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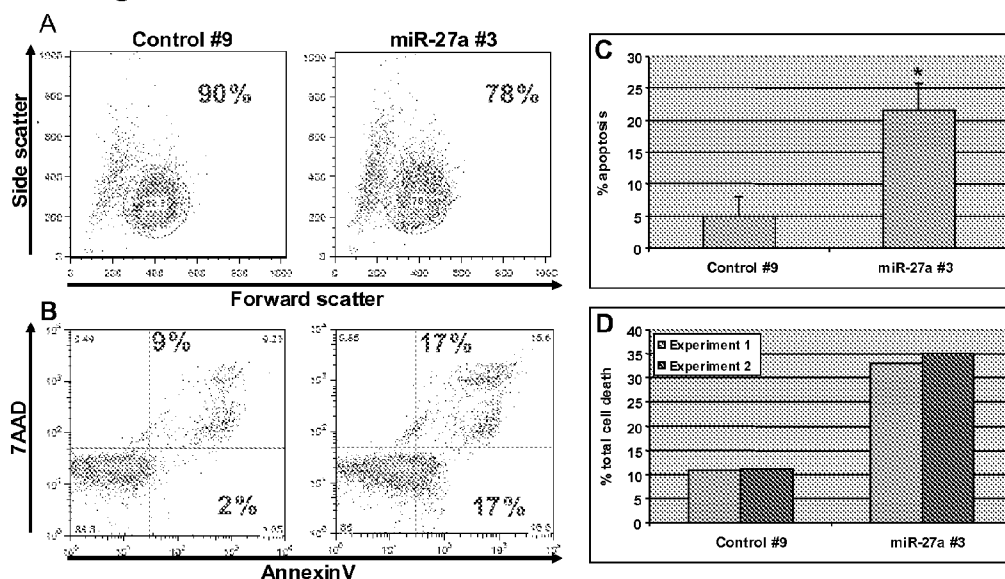
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(54) Title: MICRORNA BASED METHODS AND COMPOSITIONS FOR THE TREATMENT OF CANCER

Figure 10



(57) Abstract: The present disclosure relates to the discovery that microRNAs of the invention, in particular *miR-10a* and *miR-27a* and functional variants thereof, inhibit cancer cell growth and enhance spontaneous, chemotherapy-induced, and growth factor deprivation-induced apoptosis in hematopoietic cancer cells, but not in normal hematopoietic progenitor/stem cells. Disclosed herein are methods, compositions and kits for the use of microRNAs of the invention, *miR-10a* and *miR-27a* and functional variants thereof, in the treatment of cancer.

## MICRORNA BASED METHODS AND COMPOSITIONS FOR THE TREATMENT OF CANCER

### RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 61/011,196, filed January 15, 2008, the content of which is specifically incorporated by reference herein in its entirety.

### BACKGROUND

MicroRNAs (miRNAs) are a class of small, noncoding RNAs that function by negatively regulating the stability and/or translational efficiency of their target mRNAs (Ambros, V., Nature 431:350-55 (2004)). Hundreds of miRNAs have been identified in various species, including humans, and it has been estimated that at least 20-30% of human genes are regulated by miRNAs (Tran, N. et al., Biochem Biophys Res Commun. 358:12-17 (2007)).

Recent studies have revealed that miRNA-mediated gene regulatory mechanisms play important roles in many fundamental cellular processes, including cellular development, hematopoiesis, fat metabolism, organogenesis, proliferation and differentiation (Cheng, A. M., et al., Nucleic Acids Res. 33:1290-1297 (2005)). In mammals, aberrant expression of certain miRNAs has been implicated in several diseases, including cancer (Calin, G. A., et al., Proc. Natl. Acad. Sci. U.S.A. 99:15524-15529 (2002)). However, the cellular function of most mammalian miRNAs remains unclear, and it has thus proven difficult to develop miRNA based therapies.

Cancer remains a significant source of mortality and morbidity throughout the world. Current therapies are often ineffective in the treatment of many types of cancer. Frequently, chemotherapeutic agents that are able to induce cancer cell death are also toxic to normal, healthy cells, resulting in significant side-effects for the patient. Thus, there is a great need for novel and effective miRNA based methods and compositions for the treatment of cancer.

### SUMMARY OF THE INVENTION

The present invention relates to novel methods and compositions for the treatment of cancer. The invention is related to the discovery that certain miRNAs, *miR-10a* and *miR-27a* and functional variants thereof, inhibit cancer cell growth and enhance spontaneous, chemotherapy-induced, and growth factor deprivation-induced apoptosis in hematopoietic cancer cells, but not in normal hematopoietic progenitor/stem cells. The compositions and methods of the present invention therefore provide novel cancer therapies that enhance the effectiveness of current chemotherapeutic agents and reduce the likelihood of undesirable side-effects.

In one embodiment, the invention relates to a method for inhibiting proliferation of a cancer cell that includes contacting the cancer cell with an effective amount of a nucleic acid molecule encoding *miR-10a* or *miR-27a* or a functional variant thereof. In some embodiments the nucleic acid molecule is a miRNA, a pre-miRNA or a DNA molecule. In certain embodiments the cancer cell is transfected by the nucleic acid molecule. The invention includes embodiments in which the cancer cell is from a solid tumor, and embodiments in which the cancer cell is a leukemia cell or a lymphoma cell.

In another embodiment, the invention relates to a method for inducing apoptosis of a cancer cell that includes contacting the cancer cell with an effective amount of a nucleic acid molecule encoding of *miR-10a* or *miR-27a* or a functional variant thereof. In some embodiments the nucleic acid molecule is a miRNA, a pre-miRNA or a DNA molecule. In certain embodiments the cancer cell is transfected by the nucleic acid molecule. The invention includes embodiments in which the cancer cell is from a solid tumor, and embodiments in which the cancer cell is a leukemia cell or a lymphoma cell.

In some embodiments, the method also includes contacting the cancer cell with a chemotherapeutic agent. In certain embodiments, the chemotherapeutic agent is selected from a group consisting of: altretamine, asparaginase, BCG, bleomycin sulfate, busulfan, camptothecin, carboplatin, carmusine, chlorambucil, cisplatin, cladribine, 2-chlorodeoxyadenosine, cyclophosphamide, cytarabine, dacarbazine imidazole carboxamide, dactinomycin, daunorubicin - dunomycin, dexamethosone, doxorubicin, etoposide, floxuridine, fluorouracil, fluoxymesterone, flutamide, fludarabine, goserelin, hydroxyurea, idarubicin HCL, ifosfamide, interferon alfa, interferon alfa 2a, interferon alfa 2b, interfereon alfa n3, irinotecan, leucovorin calcium, leuprolide, levamisole, lomustine, megestrol, melphalan, L-sarcosylin, melphalan hydrochloride, MESNA, mechlorethamine, methotrexate, mitomycin, mitoxantrone, mercaptopurine, paclitaxel, plicamycin, prednisone, procarbazine, streptozocin, tamoxifen, 6-thioguanine, thiotepa, topotecan, vinblastine, vincristine and vinorelbine tartrate.

In certain embodiments, the invention relates to a method for treating cancer in a subject that includes administering to the subject an effective amount of a nucleic acid molecule encoding *miR-10a* or *miR-27a* or a functional variant thereof. In some embodiments the nucleic acid molecule is a miRNA, a pre-miRNA or a DNA molecule. In some embodiments the cancer is a solid tumor, a lymphoma or a leukemia.

In some embodiments, the method also includes administering to the patient a chemotherapeutic agent. In some instances, the chemotherapeutic agent is selected from a group consisting of altretamine, asparaginase, BCG, bleomycin sulfate, busulfan, camptothecin, carboplatin, carmusine, chlorambucil, cisplatin, cladribine, 2-chlorodeoxyadenosine,

cyclophosphamide, cytarabine, dacarbazine imidazole carboxamide, dactinomycin, daunorubicin - dunomycin, dexamethosone, doxorubicin, etoposide, floxuridine, fluorouracil, fluoxymesterone, flutamide, fludarabine, goserelin, hydroxyurea, idarubicin HCL, ifosfamide, interferon alfa, interferon alfa 2a, interferon alfa 2b, interfereon alfa n3, irinotecan, leucovorin calcium, leuprolide, levamisole, lomustine, megestrol, melphalan, L-sarcosylin, melphalan hydrochloride, MESNA, mechlorethamine, methotrexate, mitomycin, mitoxantrone, mercaptopurine, paclitaxel, plicamycin, prednisone, procarbazine, streptozocin, tamoxifen, 6-thioguanine, thiotepa, topotecan, vinblastine, vincristine and vinorelbine tartrate.

