the vector is a retroviral, adenoviral, adeno-associated viral, or lentiviral vector.

36. The vector of claim 34, wherein the vector comprises a promoter suitable for expression in a mammalian cell.

37. A cell comprising the vector of claim 34 or an inhibitory nucleic acid molecule of claim 3 or claim 4.

38. The cell of claim 37, wherein the cell is an ischemic cell in vivo.

39. A pharmaceutical composition for increasing the expression of glutaminase in a subject comprising a therapeutically effective amount of an inhibitory nucleic acid molecule that corresponds to or is complementary to at least a fragment of a miR-23a or miR-23b, in a pharmaceutically acceptable excipient, wherein the fragment is capable of decreasing the expression of glutaminase.
40. The composition of claim 39, wherein the inhibitory nucleic acid molecule is administered at a dosage of between about 100 to 300 mg/m²/day.
41. A method of decreasing glutaminase expression in a cell, the method comprising contacting the cell with an oligonucleotide comprising a nucleobase sequence having at least 85% identity to microRNA miR-23a or microRNA miR-23b, thereby decreasing glutaminase expression in the cell relative to an untreated control cell.
42. The method of claim 41, wherein the cell is a neoplastic cell.
43. A method of reducing the growth, survival or proliferation of a neoplastic cell, the method comprising contacting the cell with an oligonucleotide comprising a nucleobase sequence having at least 85% identity to microRNA miR-23a or microRNA miR-23b, thereby reducing the growth, survival or proliferation of a neoplastic cell relative to an untreated control cell.
44. A method of reducing the growth, survival or proliferation of a neoplastic cell, the method comprising contacting the cell with an expression vector encoding microRNA miR-23a or microRNA miR-23b, thereby reducing the growth, survival or proliferation of a neoplastic cell relative to an untreated control cell.
45. A method of increasing glutaminase expression in a cell, the method comprising contacting the cell with an effective amount of an inhibitory nucleic acid molecule corresponding to or complementary to at least a portion of a miR-23a or miR-23b nucleic acid molecule.
46. The method of claim 45, wherein the cell is an ischemic cell.

47. The method of any one of claims 41 - 46, wherein the cell is a mammalian cell.

48. The method of claim 47, wherein the cell is a human cell.

49. The method of claim 42, wherein the cell is a lymphoma cell, pancreatic cell or a prostate cell.

50. The method of any one of claims 43 or 44, wherein the method induces apoptosis in the neoplastic cell.

51. A method of treating neoplasia in a subject, the method comprising administering to the subject an effective amount of an oligonucleotide comprising a nucleobase sequence having at least 85% identity to microRNA miR-23a or microRNA miR-23b, thereby treating a neoplasia in the subject.

52. A method of treating neoplasia in a subject, the method comprising administering to the subject an effective amount of an expression vector encoding a microRNA selected from miR-23a or miR-23b, thereby treating the neoplasia in the subject.

53. The method of claim 51, wherein the oligonucleotide comprises a modification that enhances nuclease resistance.

54. The method of claim 51 or claim 52, wherein the subject is diagnosed as having prostate cancer or a lymphoma.

55. The method of claim 51 or claim 52, wherein the method induces apoptosis in a neoplastic cell of the subject.

56. The method of claim 51 or claim 52, wherein the effective amount is sufficient to reduce expression of glutaminase in a neoplastic cell by at least about 5% relative to an untreated control cell.

57. A method of treating a subject suffering from an ischemic event, the method comprising administering to the subject an effective amount of an inhibitory nucleic acid molecule corresponding to or complementary to one or more of miR-23a and miR-23b, wherein the inhibitory nucleic acid molecule reduces miR-23a or miR-23b expression thereby
5 treating the ischemic event.

58. The method of claim 57, wherein the ischemic event is a cardiac ischemia.

59. The method of claim 57, wherein the ischemic event is a stroke.

10 60. A method of characterizing a neoplasia, the method comprising assaying the expression of microRNA miR-23a or microRNA miR-23b.

61. The method of claim 60, wherein the method comprises assaying the expression of a
15 combination of microRNA miR-23a or microRNA miR-23b.

62. The method of claim 60, wherein the neoplasia is characterized as having Myc dysregulation.

20 63. A method of identifying an agent for the treatment of a neoplasia, the method comprising
(a) contacting a neoplastic cell with a candidate agent; and
(b) assaying the expression of microRNA miR-23a or microRNA miR-23b, wherein
an increase in said microRNA expression identifies the agent as useful for the treatment of a
25 neoplasia.

64. The method of claim 63, further comprising testing the agent in a functional assay.

65. The method of claim 63, wherein the functional assay analyses cell growth,
30 proliferation, or survival.

66. A method of identifying an agent for the protection of a cell against ischemic cell death, the method comprising:
(a) exposing a cell to ischemic conditions;

(b) contacting a cell with a candidate agent; and

(b) assaying the expression of microRNA miR-23a or microRNA miR-23b, wherein an increase in said microRNA expression identifies the agent as useful for the treatment of a neoplasia.

5

67. A primer set comprising at least two pairs of oligonucleotides, each of which pair binds to microRNA miR-23a or microRNA miR-23b, or a fragment thereof.

68. A probe set comprising at least two oligonucleotides each of which binds to
10 microRNA miR-23a or microRNA miR-23b, or a fragment thereof.

69. A microarray comprising a microRNA or nucleic acid molecule encoding microRNA miR-23a or microRNA miR-23b, or a fragment thereof.

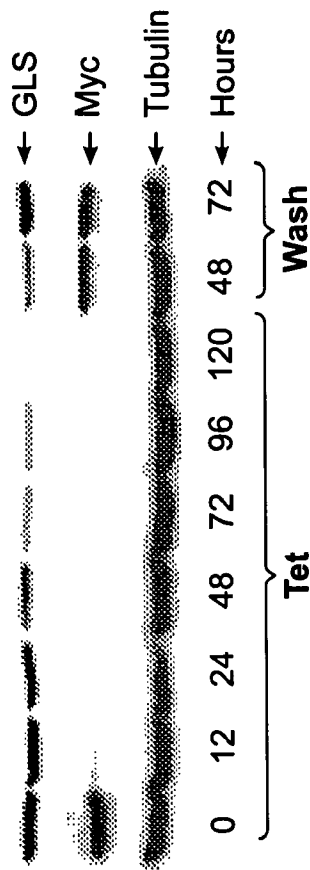


FIG. 1c

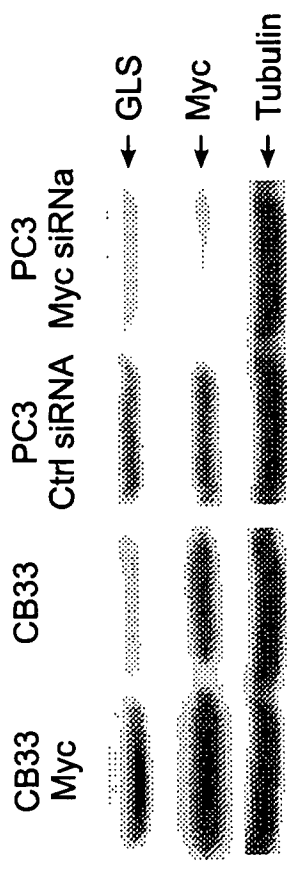


FIG. 1d

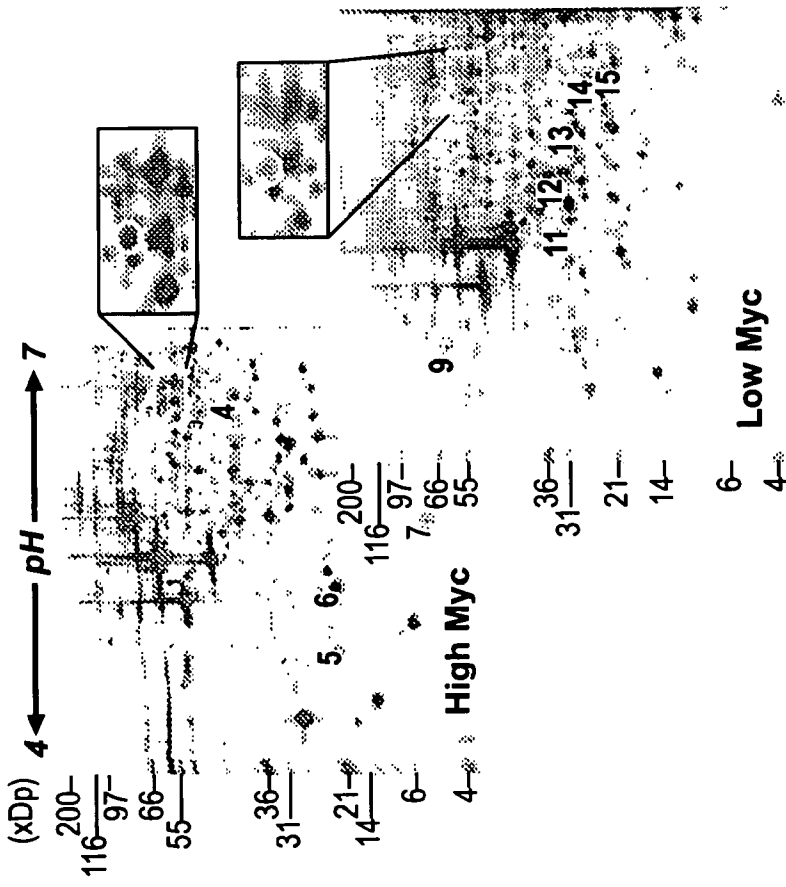


FIG. 1a

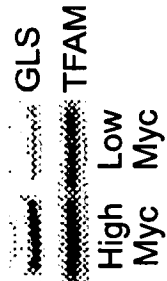


FIG. 1b

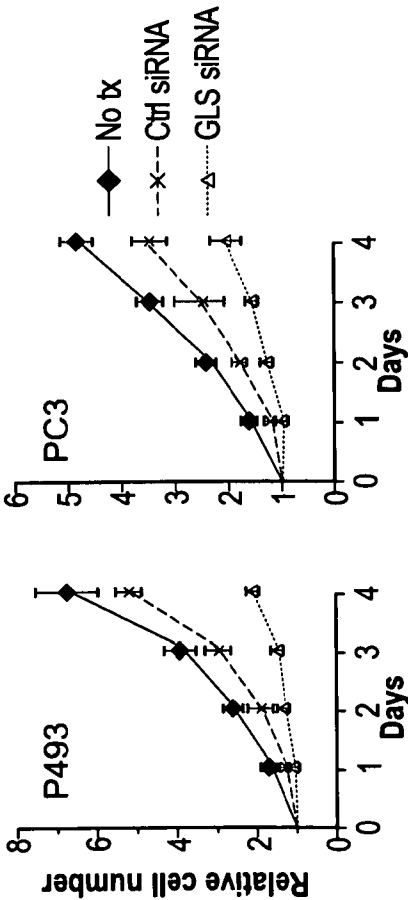
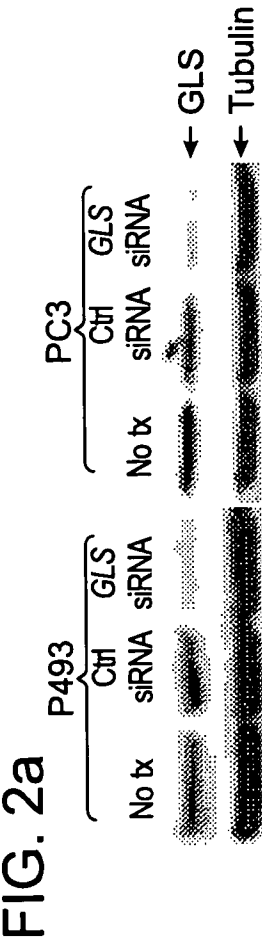


FIG. 2c

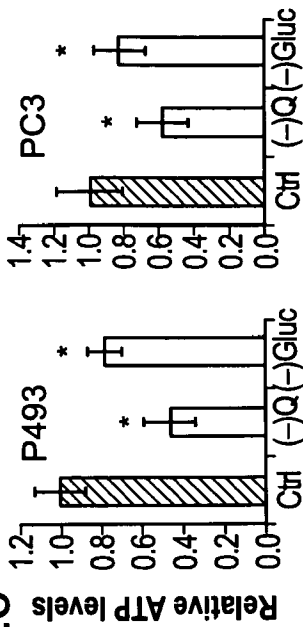


FIG. 2d

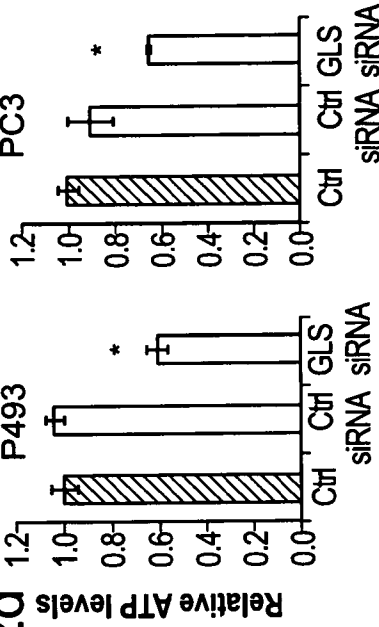
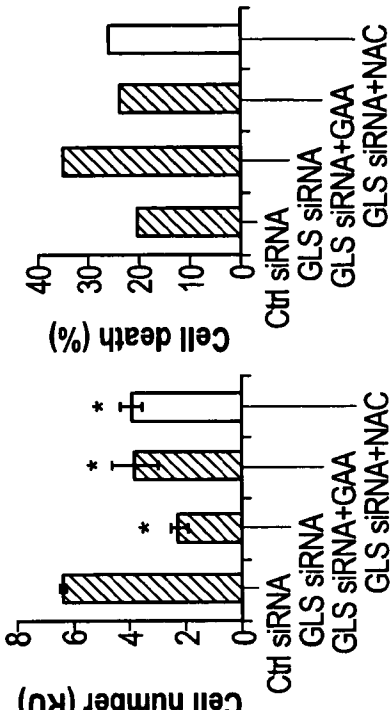