In some embodiments, the invention concerns a pharmaceutical composition that includes an isolated nucleic acid molecule encoding *miR-10a* or *miR-27a* or a functional variant thereof. The pharmaceutical composition of the invention may also include a chemotherapeutic agent and/or a pharmaceutically-acceptable carrier. In some embodiments, the chemotherapeutic agent is selected from a group consisting of: altretamine, asparaginase, BCG, bleomycin sulfate, busulfan, camptothecin, carboplatin, carmusine, chlorambucil, cisplatin, claladribine, 2-chlorodeoxyadenosine, cyclophosphamide, cytarabine, dacarbazine imidazole carboxamide, dactinomycin, daunorubicin - dunomycin, dexamethosone, doxorubicin, etoposide, floxuridine, fluorouracil, fluoxymesterone, flutamide, fludarabine, goserelin, hydroxyurea, idarubicin HCL, ifosfamide, interferon alfa, interferon alfa 2a, interferon alfa 2b, interfereon alfa n3, irinotecan, leucovorin calcium, leuprolide, levamisole, lomustine, megestrol, melphalan, L-sarcosylin, melphalan hydrochloride, MESNA, mechlorethamine, methotrexate, mitomycin, mitoxantrone, mercaptopurine, paclitaxel, plicamycin, prednisone, procarbazine, streptozocin, tamoxifen, 6-thioguanine, thiotepa, topotecan, vinblastine, vincristine and vinorelbine tartrate. In some embodiments the nucleic acid molecule is a miRNA, a pre-miRNA or a DNA molecule.

Certain embodiments of the invention relate to a kit that contains an isolated nucleic acid molecule encoding *miR-10a* or *miR-27a* or a functional variant thereof. The kit of the invention may also include a chemotherapeutic agent and/or a pharmaceutically-acceptable carrier. In some embodiments, the chemotherapeutic agent is selected from a group consisting of: altretamine, asparaginase, BCG, bleomycin sulfate, busulfan, camptothecin, carboplatin, carmusine, chlorambucil, cisplatin, claladribine, 2-chlorodeoxyadenosine, cyclophosphamide, cytarabine, dacarbazine imidazole carboxamide, dactinomycin, daunorubicin - dunomycin, dexamethosone, doxorubicin, etoposide, floxuridine, fluorouracil, fluoxymesterone, flutamide, fludarabine, goserelin, hydroxyurea, idarubicin HCL, ifosfamide, interferon alfa, interferon alfa 2a, interferon alfa 2b, interfereon alfa n3, irinotecan, leucovorin calcium, leuprolide, levamisole, lomustine, megestrol, melphalan, L-sarcosylin, melphalan hydrochloride, MESNA, mechlorethamine, methotrexate, mitomycin, mitoxantrone, mercaptopurine, paclitaxel, plicamycin, prednisone,

procarbazine, streptozocin, tamoxifen, 6-thioguanine, thiotepa, topotecan, vinblastine, vincristine and vinorelbine tartrate. In some embodiments the nucleic acid molecule is a miRNA, a pre-miRNA or a DNA molecule.

### BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1** shows the sequences of *miR-10a*, *miR\*-10a*, *pre-miR-10a*, *miR-27a*, *miR\*-27a* and *pre-miR-27a*.

**Figure 2** shows *miR-10a*, *miR-27a*, *miR-16a*, and *miR-155* expression in normal and malignant human hematopoietic cells.

**Figure 3** shows the growth rate of TF-1a (A) and K562 (B) cells transfected with *miR-10a* or lipofectamine alone as assessed by an MTT assay.

**Figure 4** shows the growth rate of TF-1a cells transfected with *miR-10a*, *miR-10a* and anti-*miR-10a* LNA or lipofectamine alone as assessed by an MTT assay.

**Figure 5** shows the growth rate of TF-1a (A) and K562 (B) cells transfected with *miR-27a* or lipofectamine alone as assessed by an MTT assay.

**Figure 6** shows the amount of apoptosis in methotrexate treated TF-1a cells transfected with lipofectamine alone (A, left) or transfected with *miR-27a* (A, right) and the percent increase in apoptosis over background in Hematopoietic Stem Progenitor Cells and TF-1a cells that had been treated with methotrexate and transfected with *miR-27a* (B).

**Figure 7** shows the amount of apoptosis in TF-1a cells that had been transfected with *miR-27a* or lipofectamine alone that had been treated with a chemotherapy vehicle mock (A), Staurosporine (B) or Camptothecin (C).

**Figure 8** shows the effect of growth factor withdrawal on TF-1a human myeloid leukemia cells that had been transfected with *miR-27a* or lipofectamine alone after 24 (A and B) or 48 (C and D) hours cultured in the absence of GM-CSF.

**Figure 9** shows the GFP expression of the human acute lymphoblastic leukemia cell line REH that had been transduced with lentivectors that express either GFP (top) or GFP and *miR-27a* (bottom) as assessed by FACS.

**Figure 10** shows the viability (A), apoptosis levels (B, C) and cell death levels (B, D) of REH cells that had been transduced with either a control lentivector or a *miR-27a* expressing lentivector.

**Figure 11** shows the level of apoptosis and cell death of REH cells that had been transduced with either a control lentivector (A, C-F) or a *miR-27a* lentivector (B, C-F) and that had been exposed to different levels of vincristine.

**Figure 12** shows the GFP expression of the human acute myeloid leukemia cell line HL60 that had been transduced with a *miR-27a* expressing lentivector or a control lentivector as assessed by FACS.

**Figure 13** shows the cell viability of HL60 cells that had been transduced with a *miR-27a* expressing lentivector or a control lentivector cells after 1 week (A, C) and after 2 weeks (B, C).

**Figure 14** shows the apoptosis levels of HL60 cells that had been transduced with a *miR-27a* expressing lentivector, transduced with a control lentivector, or unsuccessfully transduced GFP<sup>+</sup> cells.

**Figure 15** shows the cell cycle profile of HL60 cells that had been transduced with a *miR-27a* expressing lentivector, transduced with a control lentivector, or unsuccessfully transduced GFP<sup>+</sup> cells as assessed by propidium iodide staining and FACS (A) and a table summarizing the results (B).

**Figure 16** shows the apoptosis and cell death levels of TF1 human Erythroleukemia cells that had been transduced with a *miR-27a* expressing lentivector or a control lentivector (A) and a table summarizing the results (B).

**Figure 17** shows the GFP expression of the human myeloid leukemia cell line K562 that had been transduced with a *miR-27a* expressing lentivector (A, B) or a control lentivector (B) as assessed by FACS.

**Figure 18** shows the viability of K562 cells that had been transduced with *miR-27a* expressing lentivector and unsuccessfully transduced K562 cells (A) and a table summarizing the results (B).

**Figure 19** shows the cell death levels of K562 cells that had been transduced with *miR-27a* expressing lentivector and unsuccessfully transduced K562 cells (A) and a table summarizing the results (B).

**Figure 20** shows the apoptosis levels of K562 cells that had been transduced with a *miR-27a* expressing lentivector, transduced with a control lentivector, or unsuccessfully transduced GFP<sup>+</sup> cells (A) and a table summarizing the results (B).

**Figure 21** shows the cell cycle profile of K562 cells that had been transduced with a *miR-27a* expressing lentivector, or unsuccessfully transduced GFP<sup>+</sup> cells as assessed by propidium iodide staining and FACS (A) and a table summarizing the results (B).

**Figure 22** shows the results of luciferase reporter assays that demonstrate that *miR-27a* binds to the 3'-untranslated region of YWHAQ (14-3-30) (A), Polo-like kinase 2 (PLK2) (B) and the multi-drug resistant transporter ABCC4 (C, D).

## DETAILED DESCRIPTION OF THE INVENTION

### I. Definitions

The present invention is directed to novel methods and compositions relating to the use of miRNAs in the treatment of cancer. In order for the present invention to be more readily understood, certain terms and phrases are defined below and throughout the specification.

As used herein, the term "miRNA" or "miR" means a non-coding RNA between 17 and 25 nucleobases in length which hybridizes to and regulates the expression of a coding RNA. A 17-25 nucleotide miRNA molecule can be obtained from a miR precursor through natural processing routes (e.g., using intact cells or cell lysates) or by synthetic processing routes (e.g., using isolated processing enzymes, such as isolated Dicer, Argonaut, or RNAase III). It is understood that the 17-25 nucleotide RNA molecule can also be produced directly by biological or chemical syntheses, without having been processed from a miR precursor. For ease of discussion, the phrase "miR gene expression products" encompasses both miRNAs produced through pre-miRNA processing and miRNAs produced through direct biological or chemical synthesis.

As used herein, the term "miR precursor," "pre-miRNA" or "pre-miR" means a non-coding RNA having a hairpin structure, which contains a miRNA. In certain embodiments, a pre-miRNA is the product of cleavage of a primary mi-RNA transcript, or "pri-miR" by the double-stranded RNA-specific ribonuclease known as Drosha, but a pre-miRNAs can also be produced directly by biological or chemical synthesis without having been processed from a pri-miR.

As used herein, the term "subject" means a human or non-human animal selected for treatment or therapy.

As used herein, the term "subject suspected of having" means a subject exhibiting one or more clinical indicators of a disease or condition. In certain embodiments, the disease or condition is cancer. In certain embodiments, the cancer is leukemia or lymphoma.

As used herein, the term "subject in need thereof" means a subject identified as in need of a therapy or treatment.

As used herein, the term “preventing” or “prevention” refers to delaying or forestalling the onset, development or progression of a condition or disease for a period of time, including weeks, months, or years.

As used herein, the term “treatment” or “treat” means the application of one or more procedures used for the amelioration of a disease. In certain embodiments the specific procedure is the administration of one or more pharmaceutical agents. In some embodiments, the pharmaceutical agents include microRNA. In some embodiments the pharmaceutical agent is *miR-10a* or *miR-27a* or functional variants thereof.

As used herein, the term “amelioration” means a lessening of severity of at least one indicator of a condition or disease. In certain embodiments, amelioration includes a delay or slowing in the progression of one or more indicators of a condition or disease. The severity of indicators may be determined by subjective or objective measures which are known to those skilled in the art.

As used herein, the term “cancer” includes, but is not limited to, solid tumors and blood borne tumors, including leukemia and lymphoma. The term cancer refers to diseases of the skin, tissues, organs, bone, cartilage, blood and vessels. The term “cancer” further encompasses primary and metastatic cancers.

As used herein, the term “administering” means providing a pharmaceutical agent or composition to a subject, and includes, but is not limited to, administering by a medical professional and self-administering.

As used herein, the term “pharmaceutical composition” means a mixture of substances suitable for administering to an individual that includes a pharmaceutical agent. For example, a pharmaceutical composition may comprise a miRNA encoding oligonucleotide and a sterile aqueous solution.

As used herein, the term “pharmaceutical agent” refers to a substance that provides a therapeutic effect when administered to a subject.

As used herein, the term “modulation” means to a perturbation of function or activity. In certain embodiments, modulation means an increase in gene expression, mRNA translation or protein function. In certain embodiments, modulation means a decrease in gene expression, mRNA translation or protein function.

As used herein, the term “expression” means any functions and steps by which a gene's coded information is converted into structures present and operating in a cell.

As used herein, the term “complementary” means a first nucleobase sequence is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98% or 99% identical, or is 100% identical, to the complement of a second nucleobase sequence over a region of 8, 9, 10, 11, 12, 13, 14, 15, 16,

17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more nucleobases, or that the two sequences hybridize under stringent hybridization conditions. In certain embodiments an oligonucleotide that contains a nucleobase sequence which is 100% complementary to a miRNA, or precursor thereof, may not be 100% complementary to the miRNA, or precursor thereof, over the entire length of the oligonucleotide.

As used herein, the term "complementarity" means the nucleobase pairing ability between a first nucleic acid and a second nucleic acid.

As used herein, the term "full-length complementarity" means each nucleobase of a first nucleic acid is capable of pairing with each nucleobase at a corresponding position in a second nucleic acid. For example, in certain embodiments, an oligonucleotide wherein each nucleobase has complementarity to a nucleobase in a miRNA has full-length complementarity to the miRNA.

As used herein, the term "percent complementary" means the number of complementary nucleobases in a nucleic acid divided by the length of the nucleic acid. In certain embodiments, percent complementarity of an oligonucleotide means the number of nucleobases that are complementary to the target nucleic acid, divided by the number of nucleobases of the oligonucleotide. In certain embodiments, percent complementarity of an oligonucleotide means the number of nucleobases that are complementary to a miRNA, divided by the number of nucleobases of the oligonucleotide.

As used herein, the term "percent identity" means the number of nucleobases in a first nucleic acid that are identical to nucleobases at corresponding positions in a second nucleic acid, divided by the total number of nucleobases in the first nucleic acid.

As used herein, the term "substantially identical" may mean that a first and second nucleobase sequence are at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98% or 99% identical, or 100% identical, over a region of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more nucleobases.

As used herein, the term "identical" means having the same nucleobase sequence.

As used herein, the term "oligonucleotide" means a polymer of linked nucleosides, each of which can be modified or unmodified, independent from one another.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a

host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors".

## II. MicroRNA

MicroRNA molecules ("miRNAs") are single-stranded RNA molecules that are generally 21 to 23 nucleotides in length, though lengths of 17 to 25 nucleotides have been reported. Individual miRNAs are derived from a longer precursor RNA molecule ("precursor miRNA," or "pre-miRNA"). The precursor miRNAs have two regions of complementarity that enables them to form a stem-loop- or fold-back-like structure, which is cleaved by an enzyme called Dicer in animals. Dicer is ribonuclease III-like nuclease. The processed miRNA is typically a portion of the stem.

Processed miRNA (also referred to as "mature miRNA" or "miRNA") become part of a large complex to down-regulate a particular target gene. Examples of animal miRNAs include those that imperfectly basepair with a portion of a target mRNA, which halts the translation of the target (Olsen et al., 1999; Seggerson et al., 2002). In other cases, binding of miRNA to a target mRNA may result in target degradation.

The present invention is directed to compositions and methods related to the use of miRNAs in the treatment of cancer. Specifically, the present invention is based on the inventor's discovery that certain miRNAs, in particular *miR-10a* and *miR-27a*, and functional variants thereof are expressed in normal cells but are not expressed in cancer cells, and that introduction of any of these miRNAs into cancer cells results in reduced proliferation, increased apoptosis, and increased sensitivity to chemotherapeutic agents in cancer cells. On the other hand, normal hematopoietic cells are not affected by introduction of these miRNAs.

The sequence for human *miR-10a* is provided in Figure 1 and SEQ ID NO: 1. Endogenous *miR-10a* is derived from *pre-miR-10a* (SEQ ID NO: 3), which forms a stem-loop secondary structure in which the *miR-10a* sequence hybridizes to the complementary strand *miR\*-10a* (SEQ ID NO: 2). Human *miR-10a* is located on chromosome 17 within a cluster of homobox (Hox) genes, and may be involved in Hox gene regulation (Mansfield *et al.* Nature Genetics, 36(10): 1079-83 (2004)) and megakaryocyte differentiation (Garzon *et al.* PNAS, 103(13): 5078-83 (2006)).

The sequence for human *miR-27a* is provided in Figure 1 and SEQ ID NO: 4. Endogenous *miR-27a* is derived from *pre-miR-27a* (SEQ ID NO: 6), which forms a stem-loop secondary structure in which the *miR-27a* sequence hybridizes to the complementary strand *miR\*-27a* (SEQ ID NO: 5). Suspected targets of *miR-27a* include *ZBTB10* and *Myt-1* (Scott *et al.* Cancer Research, 66(3):1277-81 (2006); Mertens-Talcott *et al.* Cancer Research, 67(22):1001-11 (2007)).

It is well known in the art that modifications can be made to the sequence of a miRNA or a pre-miRNA without disrupting miRNA activity. As used herein, the term "functional variant" of a miRNA sequence refers to an oligonucleotide sequence that varies from the natural miRNA sequence, but retains one or more functional characteristics of the miRNA (*e.g.* cancer cell proliferation inhibition, induction of cancer cell apoptosis, enhancement of cancer cell susceptibility to chemotherapeutic agents, specific miRNA target inhibition). In some embodiments, a functional variant of a miRNA sequence retains all of the functional characteristics of the miRNA. In certain embodiments, a functional variant of a miRNA has a nucleobase sequence that is at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the miRNA or precursor thereof over a region of about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more nucleobases, or that the functional variant hybridizes to the complement of the miRNA or precursor thereof under stringent hybridization conditions. Accordingly, in certain embodiments the nucleobase sequence of a functional variant may be capable of hybridizing to one or more target sequences of the miRNA.

It is understood that any nucleobase sequences set forth herein are independent of any modification to a sugar moiety, an internucleoside linkage, or a nucleobase. It is further understood that a nucleobase sequence comprising U's also encompasses the same nucleobase sequence wherein 'U' is replaced by 'T' at one or more positions having 'U.' Conversely, it is understood that a nucleobase sequence comprising T's also encompasses the same nucleobase sequence wherein 'T' is replaced by 'U' at one or more positions having 'T.'

Nucleobase sequences miRNAs and their corresponding stem-loop sequences described herein may be found in miRBase, an online searchable database of miRNA sequences and annotation, found at <http://microrna.sanger.ac.uk/>. Entries in the miRBase Sequence database represent a predicted hairpin portion of a miRNA transcript (the stem-loop), with information on the location and sequence of the mature miRNA sequence. The miRNA stem-loop sequences in the database are not strictly precursor miRNAs (pre-miRNAs), and may in some instances include the pre-miRNA and some flanking sequence from the presumed primary transcript. The miRNA nucleobase sequences described herein encompass any version of the miRNA, including the sequences described in Release 10.0 of the miRBase sequence database and sequences described in any earlier Release of the miRBase sequence database. A sequence database release may result in the re-naming of certain miRNAs. A sequence database release may result in a variation of a mature miRNA sequence.