FIG. 2e



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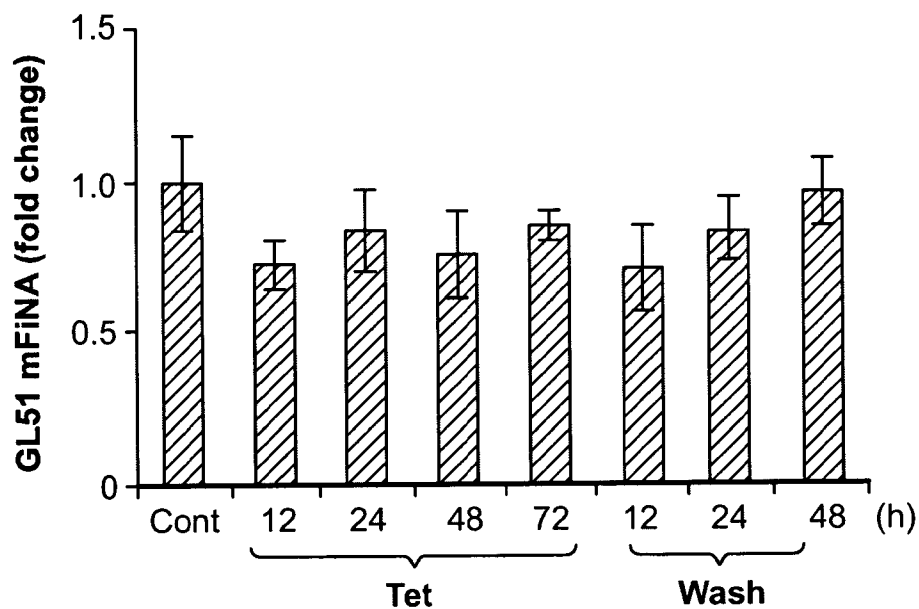


FIG. 3a

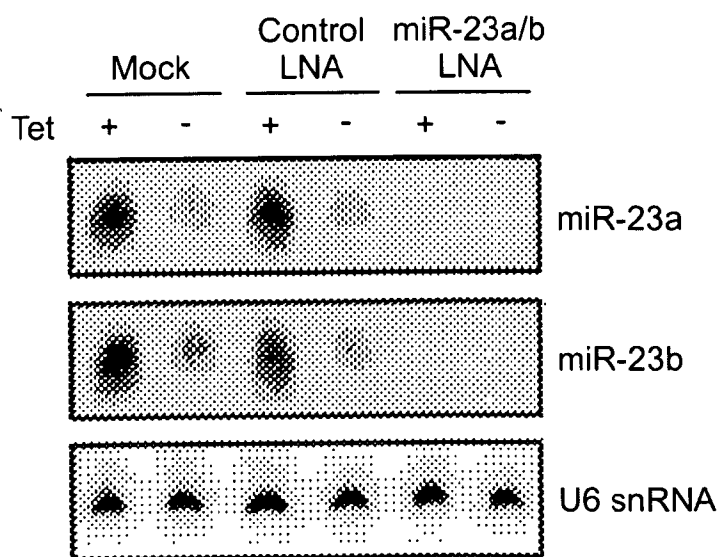


FIG. 3b

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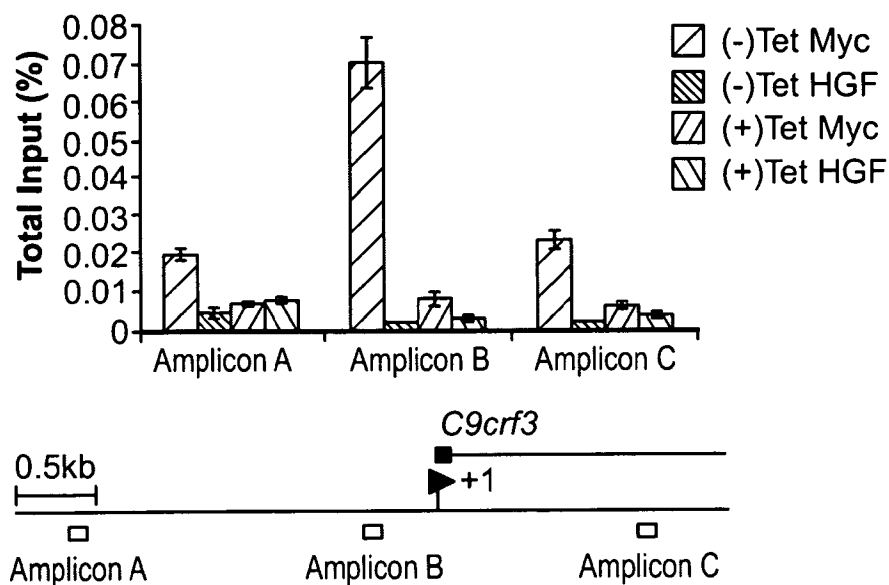
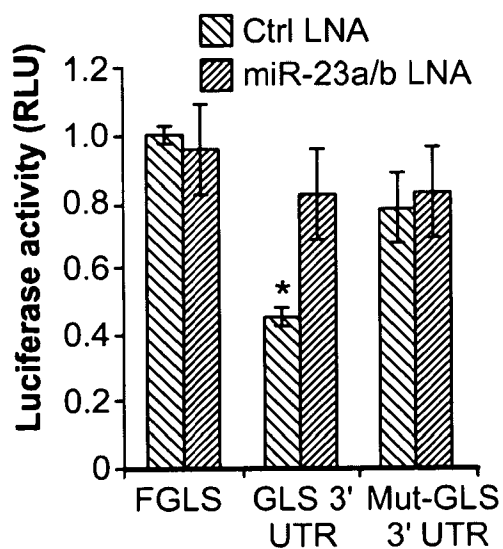