In some embodiments, miRNA sequences of the invention (*e.g.* *miR-10a* and *miR-27a* and functional variants thereof) may be associated with a second RNA sequence that may be located on

the same RNA molecule or on a separate RNA molecule as the miRNA sequence. In such cases, the miRNA sequence may be referred to as the active strand, while the second RNA sequence, which is at least partially complementary to the miRNA sequence, may be referred to as the complementary strand. The active and complementary strands are hybridized to create a double-stranded RNA that is similar to a naturally occurring miRNA precursor. The activity of a miRNA may be optimized by maximizing uptake of the active strand and minimizing uptake of the complementary strand by the miRNA protein complex that regulates gene translation. This can be done through modification and/or design of the complementary strand.

In some embodiments, the complementary strand is modified so that a chemical group other than a phosphate or hydroxyl at its 5' terminus. The presence of the 5' modification apparently eliminates uptake of the complementary strand and subsequently favors uptake of the active strand by the miRNA protein complex. The 5' modification can be any of a variety of molecules known in the art, including  $\text{NH}_2$ ,  $\text{NHCOCH}_3$ , and biotin.

In another embodiment, the uptake of the complementary strand by the miRNA pathway is reduced by incorporating nucleotides with sugar modifications in the first 2-6 nucleotides of the complementary strand. It should be noted that such sugar modifications can be combined with the 5' terminal modifications described above to further enhance miRNA activities.

In some embodiments, the complementary strand is designed so that nucleotides in the 3' end of the complementary strand are not complementary to the active strand. This results in double-strand hybrid RNAs that are stable at the 3' end of the active strand but relatively unstable at the 5' end of the active strand. This difference in stability enhances the uptake of the active strand by the miRNA pathway, while reducing uptake of the complementary strand, thereby enhancing miRNA activity.

### **III. Nucleic Acid Molecules**

One aspect of the invention pertains to pharmaceutical compositions containing nucleic acid molecules. As used herein, the term "nucleic acid molecule" is intended to include DNA molecules and RNA molecules and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, and may be generated using purified enzymes or by chemical synthesis. They may be crude or purified. The term "miRNA," unless otherwise indicated, refers to the mature miRNA sequence.

The term "miRNA" generally refers to a single stranded molecule, but in specific embodiments, molecules implemented in the invention will also encompass a region or an additional strand that is partially (between 10% and 50% complementarity across the length of the strand), substantially (greater than 50% but less than 100% complementary across the length of the

strand) or fully complementary to the miRNA on the same single-stranded molecule or on another nucleic acid.

In still another embodiment, an nucleic acid molecule of the present invention comprises a nucleotide sequence which is at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the entire length of *miR-10a* (SEQ ID NO: 1) or *miR-27a* (SEQ ID NO: 4), or a portion of any of these nucleotide sequences.

The skilled artisan will further appreciate that changes can be introduced by mutation into a nucleic acid molecule of the present invention, without altering the functional activity of the nucleic acid molecule. Accordingly, another aspect of the invention pertains to nucleic acid molecules that contain changes in nucleotide residues that are not essential for activity. Such polynucleotides differ in nucleotide sequence from those of *miR-10a* (SEQ ID NO: 1) or *miR-27a* (SEQ ID NO: 4), yet retain biological activity. In one embodiment, the isolated nucleic acid molecule comprises a nucleotide sequence wherein nucleic acid sequence at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to those in SEQ ID NO:1 or SEQ ID NO: 4.

The expression characteristics of a nucleic acid molecules of the present invention (*e.g.*, SEQ ID NO:1 or SEQ ID NO: 4 or variants thereof) within a cell may be modified by inserting a heterologous regulatory element such that the inserted regulatory element is operatively linked with a nucleic acid molecule of the present invention. For example, a heterologous regulatory element may be inserted such that it is operatively linked with a nucleic acid molecule of the present invention using techniques which are well known to those of skill in the art, and described, *e.g.*, in Chappel, U.S. Pat. No. 5,272,071; PCT publication No. WO 91/06667, published May 16, 1991, each of which is incorporated by reference in its entirety.

A nucleic acid may be made by any technique known to one of ordinary skill in the art, such as, for example, chemical synthesis, enzymatic production or biological production. Non-limiting examples of nucleic acid synthesis are included in U.S. Pat. Nos. 4,704,362, 5,221,619, 5,705,629 4,659,774, 4,816,571, 5,141,813, 5,264,566, 4,959,463, 5,428,148, 5,554,744, 5,574,146, 5,602,244 and 5,583,013, each of which are incorporated by reference in their entirety.

In some embodiments, the nucleic acid molecule of the invention is an isolated miRNA. As used herein, an "isolated" miRNA is one which is synthesized, or altered or removed from the natural state through human intervention. For example, a miRNA gene product naturally present in a living animal is not "isolated." A synthetic miRNA, or a miRNA partially or completely separated from the coexisting materials of its natural state, is "isolated." An isolated miRNA can exist in substantially purified form, or can exist in a cell into which the miRNA has been delivered. Thus, a miRNA which is deliberately delivered to, or expressed in, a cell is considered an "isolated"

miRNA. A miRNA produced inside a cell by a miRNA precursor molecule is also considered to be an "isolated" molecule.

Isolated miRNAs can be obtained using a number of standard techniques. For example, an isolated miRNA can be chemically synthesized or recombinantly produced using methods known in the art. In some instances, miRNA are chemically synthesized using appropriately protected ribonucleoside phosphoramidites and a conventional DNA/RNA synthesizer. Commercial suppliers of synthetic RNA molecules or synthesis reagents include, e.g., Proligo (Hamburg, Germany), Dharmacon Research (Lafayette, Colo., USA), Pierce Chemical (part of Perbio Science, Rockford, Ill., USA), Glen Research (Sterling, Va., USA), ChemGenes (Ashland, Mass., USA) and Cruachem (Glasgow, UK).

Alternatively, the miRNAs can be expressed from recombinant circular or linear DNA plasmids using any suitable promoter. Suitable promoters for expressing RNA from a plasmid include, e.g., the U6 or H1 RNA pol III promoter sequences, or the cytomegalovirus promoters. Selection of other suitable promoters is within the skill in the art. The recombinant plasmids of the invention can also comprise inducible or regulatable promoters for the expression of the miRNAs in cancer cells.

The miRNAs that are expressed from recombinant plasmids can be isolated from cultured cell expression systems by standard techniques. The miRNAs which are expressed from recombinant plasmids can also be delivered to, and expressed directly in, cancer cells. The use of recombinant plasmids to deliver the miRNAs to cancer cells is discussed in more detail below.

In embodiments of the invention in which multiple miRNAs are used, the miRNAs can be expressed from separate recombinant plasmids, or can be expressed from the same recombinant plasmid. In some embodiments, the miRNAs are expressed as the RNA precursor molecules from a single plasmid, and the precursor molecules are processed into the functional miRNA by a suitable processing system, including processing systems extant within a cancer cell. Other suitable processing systems include, e.g., the in vitro *Drosophila* cell lysate system as described in U.S. published application 2002/0086356 to Tuschl et al. and the *E. coli* RNase III system described in U.S. published patent application 2004/0014113 to Yang et al., the entire disclosures of which are herein incorporated by reference in their entirety.

Selection of plasmids suitable for expressing the miRNAs, methods for inserting nucleic acid sequences into the plasmid, and methods of delivering the recombinant plasmid to the cells of interest are within the skill in the art. See, for example, Zeng et al. (2002), *Molecular Cell* 9:1327-1333; Tuschl (2002), *Nat. Biotechnol.* 20:446-448; Brummelkamp et al. (2002), *Science* 296:550-553; Miyagishi et al. (2002), *Nat. Biotechnol.* 20:497-500; Paddison et al. (2002), *Genes Dev.*

16:948-958; Lee et al. (2002), Nat. Biotechnol. 20:500-505; and Paul et al. (2002), Nat. Biotechnol. 20:505-508, the entire disclosures of which are herein incorporated by reference.

In one embodiment, a plasmid expressing a miRNA sequence includes a sequence encoding a miRNA precursor RNA under the control of a promoter. As used herein, "under the control" of a promoter means that the nucleic acid sequences encoding the miRNA are operatively linked to the promoter, so that the promoter can initiate transcription of the miRNA coding sequences.

The miRNAs can also be expressed from recombinant viral vectors. The RNA expressed from the recombinant viral vectors can either be isolated from cultured cell expression systems by standard techniques, or can be expressed directly in cancer cells. The use of recombinant viral vectors to deliver the miRNAs to cancer cells is discussed in more detail below.

The recombinant viral vectors of the invention comprise sequences encoding the miRNAs and any suitable promoter for expressing the RNA sequences. Suitable promoters include, for example, the U6 or H1 RNA pol III promoter sequences, or the cytomegalovirus promoters. Selection of other suitable promoters is within the skill in the art. The recombinant viral vectors of the invention can also comprise inducible or regulatable promoters for expression of the miRNAs in a cancer cell.