FIG. 3c



CCUUAAGGGACCG-OUACACUA miR-23a

3' CCAUUAGGGACCG-OUACACUA miR-23a

||||| |||||

3' ..ACAAUCUCCCOUCAUAAUGUGAG..GLS3'UTR

3' ..ACAAUCUCCCOUCAUAAUGUGAG..Mur-GLS3'UTR

FIG. 3d

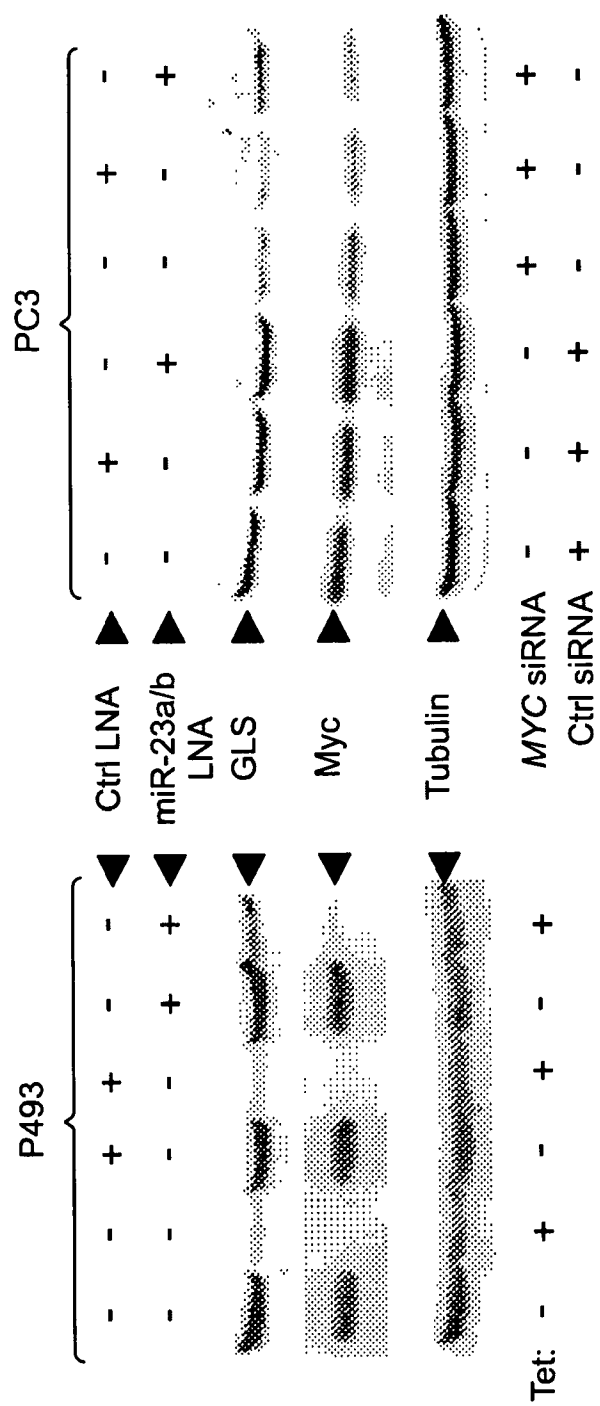


FIG. 3e

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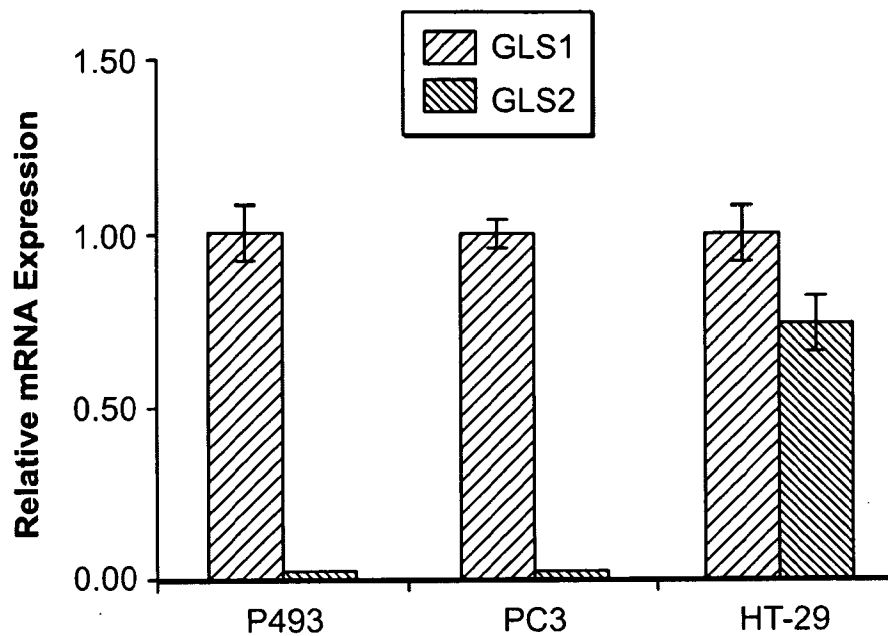


FIG. 4

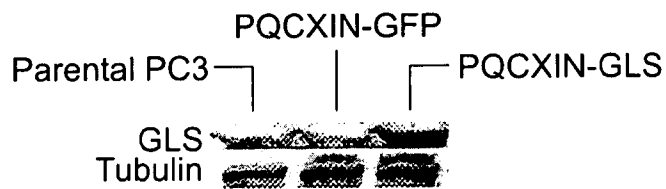


FIG. 5a

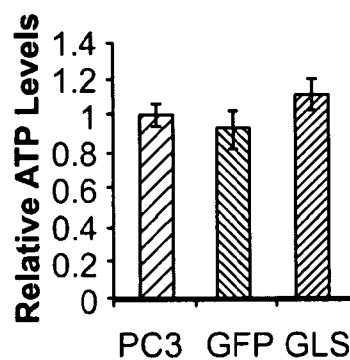


FIG. 5b

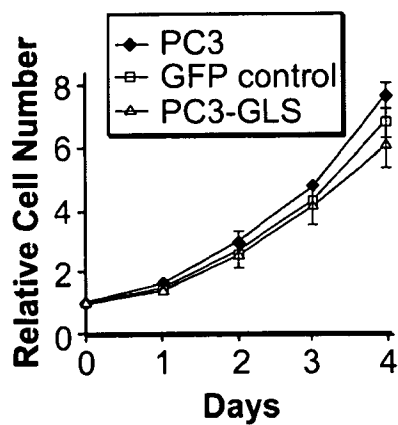


FIG. 5c

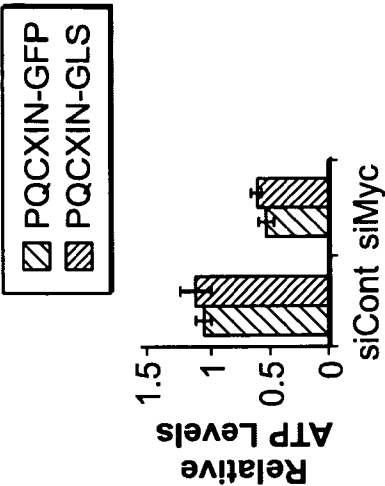


FIG. 5e

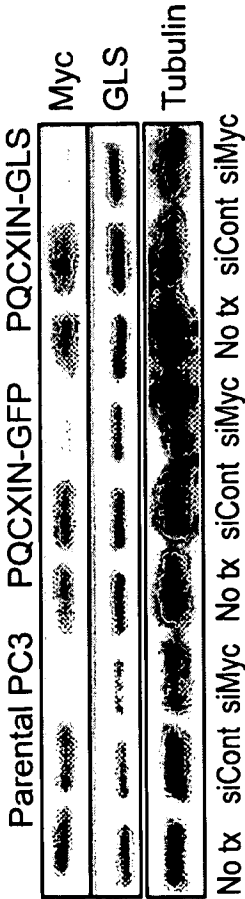


FIG. 5d

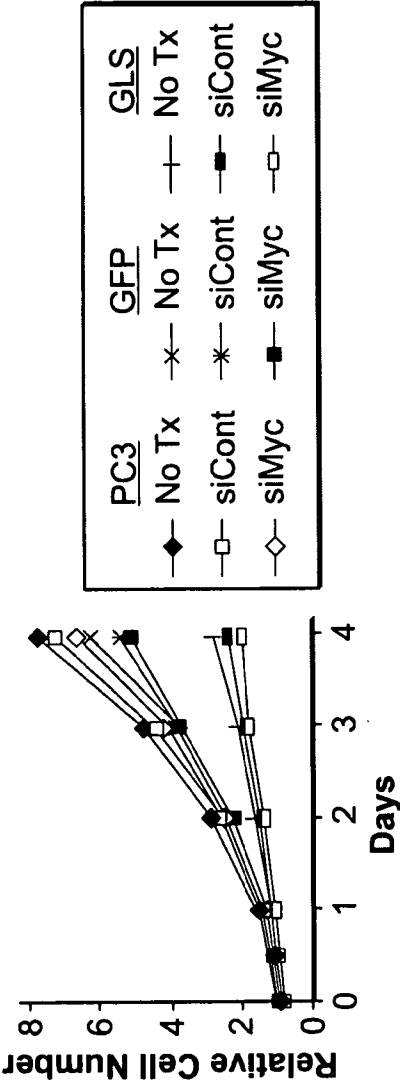
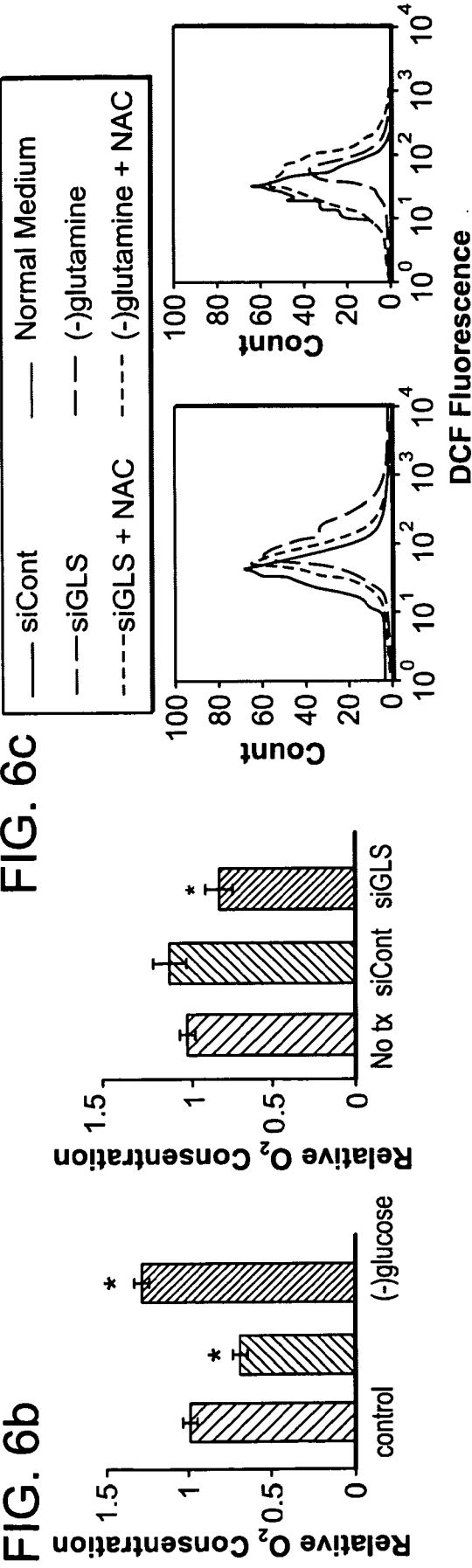
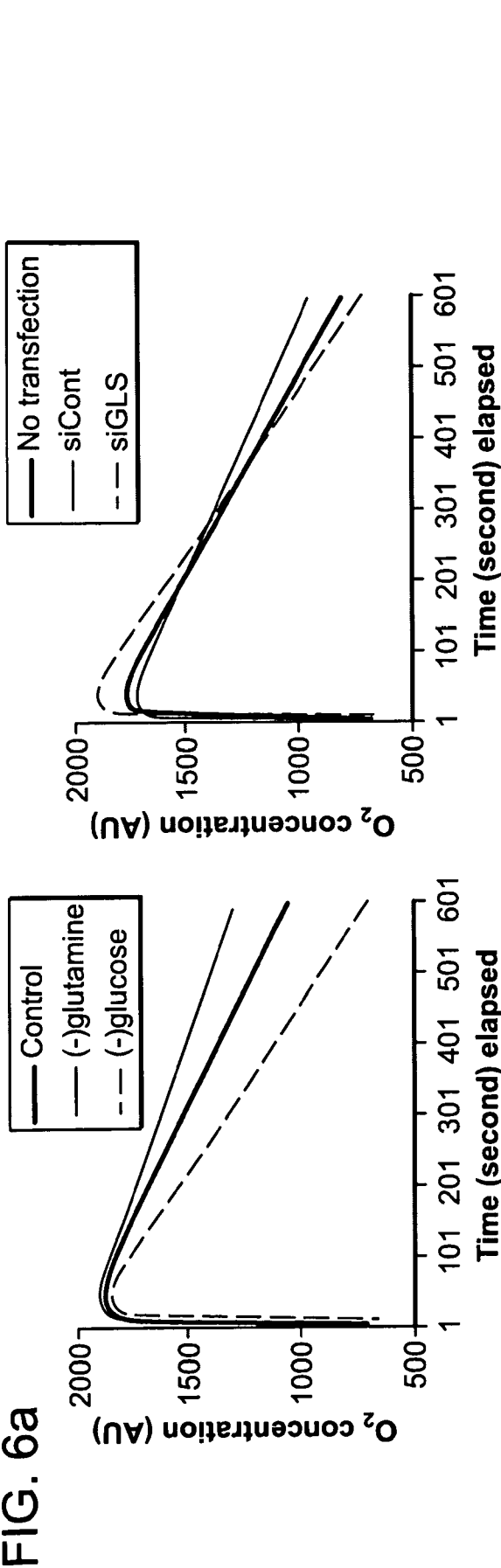
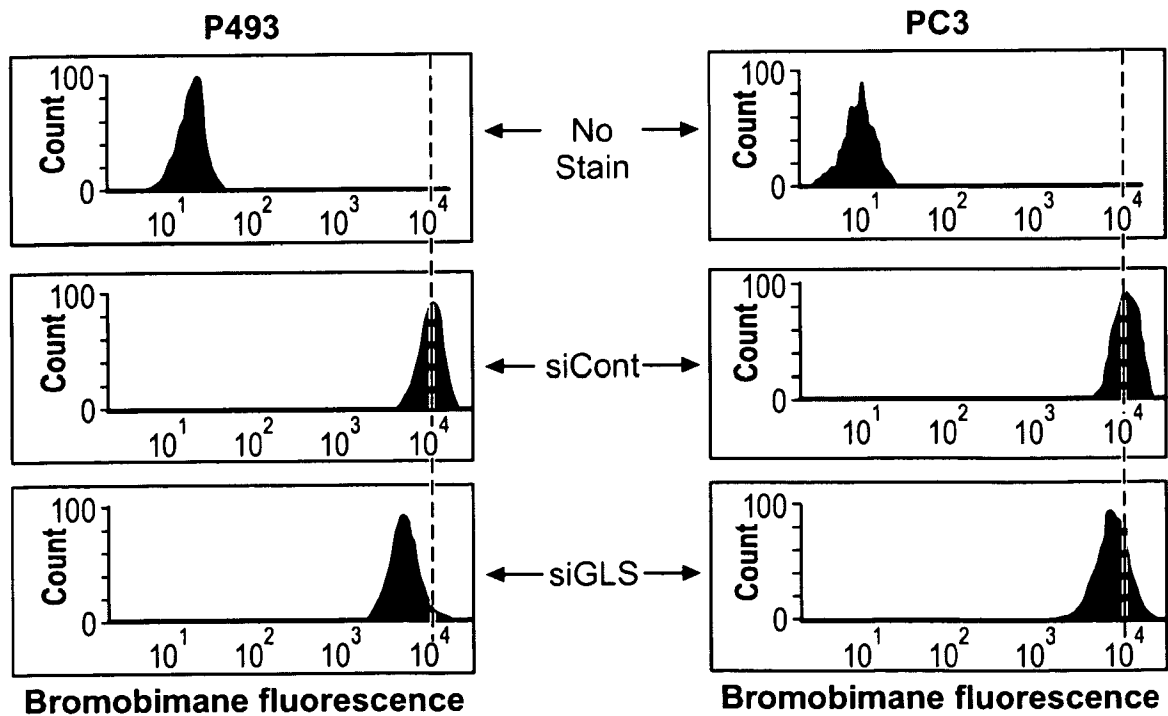
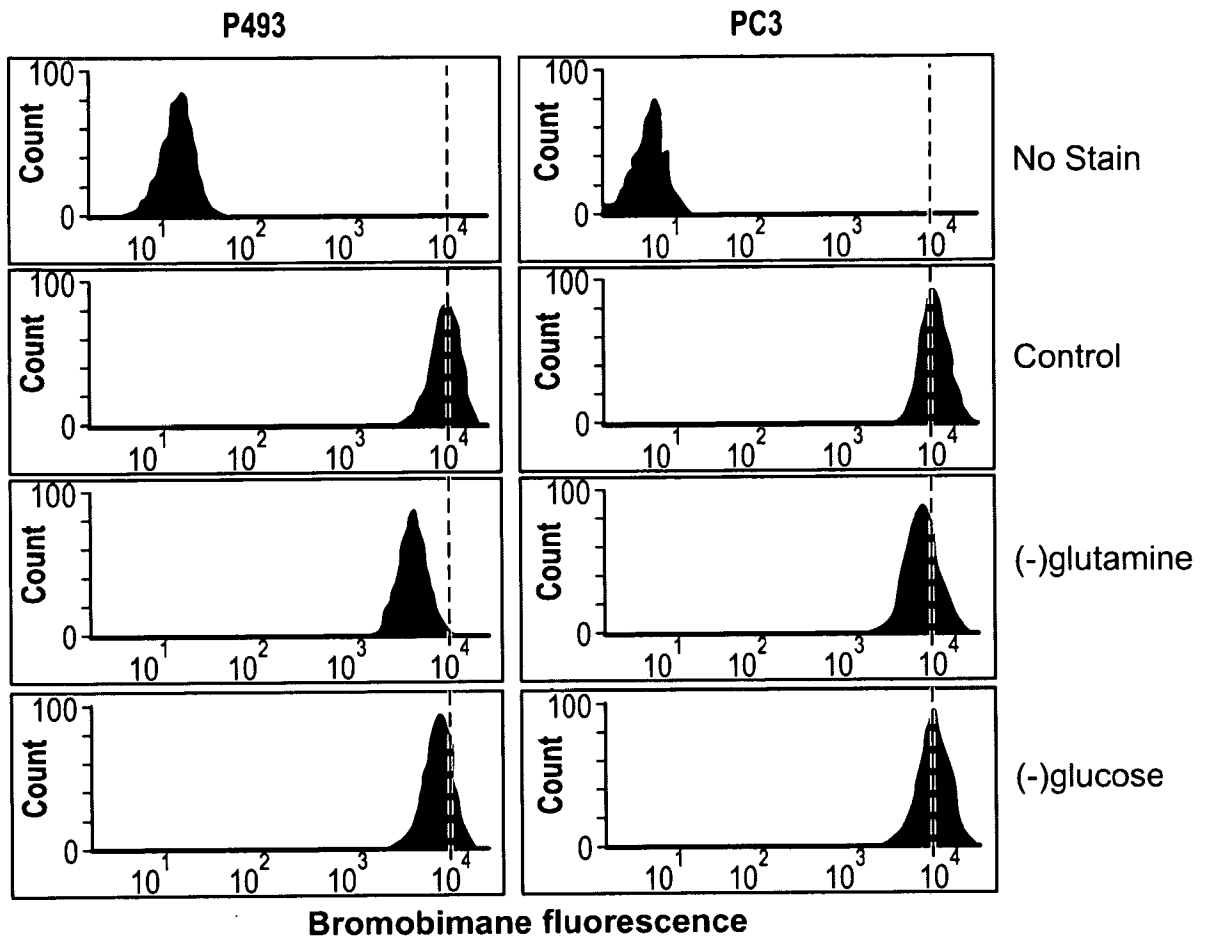