Any viral vector capable of accepting the coding sequences for the miRNAs can be used; for example, vectors derived from adenovirus (AV); adeno-associated virus (AAV); retroviruses (e.g., lentiviruses, Rhabdoviruses, murine leukemia virus); herpes virus, and the like. The tropism of the viral vectors can be modified by pseudotyping the vectors with envelope proteins or other surface antigens from other viruses, or by substituting different viral capsid proteins, as appropriate.

For example, lentiviral vectors (lentivector) encoding miRNAs of the invention can be pseudotyped with surface proteins from vesicular stomatitis virus (VSV), rabies, Ebola, Mokola, and the like. Lentivectors can be prepared, for example, by transfecting a suitable packaging host cell with appropriate nucleic acid vectors using methods known in the art. The design and use of lentiviral vectors suitable for gene therapy is described, for example, in U.S. Pat. Nos. 6,531,123, 6,207,455 and 6,165,782, the disclosures of which are hereby incorporated by reference. The use of lentivector-delivered RNA interference in silencing gene expression in transgenic mice is described by Rubinson et al. (Nat. Genet. 33:401-406, 2003).

AAV vectors of the invention can be made to target different cells by engineering the vectors to express different capsid protein serotypes. For example, an AAV vector expressing a serotype 2 capsid on a serotype 2 genome is called AAV 2/2. This serotype 2 capsid gene in the AAV 2/2 vector can be replaced by a serotype 5 capsid gene to produce an AAV 2/5 vector. Techniques for constructing AAV vectors which express different capsid protein serotypes are

within the skill in the art; see, e.g., Rabinowitz J. E. et al. (2002), J Virol 76:791-801, the entire disclosure of which is herein incorporated by reference.

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

Suitable viral vectors include those derived from AV and AAV. A suitable AV vector for expressing the miRNAs, a method for constructing the recombinant AV vector, and a method for delivering the vector into target cells, are described in Xia et al. (2002), Nat. Biotech. 20:1006-1010, the entire disclosure of which is herein incorporated by reference. Suitable AAV vectors for expressing the miRNAs, methods for constructing the recombinant AAV vector, and methods for delivering the vectors into target cells are described in Samulski et al. (1987), J. Virol. 61:3096-3101; Fisher et al. (1996), J. Virol., 70:520-532; Samulski et al. (1989), J. Virol. 63:3822-3826; U.S. Pat. No. 5,252,479; U.S. Pat. No. 5,139,941; International Patent Application No. WO 94/13788; and International Patent Application No. WO 93/24641, the entire disclosures of which are herein incorporated by reference.

#### **IV. Pharmaceutical Compositions**

In another aspect, the present invention provides a composition, *e.g.*, a pharmaceutical composition, containing one or a combination nucleic acid molecules containing sequences that encode miRNAs of the invention (*e.g.* *miR-10a* and/or *miR-27a*), or a functional variant thereof formulated together with a pharmaceutically acceptable carrier. In some embodiments the pharmaceutical compositions nucleic acid molecules within the pharmaceutical composition are miRNAs or pre-miRNAs.

In some embodiments, pharmaceutical compositions of the invention also contain other agents or can be administered in combination therapy, *i.e.*, combined with other agents. For example, the combination therapy can combine a composition of the present invention with at least one or more additional therapeutic agents, such as chemotherapeutic agents. The pharmaceutical compositions of the invention can also be administered in conjunction with radiation therapy.

The term chemotherapeutic agent includes, without limitation, platinum-based agents, such as carboplatin and cisplatin; nitrogen mustard alkylating agents; nitrosourea alkylating agents, such as carmustine (BCNU) and other alkylating agents; antimetabolites, such as methotrexate; purine analog antimetabolites; pyrimidine analog antimetabolites, such as fluorouracil (5-FU) and

gemcitabine; hormonal antineoplastics, such as goserelin, leuprolide, and tamoxifen; natural antineoplastics, such as taxanes (e.g., docetaxel and paclitaxel), aldesleukin, interleukin-2, etoposide (VP-16), interferon alfa, and tretinoin (ATRA); antibiotic natural antineoplastics, such as bleomycin, dactinomycin, daunorubicin, doxorubicin, and mitomycin; and vinca alkaloid natural antineoplastics, such as vinblastine and vincristine.

Further, the following additional drugs may also be used in combination with an antineoplastic agent, even if not considered antineoplastic agents themselves: dactinomycin; daunorubicin HCl; docetaxel; doxorubicin HCl; epoetin alfa; etoposide (VP-16); ganciclovir sodium; gentamicin sulfate; interferon alfa; leuprolide acetate; meperidine HCl; methadone HCl; ranitidine HCl; vinblastin sulfate; and zidovudine (AZT). For example, fluorouracil has recently been formulated in conjunction with epinephrine and bovine collagen to form a particularly effective combination.

Still further, the following listing of amino acids, peptides, polypeptides, proteins, polysaccharides, and other large molecules may also be used: interleukins 1 through 18, including mutants and analogues; interferons or cytokines, such as interferons  $\alpha$ ,  $\beta$ , and  $\gamma$ ; hormones, such as luteinizing hormone releasing hormone (LHRH) and analogues and, gonadotropin releasing hormone (GnRH); growth factors, such as transforming growth factor- $\beta$  (TGF- $\beta$ ), fibroblast growth factor (FGF), nerve growth factor (NGF), growth hormone releasing factor (GHRF), epidermal growth factor (EGF), fibroblast growth factor homologous factor (FGFHF), hepatocyte growth factor (HGF), and insulin growth factor (IGF); tumor necrosis factor- $\alpha$  &  $\beta$  (TNF- $\alpha$  &  $\beta$ ); invasion inhibiting factor-2 (IIF-2); bone morphogenetic proteins 1-7 (BMP 1-7); somatostatin; thymosin-  $\alpha$  - 1;  $\gamma$ -globulin; superoxide dismutase (SOD); complement factors; anti-angiogenesis factors; antigenic materials; and pro-drugs.

Chemotherapeutic agents for use with the compositions and methods of treatment described herein include, but are not limited to alkylating agents such as thiotepa and cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine,

trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the enediyne antibiotics (e.g., calicheamicin, especially calicheamicin gammall and calicheamicin omega11; dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores, aclacinomysins, actinomycin, authrarnycin, azaserine, bleomycins, cactinomycin, carabacin, caminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitio stanol, mepitio stanane, testolactone; anti-adrenals such as aminogluthethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK polysaccharide complex); razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g., paclitaxel and doxetaxel; chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum coordination complexes such as cisplatin, oxaliplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (e.g., CPT-11); topoisomerase inhibitor RFS 2000; difluoromethylomithine (DMFO); retinoids such as retinoic acid; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

In another embodiment, the composition of the invention may comprise other biologically active substances, including therapeutic drugs or pro-drugs, for example, other chemotherapeutic agents, scavenger compounds, antibiotics, anti-virals, anti-fungals, anti-inflammatories,

vasoconstrictors and anticoagulants, antigens useful for cancer vaccine applications or corresponding pro-drugs.

Exemplary scavenger compounds include, but are not limited to thiol-containing compounds such as glutathione, thiourea, and cysteine; alcohols such as mannitol, substituted phenols; quinones, substituted phenols, aryl amines and nitro compounds.

Various forms of the chemotherapeutic agents and/or other biologically active agents may be used. These include, without limitation, such forms as uncharged molecules, molecular complexes, salts, ethers, esters, amides, and the like, which are biologically activated when implanted, injected or otherwise inserted into the tumor.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. The carrier may be suitable, for example, for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (*e.g.*, by injection or infusion). In other embodiments, the carrier may be suitable for treatment of hematopoietic cells for use in autologous transplantation. Depending on the route of administration, the active compound (*i.e.*, the miRNA or miRNA encoding nucleic acid molecule) may be coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound.

Regardless of the route of administration selected, the compounds of the present invention (*i.e.*, the miRNA or miRNA encoding nucleic acid molecule) may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, may be formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of the present invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts. A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the

invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. In general, a suitable daily dose of compositions of the invention will be that amount of the compound which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. It is preferred that administration be intravenous, intramuscular, intraperitoneal, or subcutaneous, preferably administered proximal to the site of the target. If desired, the effective daily dose of a therapeutic composition may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms.

In certain embodiments, a pharmaceutical agent is sterile lyophilized oligonucleotide that is reconstituted with a suitable diluent, e.g., sterile water or sterile saline. The reconstituted product may be administered as a subcutaneous injection or as an intravenous infusion after dilution into saline. In some embodiments, the lyophilized drug product consists of an oligonucleotide which has been prepared in water for injection, or in saline for injection, adjusted to pH 7.0-9.0 with acid or base during preparation, and then lyophilized.

In certain embodiments, a pharmaceutical composition of the present invention comprises a delivery system. Any compounds useful for delivery of nucleic acid molecules in general, or siRNA and miRNA in particular, may be present in the pharmaceutical compositions of the invention. Examples of delivery systems include, but are not limited to, liposomes, cationic polymers, antibody-protamine fusions, atelocollagen and nanoparticles.

In certain embodiments, a pharmaceutical composition of the present invention comprises one or more tissue-specific delivery molecules designed to deliver the one or more pharmaceutical agents of the present invention to specific tissues or cell types. For example, in certain embodiments, pharmaceutical compositions include liposomes coated with a tissue-specific antibody.

In certain embodiments, a compound comprises an oligonucleotide (*i.e.* a miRNA or miRNA encoding oligonucleotide) conjugated to one or more moieties which enhance the activity, cellular distribution or cellular uptake of the resulting oligonucleotide. In certain such embodiments, the moiety is a cholesterol moiety or a lipid moiety. Additional moieties for conjugation include carbohydrates, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. In certain embodiments, a conjugate group is attached directly to the oligonucleotide. In certain embodiments, a conjugate group is attached to the oligonucleotide by a linking moiety selected from amino, hydroxyl, carboxylic acid, thiol, unsaturations (e.g., double or triple bonds), 8-amino-3,6-dioxaoctanoic acid (ADO), succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), 6-

aminohexanoic acid (AHEx or AHA), substituted C1-C10 alkyl, substituted or unsubstituted C2-C10 alkenyl, and substituted or unsubstituted C2-C10 alkynyl. In certain such embodiments, a substituent group is selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl.

In certain such embodiments, the compound comprises the oligonucleotide having one or more stabilizing groups that are attached to one or both termini of the oligonucleotide to enhance properties such as, for example, nuclease stability. Included in stabilizing groups are cap structures. These terminal modifications protect the oligonucleotide from exonuclease degradation, and can help in delivery and/or localization within a cell. The cap can be present at the 5'-terminus (5'-cap), or at the 3'-terminus (3'-cap), or can be present on both termini. Cap structures include, for example, inverted deoxy abasic caps.

Suitable cap structures include a 4',5'-methylene nucleotide, a 1-(beta-D-erythrofuransyl) nucleotide, a 4'-thio nucleotide, a carbocyclic nucleotide, a 1,5-anhydrohexitol nucleotide, an L-nucleotide, an alpha-nucleotide, a modified base nucleotide, a phosphorodithioate linkage, a threo-pentofuransyl nucleotide, an acyclic 3',4'-seco nucleotide, an acyclic 3,4-dihydroxybutyl nucleotide, an acyclic 3,5-dihydroxypentyl nucleotide, a 3'-3'-inverted nucleotide moiety, a 3'-3'-inverted abasic moiety, a 3'-2'-inverted nucleotide moiety, a 3'-2'-inverted abasic moiety, a 1,4-butanediol phosphate, a 3'-phosphoramidate, a hexylphosphate, an aminohexyl phosphate, a 3'-phosphate, a 3'-phosphorothioate, a phosphorodithioate, a bridging methylphosphonate moiety, and a non-bridging methylphosphonate moiety 5'-amino-alkyl phosphate, a 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate, a 6-aminohexyl phosphate, a 1,2-aminododecyl phosphate, a hydroxypropyl phosphate, a 5'-5'-inverted nucleotide moiety, a 5'-5'-inverted abasic moiety, a 5'-phosphoramidate, a 5'-phosphorothioate, a 5'-amino, a bridging and/or non-bridging 5'-phosphoramidate, a phosphorothioate, and a 5'-mercapto moiety.

In certain embodiments, the compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for

influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the oligonucleotide(s) of the formulation.

In certain embodiments, pharmaceutical compositions of the present invention comprise one or more miRNAs or miRNA encoding oligonucleotides and one or more excipients. Examples of excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylase, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose and polyvinylpyrrolidone.

In certain embodiments, a pharmaceutical composition of the present invention is a liquid (e.g., a suspension, elixir and/or solution). In certain of such embodiments, a liquid pharmaceutical composition is prepared using ingredients known in the art, including, but not limited to, water, glycols, oils, alcohols, flavoring agents, preservatives, and coloring agents.

In certain embodiments, a pharmaceutical composition of the present invention is a solid (e.g., a powder, tablet, and/or capsule). In certain of such embodiments, a solid pharmaceutical composition comprising one or more oligonucleotides is prepared using ingredients known in the art, including, but not limited to, starches, sugars, diluents, granulating agents, lubricants, binders, and disintegrating agents.

In certain embodiments, a pharmaceutical composition of the present invention is formulated as a depot preparation. Certain such depot preparations are typically longer acting than non-depot preparations. In certain embodiments, such preparations are administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. In certain embodiments, depot preparations are prepared using suitable polymeric or hydrophobic materials (for example an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

In certain embodiments, a pharmaceutical composition of the present invention comprises a co-solvent system. Certain of such co-solvent systems comprise, for example, benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. In certain embodiments, such co-solvent systems are used for hydrophobic compounds. A non-limiting example of such a co-solvent system is the VPD co-solvent system, which is a solution of absolute ethanol comprising 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant Polysorbate 80<sup>TM</sup> and 65% w/v polyethylene glycol 300. The proportions of such co-solvent systems may be varied considerably without significantly altering their solubility and toxicity characteristics. Furthermore, the identity of co-solvent components may be varied: for example, other surfactants may be used instead of Polysorbate 80<sup>TM</sup>; the fraction size of polyethylene glycol may be varied; other biocompatible polymers may replace polyethylene glycol, e.g., polyvinyl pyrrolidone; and other sugars or polysaccharides may substitute for dextrose.

In certain embodiments, a pharmaceutical composition of the present invention comprises a sustained-release system. A non-limiting example of such a sustained-release system is a semi-permeable matrix of solid hydrophobic polymers. In certain embodiments, sustained-release systems may, depending on their chemical nature, release pharmaceutical agents over a period of hours, days, weeks or months.

In certain embodiments, a pharmaceutical composition of the present invention is prepared for oral administration. In certain of such embodiments, a pharmaceutical composition is formulated by combining one or more compounds comprising oligonucleotide (*i.e.*, the miRNA or miRNA encoding oligonucleotide) with one or more pharmaceutically acceptable carriers. Certain of such carriers enable pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject. In certain embodiments, pharmaceutical compositions for oral use are obtained by mixing the oligonucleotide and one or more solid excipient. Suitable excipients include, but are not limited to, fillers, such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). In certain embodiments, such a mixture is optionally ground and auxiliaries are optionally added. In certain embodiments, pharmaceutical compositions are formed to obtain tablets or dragee cores. In certain embodiments, disintegrating agents (e.g., cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate) are added.

In certain embodiments, a pharmaceutical composition is prepared for administration by injection (e.g., intravenous, subcutaneous, intramuscular, etc.). In certain of such embodiments, a pharmaceutical composition comprises a carrier and is formulated in an aqueous solution, such as water or physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. In certain embodiments, other ingredients are included (e.g., ingredients that aid in solubility or serve as preservatives). In certain embodiments, injectable suspensions are prepared using appropriate liquid carriers, suspending agents and the like. Certain pharmaceutical compositions for injection are presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers. Certain pharmaceutical compositions for injection are suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Certain solvents suitable for use in pharmaceutical compositions for injection include, but are not limited to, lipophilic solvents and fatty oils, such as sesame oil, synthetic fatty acid esters, such as ethyl oleate or triglycerides, and liposomes. Aqueous injection suspensions may contain substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, such suspensions may also

contain suitable stabilizers or agents that increase the solubility of the pharmaceutical agents to allow for the preparation of highly concentrated solutions.

Therapeutic compositions can be administered with medical devices known in the art. For example, in one embodiment, a therapeutic composition of the invention can be administered with a needleless hypodermic injection device, such as the devices disclosed in U.S. Pat. Nos. 5,399,163, 5,383,851, 5,312,335, 5,064,413, 4,941,880, 4,790,824, or 4,596,556, each of which is incorporated by reference in their entirety. Examples of well-known implants and modules useful in the present invention include: U.S. Pat. No. 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; U.S. Pat. No. 4,486,194, which discloses a therapeutic device for administering medicants through the skin; U.S. Pat. No. 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion rate; U.S. Pat. No. 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; U.S. Pat. No. 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments; and U.S. Pat. No. 4,475,196, which discloses an osmotic drug delivery system, each of which are incorporated by reference in their entirety. Many other such implants, delivery systems, and modules are known to those skilled in the art.