FIG. 5f



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FIG. 7



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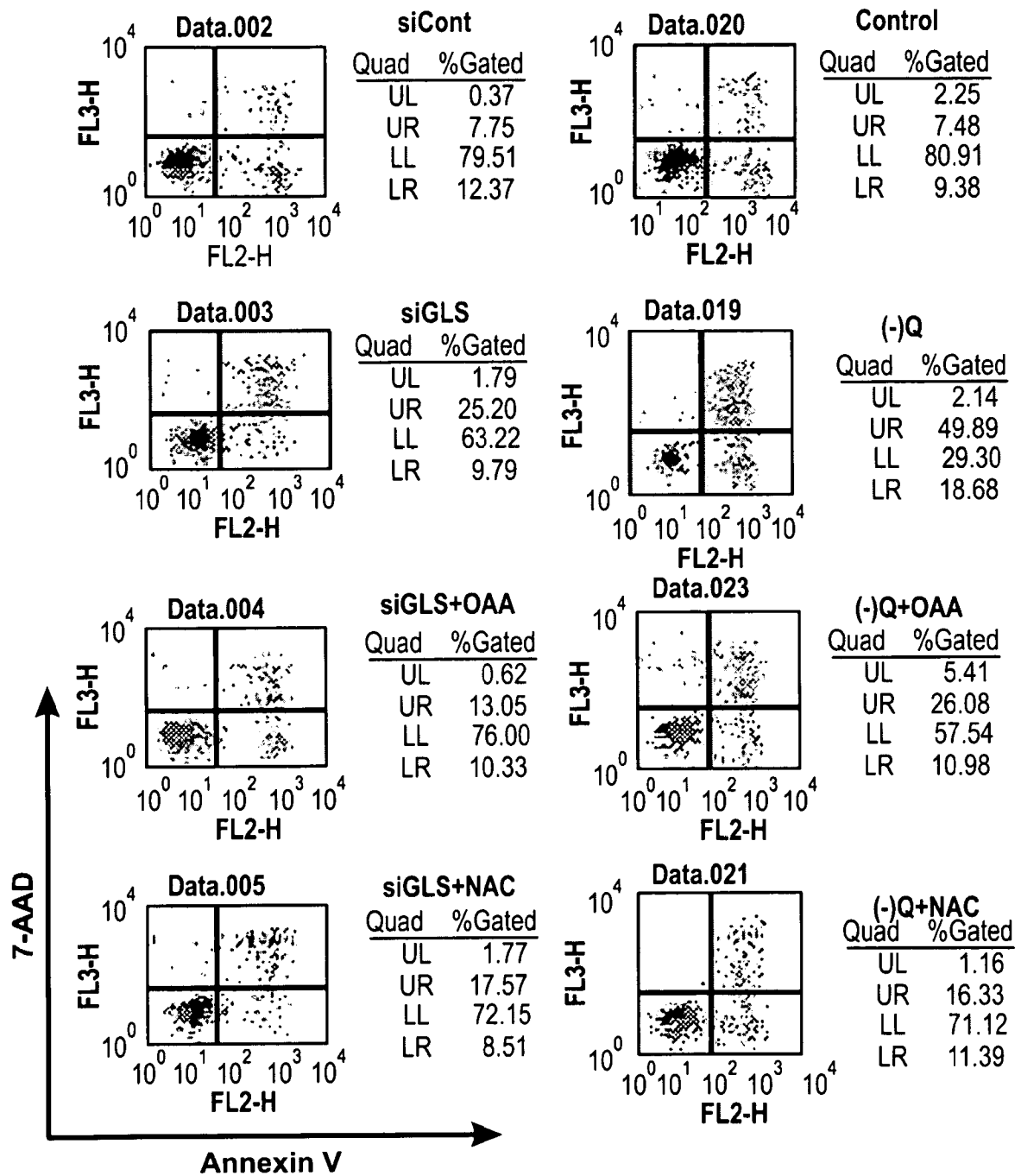


FIG. 8

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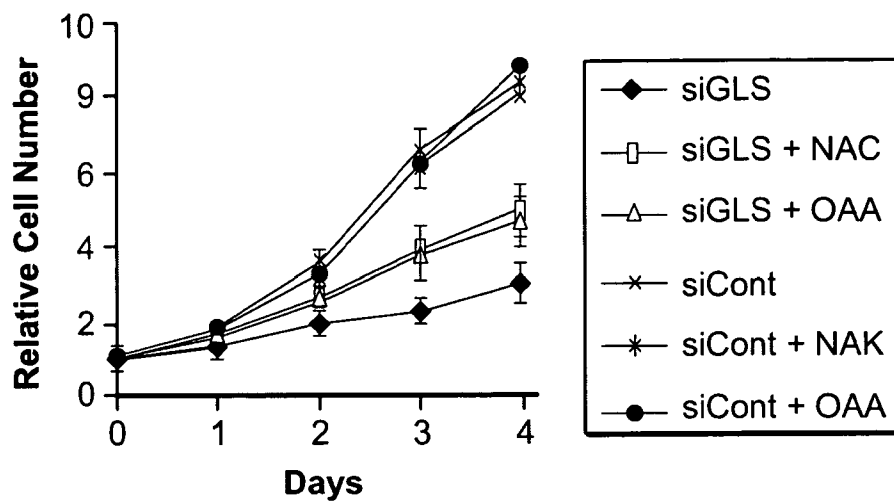