In certain embodiments, the miRNAs of the invention can be formulated to ensure proper distribution *in vivo*. For example, they can be formulated, in liposomes. For methods of manufacturing liposomes, see, *e.g.*, U.S. Pat. Nos. 4,522,811; 5,374,548; and 5,399,331, each of which is incorporated by reference in their entirety. The liposomes may comprise one or more moieties which are selectively transported into specific cells or organs, thus enhance targeted drug delivery (see, *e.g.*, V. V. Ranade (1989) *J. Clin. Pharmacol.* 29:685). Exemplary targeting moieties include folate or biotin (see, *e.g.*, U.S. Pat. No. 5,416,016 to Low *et al.*); mannosides (Umezawa *et al.*, (1988) *Biochem. Biophys. Res. Commun.* 153:1038); antibodies (P. G. Bloeman *et al.* (1995) *FEBS Lett.* 357:140; M. Owais *et al.* (1995) *Antimicrob. Agents Chemother.* 39:180); surfactant protein A receptor (Briscoe *et al.* (1995) *Am. J. Physiol.* 1233:134), different species of which may comprise the formulations of the inventions, as well as components of the invented molecules; p120 (Schreier *et al.* (1994) *J. Biol. Chem.* 269:9090); see also K. Keinänen; M. L. Laukkanen (1994) *FEBS Lett.* 346:123; J. J. Killian; I. J. Fidler (1994) *Immunomethods* 4:273. In one embodiment of the invention, the therapeutic compounds of the invention are formulated in liposomes; in another embodiment, the liposomes include a targeting moiety. In yet another embodiment, the therapeutic compounds in the liposomes are delivered by bolus injection to a site proximal to the tumor.

In certain embodiments, a pharmaceutical composition of the present invention comprises an oligonucleotide (*i.e.* a miRNA or miRNA encoding oligonucleotide) in a therapeutically effective amount. In certain embodiments, the therapeutically effective amount is sufficient to prevent,

alleviate or ameliorate symptoms of a disease or to prolong the survival of the subject being treated. Determination of a therapeutically effective amount is well within the capability of those skilled in the art.

In certain such embodiments, the pharmaceutical composition of the invention comprises an oligonucleotide (*i.e.* a miRNA or miRNA encoding oligonucleotide) hybridized to a complementary strand, *i.e.* a double-stranded oligomeric compound.

## V. Uses and Methods of the Invention

The present invention further provides novel therapeutic methods of treating cancer comprising administering to a subject, *e.g.*, a subject in need thereof, an effective amount of an oligonucleotide containing a *miR-10a* and/or *miR-27a* sequence, or functional variants thereof.

In some embodiments, the methods and compositions of the present invention may be used to treat a hematological cancer, such as a leukemia or lymphoma. In some embodiments, methods and compositions of the present invention may be used to treat any cancer. Cancers that may be treated by methods and compositions of the invention include, but are not limited to, cancer cells from the bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, gastrointestinal, gum, head, kidney, liver, lung, nasopharynx, neck, ovary, prostate, skin, stomach, testis, tongue, or uterus. In addition, the cancer may specifically be of the following histological type, though it is not limited to these: neoplasm, malignant; carcinoma; carcinoma, undifferentiated; giant and spindle cell carcinoma; small cell carcinoma; papillary carcinoma; squamous cell carcinoma; lymphoepithelial carcinoma; basal cell carcinoma; pilomatrix carcinoma; transitional cell carcinoma; papillary transitional cell carcinoma; adenocarcinoma; gastrinoma, malignant; cholangiocarcinoma; hepatocellular carcinoma; combined hepatocellular carcinoma and cholangiocarcinoma; trabecular adenocarcinoma; adenoid cystic carcinoma; adenocarcinoma in adenomatous polyp; adenocarcinoma, familial polyposis coli; solid carcinoma; carcinoid tumor, malignant; bronchiolo-alveolar adenocarcinoma; papillary adenocarcinoma; chromophobe carcinoma; acidophil carcinoma; oxyphilic adenocarcinoma; basophil carcinoma; clear cell adenocarcinoma; granular cell carcinoma; follicular adenocarcinoma; papillary and follicular adenocarcinoma; nonencapsulating sclerosing carcinoma; adrenal cortical carcinoma; endometroid carcinoma; skin appendage carcinoma; apocrine adenocarcinoma; sebaceous adenocarcinoma; ceruminous adenocarcinoma; mucoepidermoid carcinoma; cystadenocarcinoma; papillary cystadenocarcinoma; papillary serous cystadenocarcinoma; mucinous cystadenocarcinoma; mucinous adenocarcinoma; signet ring cell carcinoma; infiltrating duct carcinoma; medullary carcinoma; lobular carcinoma; inflammatory carcinoma; paget's disease, mammary; acinar cell carcinoma; adenosquamous carcinoma; adenocarcinoma w/squamous metaplasia; thymoma, malignant; ovarian stromal tumor, malignant;

thecoma, malignant; granulosa cell tumor, malignant; androblastoma, malignant; sertoli cell carcinoma; leydig cell tumor, malignant; lipid cell tumor, malignant; paraganglioma, malignant; extra-mammary paraganglioma, malignant; pheochromocytoma; glomangiosarcoma; malignant melanoma; amelanotic melanoma; superficial spreading melanoma; malig melanoma in giant pigmented nevus; epithelioid cell melanoma; blue nevus, malignant; sarcoma; fibrosarcoma; fibrous histiocytoma, malignant; myxosarcoma; liposarcoma; leiomyosarcoma; rhabdomyosarcoma; embryonal rhabdomyosarcoma; alveolar rhabdomyosarcoma; stromal sarcoma; mixed tumor, malignant; mullerian mixed tumor; nephroblastoma; hepatoblastoma; carcinosarcoma; mesenchymoma, malignant; brenner tumor, malignant; phyllodes tumor, malignant; synovial sarcoma; mesothelioma, malignant; dysgerminoma; embryonal carcinoma; teratoma, malignant; struma ovarii, malignant; choriocarcinoma; mesonephroma, malignant; hemangiosarcoma; hemangioendothelioma, malignant; kaposi's sarcoma; hemangiopericytoma, malignant; lymphangiosarcoma; osteosarcoma; juxtacortical osteosarcoma; chondrosarcoma; chondroblastoma, malignant; mesenchymal chondrosarcoma; giant cell tumor of bone; ewing's sarcoma; odontogenic tumor, malignant; ameloblastic odontosarcoma; ameloblastoma, malignant; ameloblastic fibrosarcoma; pinealoma, malignant; chordoma; glioma, malignant; ependymoma; astrocytoma; protoplasmic astrocytoma; fibrillary astrocytoma; astroblastoma; glioblastoma; oligodendroglioma; oligodendroblastoma; primitive neuroectodermal; cerebellar sarcoma; ganglioneuroblastoma; neuroblastoma; retinoblastoma; olfactory neurogenic tumor; meningioma, malignant; neurofibrosarcoma; neurilemmoma, malignant; granular cell tumor, malignant; malignant lymphoma; Hodgkin's disease; Hodgkin's lymphoma; paragranuloma; malignant lymphoma, small lymphocytic; malignant lymphoma, large cell, diffuse; malignant lymphoma, follicular; mycosis fungoides; other specified non-Hodgkin's lymphomas; malignant histiocytosis; multiple myeloma; mast cell sarcoma; immunoproliferative small intestinal disease; leukemia; lymphoid leukemia; plasma cell leukemia; erythroleukemia; lymphosarcoma cell leukemia; myeloid leukemia; basophilic leukemia; eosinophilic leukemia; monocytic leukemia; mast cell leukemia; megakaryoblastic leukemia; myeloid sarcoma; and hairy cell leukemia. Moreover, miRNA can be evaluated in precancers, such as metaplasia, dysplasia, and hyperplasia.

In some embodiments, the methods and compositions of the invention may be used to treat cancers that do not express *miR-10a* and/or *miR-27a*. Any techniques for determining RNA expression known in the art can be used to determine whether a cancer expresses *miR-10a* and/or *miR-27a*. Examples of such techniques include, but are not limited to, RT-PCR, Northern blot and miRNA expression microarrays.

In certain embodiments, the methods of treating a cancer comprise administering a miRNA of the present invention or a nucleic acid encoding a miRNA of the present invention in conjunction

with a second agent to the subject. Such methods, in certain embodiments, comprise administering pharmaceutical compositions comprising *miR-10a* and/or *miR-27a*, or functional variants thereof, in conjunction with one or more chemotherapeutic agents. Conjunctive therapy includes sequential, simultaneous and separate, or co-administration of the active compound. In one embodiment, the second agent is a chemotherapeutic agent. In another embodiment, the second agent is radiation therapy. In a further embodiment, radiation therapy may be administered in addition to the miRNA and a second agent. In certain embodiments, the second agent may be co-formulated in the same pharmaceutical composition as the miRNA or miRNA encoding oligonucleotide.

Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the enediyne antibiotics (e.g., calicheamicin, especially calicheamicin gammall and calicheamicin omegall; dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores, aclacinomysins, actinomycin, authrarnycin, azaserine, bleomycins, cactinomycin, carabacin, caminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane,

testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK polysaccharide complex); razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2''-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g., paclitaxel and doxetaxel; chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum coordination complexes such as cisplatin, oxaliplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (e.g., CPT-11); topoisomerase inhibitor RFS 2000; difluoromethylomithine (DMFO); retinoids such as retinoic acid; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen, raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY17018, onapristone, and toremifene; aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, megestrol acetate, exemestane, formestane, fadrozole, vorozole, letrozole, and anastrozole; and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; as well as troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those which inhibit expression of genes in signaling pathways implicated in aberrant cell proliferation, such as, for example, PKC- $\alpha$ , Ralf and H-Ras; ribozymes such as a VEGF expression inhibitor and a HER2 expression inhibitor; vaccines such as gene therapy vaccines and pharmaceutically acceptable salts, acids or derivatives of any of the above.