FIG. 9a

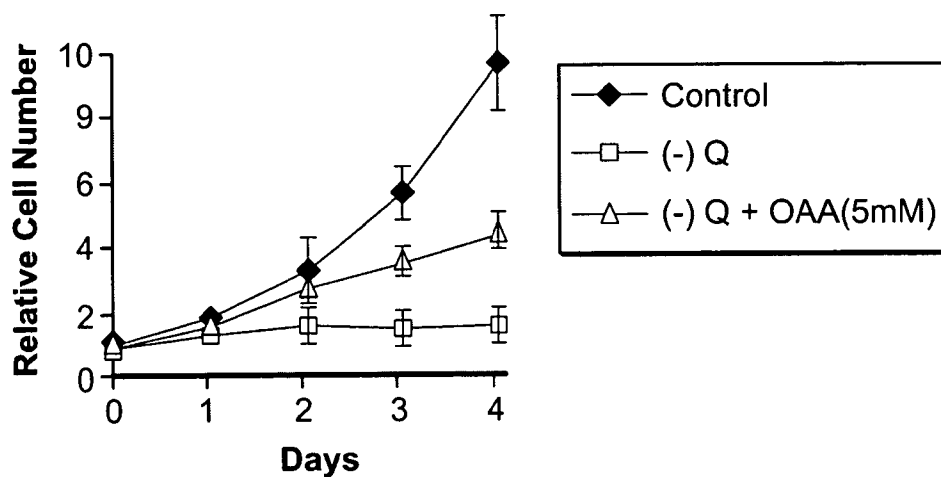


FIG. 9b

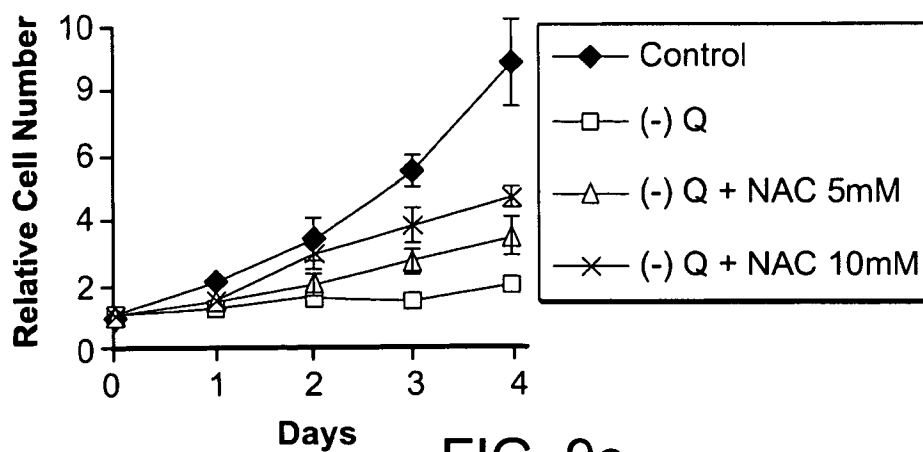


FIG. 9c

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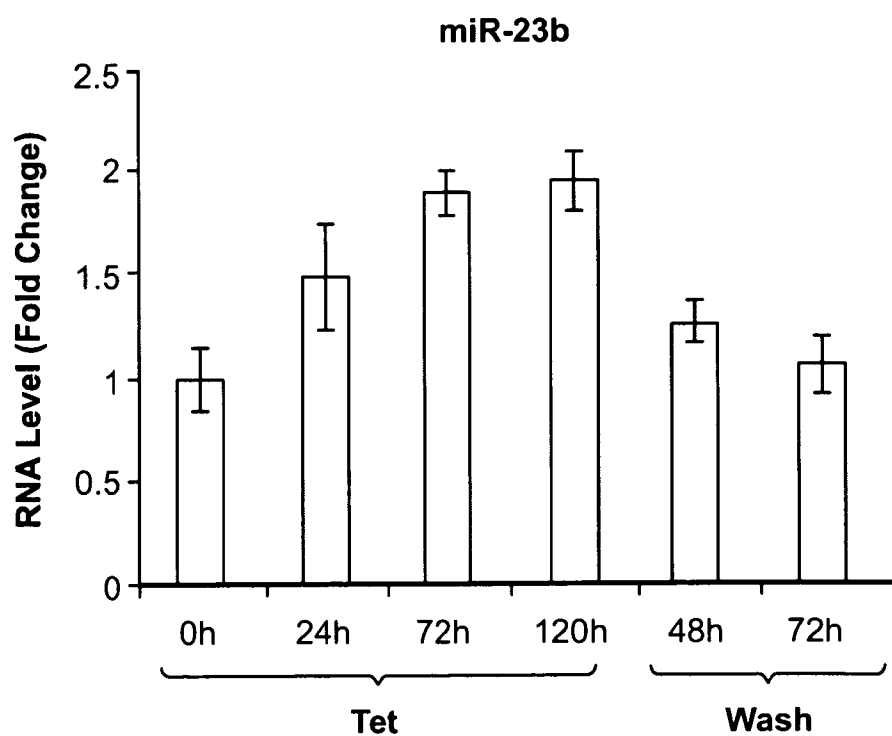
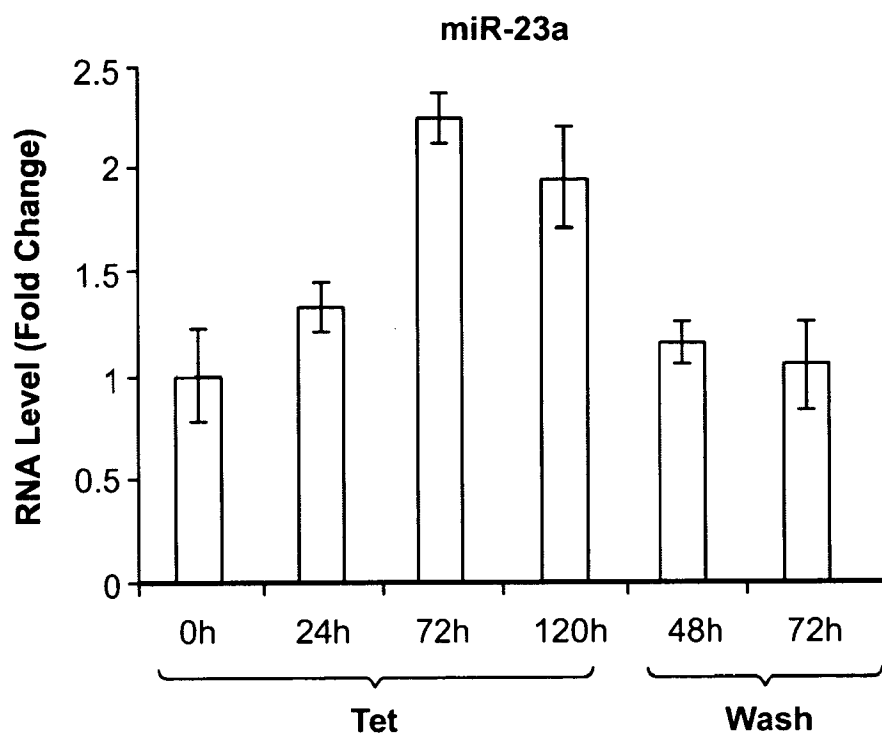


FIG. 10

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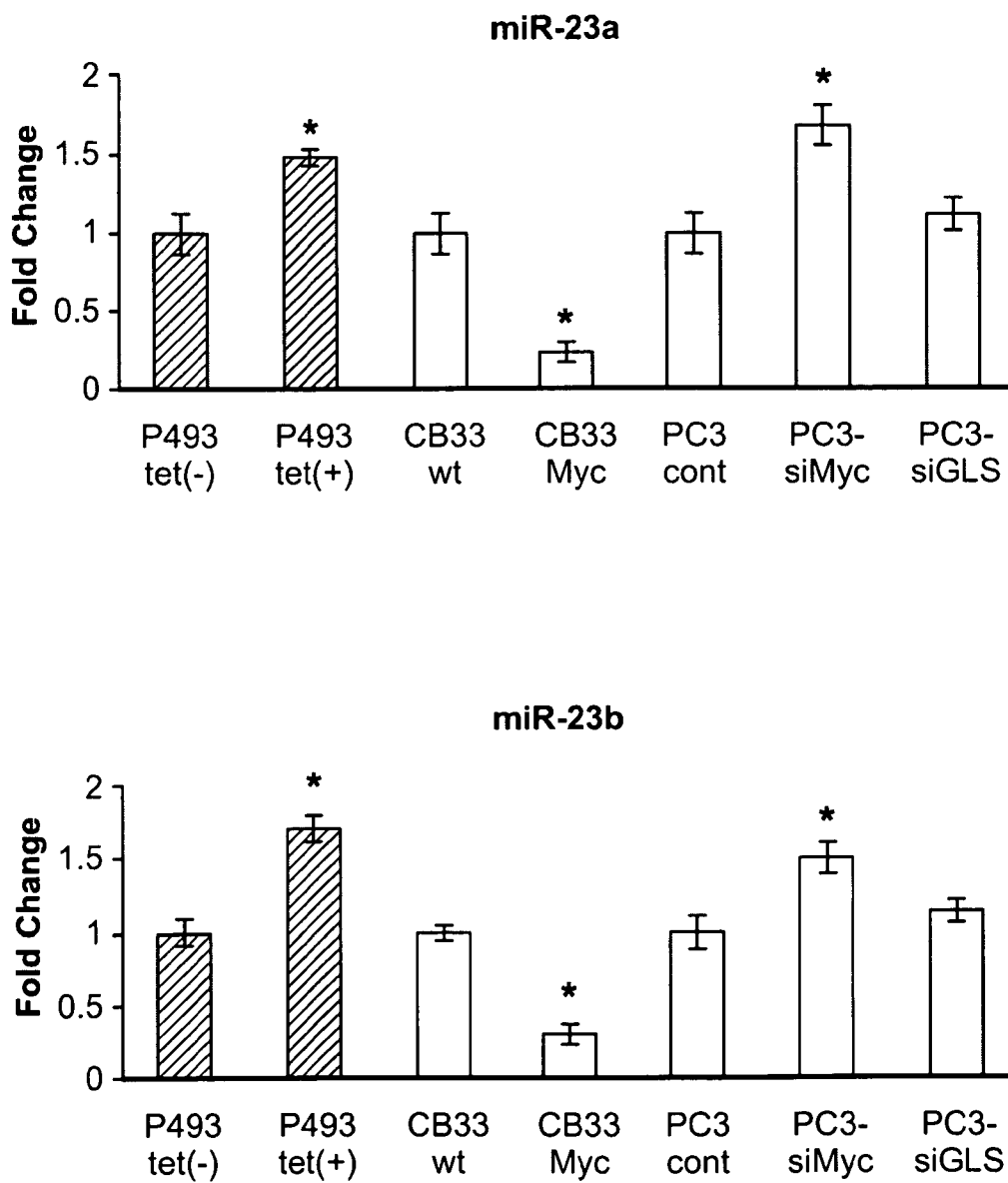


FIG. 11

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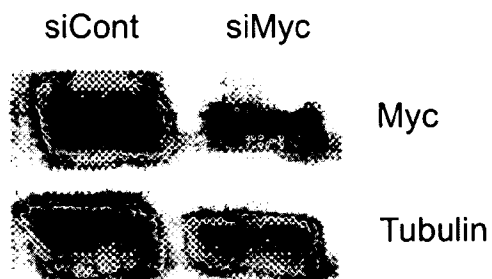


FIG. 12a

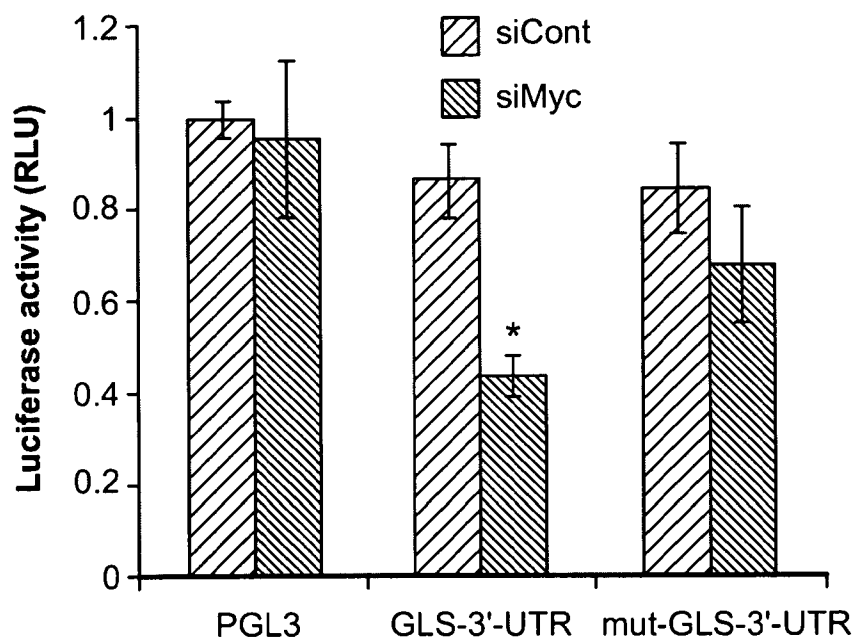


FIG. 12b

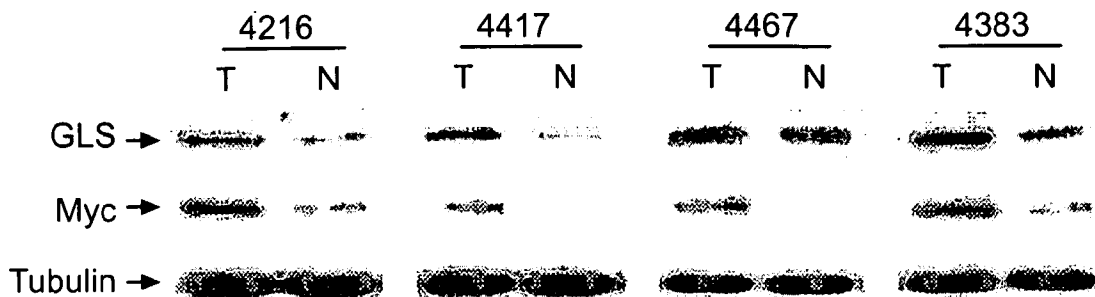


FIG. 13