In most embodiments, pharmaceutical compositions will be delivered in a therapeutically effective amount of an incorporated therapeutic agent as part of a prophylactic or therapeutic treatment. The desired concentration of active compound in delivered will depend on absorption, inactivation, and excretion rates of the pharmaceutical agent, as well as the delivery rate of the compound. It is to be noted that dosage values may also vary with the severity of the condition to

be alleviated. It is to be further understood that for any particular subject, specific dosage regimens 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. Typically, dosing will be determined using techniques known to one skilled in the art.

In some embodiments of the present treatment methods, an effective amount of at least one miRNA or miRNA encoding oligonucleotide can be administered to a subject. One skilled in the art can readily determine an effective amount of a miRNA or miRNA encoding oligonucleotide to be administered to a given subject, by taking into account factors such as the size and weight of the subject; the extent of disease penetration; the age, health and sex of the subject; the route of administration; and whether the administration is regional or systemic.

For example, an effective amount of a miRNA or miRNA encoding oligonucleotide can be based on the approximate weight of a tumor mass to be treated. The approximate weight of a tumor mass can be determined by calculating the approximate volume of the mass, wherein one cubic centimeter of volume is roughly equivalent to one gram. In some embodiments the effective amount based on the weight of the tumor mass may be injected directly into the tumor. An effective amount of a miRNA or miRNA encoding oligonucleotide can also be based on the approximate or estimated body weight of a subject to be treated.

One skilled in the art can also readily determine an appropriate dosage regimen for the administration of a miRNA or miRNA encoding oligonucleotide to a given subject. For example, a miRNA or miRNA encoding oligonucleotide can be administered to the subject once (e.g., as a single injection or deposition). Alternatively, a miRNA or miRNA encoding oligonucleotide can be administered in multiple administrations over a period of time. Where a dosage regimen comprises multiple administrations, it is understood that the effective amount of the miRNA or miRNA encoding oligonucleotide administered to the subject can comprise the total amount of gene product administered over the entire dosage regimen.

The miRNA or miRNA encoding oligonucleotide can be administered to a subject by any means suitable for delivering these compounds to cancer cells of the subject. For example, the miRNA or miRNA encoding oligonucleotide can be administered by any methods suitable to transfect cells of the subject with the miRNAs of the invention or nucleic acids encoding the miRNAs of the invention.

A miRNA or miRNA encoding oligonucleotide can be administered to a subject by any suitable administration route. Suitable enteral administration routes for the present methods include, e.g., oral, rectal, or intranasal delivery. Suitable parenteral administration routes include, e.g., intravascular administration (e.g., intravenous bolus injection, intravenous infusion, intra-arterial bolus injection, intra-arterial infusion and catheter instillation into the vasculature); peri- and

intra-tissue injection (e.g., peri-tumoral and intra-tumoral injection, intra-retinal injection, or subretinal injection); subcutaneous injection or deposition, including subcutaneous infusion (such as by osmotic pumps); direct application to the tissue of interest, for example by a catheter or other placement device (e.g., a retinal pellet or a suppository or an implant comprising a porous, non-porous, or gelatinous material); and inhalation. In some embodiments, administration routes are injection, infusion and direct injection into the tumor.

In some embodiments, the methods and compositions of the present invention will be used in conjunction with autologous transplantation. For example, in some embodiments, a population of cells that includes hematopoietic stem/progenitor cells (e.g. bone marrow) will be isolated from a subject that has or is suspected of having cancer. The subject will be treated with a chemotherapeutic agent and/or radiation, while the isolated cell population will be treated with methods and/or compositions of the present invention (e.g. *miR-10a* and/or *miR-27a* or functional variants thereof). In some embodiments, the isolated cell population will be treated with additional chemotherapeutic agents and/or radiation. The treated isolated cell population will be reintroduced into the subject such that the autologously transplanted cells can reconstitute the subject's hematopoietic system.

The miRNA or miRNA encoding oligonucleotide can be administered to a subject by any means suitable for delivering these compounds to cancer cells of the subject. For example, the miRNA or miRNA encoding oligonucleotide can be administered by any methods suitable to transfect cells of the subject with the miRNAs of the invention or nucleic acids encoding the miRNAs of the invention.

In the present methods, a miRNA or miRNA encoding oligonucleotide can be administered to the subject either as naked RNA, in combination with a delivery reagent, or as a nucleic acid (e.g., a recombinant plasmid or viral vector) comprising sequences that express the a miRNA or miRNA encoding oligonucleotide. Any nucleic acid delivery method known in the art can be used in the present invention. Suitable delivery reagents include, but are not limited to, e.g, the Mirus Transit TKO lipophilic reagent; lipofectin; lipofectamine; cellfectin; polycations (e.g., polylysine), atelocollagen, nanoplexes and liposomes.

Recombinant plasmids and viral vectors comprising sequences that express the miRNA and techniques for delivering such plasmids and vectors to cancer cells, are discussed above.

The use of atelocollagen as a delivery vehicle for nucleic acid molecules is described in Minakuchi *et al.* Nucleic Acids Res., 32(13):e109 (2004); Hanai *et al.* Ann NY Acad Sci., 1082:9-17 (2006); and Kawata *et al.* Mol Cancer Ther., 7(9):2904-12 (2008); each of which is incorporated herein in their entirety.

In one embodiment of the invention, liposomes are used to deliver a miRNA or miRNA encoding oligonucleotide to a subject. Liposomes can also increase the blood half-life of the miRNA or miRNA encoding oligonucleotide.

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

The liposomes for use in the present methods can comprise a ligand molecule that targets the liposome to cancer cells. Ligands which bind to receptors prevalent in cancer cells, such as monoclonal antibodies that bind to tumor cell antigens, are preferred.

The liposomes for use in the present methods can also be modified so as to avoid clearance by the mononuclear macrophage system ("MMS") and reticuloendothelial system ("RES"). Such modified liposomes have opsonization-inhibition moieties on the surface or incorporated into the liposome structure. In an embodiment, a liposome of the invention can comprise both opsonization-inhibition moieties and a ligand.

Opsonization-inhibiting moieties for use in preparing the liposomes of the invention are typically large hydrophilic polymers that are bound to the liposome membrane. As used herein, an opsonization inhibiting moiety is "bound" to a liposome membrane when it is chemically or physically attached to the membrane, e.g., by the intercalation of a lipid-soluble anchor into the membrane itself, or by binding directly to active groups of membrane lipids. These opsonization-inhibiting hydrophilic polymers form a protective surface layer that significantly decreases the uptake of the liposomes by the MMS and RES; e.g., as described in U.S. Pat. No. 4,920,016, the entire disclosure of which is herein incorporated by reference.

Opsonization inhibiting moieties suitable for modifying liposomes are preferably water-soluble polymers with a number-average molecular weight from about 500 to about 40,000 daltons, and more preferably from about 2,000 to about 20,000 daltons. Such polymers include polyethylene glycol (PEG) or polypropylene glycol (PPG) derivatives; e.g., methoxy PEG or PPG, and PEG or PPG stearate; synthetic polymers such as polyacrylamide or poly N-vinyl pyrrolidone; linear, branched, or dendrimeric polyamidoamines; polyacrylic acids; polyalcohols, e.g., polyvinylalcohol and polyxylitol to which carboxylic or amino groups are chemically linked, as well as gangliosides, such as ganglioside GM1. Copolymers of PEG, methoxy PEG, or methoxy PPG, or derivatives thereof, are also suitable. In addition, the opsonization inhibiting polymer can be a block copolymer

of PEG and either a polyamino acid, polysaccharide, polyamidoamine, polyethyleneamine, or polynucleotide. The opsonization inhibiting polymers can also be natural polysaccharides containing amino acids or carboxylic acids, e.g., galacturonic acid, glucuronic acid, mannuronic acid, hyaluronic acid, pectic acid, neuraminic acid, alginic acid, carrageenan; aminated polysaccharides or oligosaccharides (linear or branched); or carboxylated polysaccharides or oligosaccharides, e.g., reacted with derivatives of carbonic acids with resultant linking of carboxylic groups. Preferably, the opsonization-inhibiting moiety is a PEG, PPG, or derivatives thereof. Liposomes modified with PEG or PEG-derivatives are sometimes called "PEGylated liposomes."

The opsonization inhibiting moiety can be bound to the liposome membrane by any one of numerous well-known techniques. For example, an N-hydroxysuccinimide ester of PEG can be bound to a phosphatidyl-ethanolamine lipid-soluble anchor, and then bound to a membrane. Similarly, a dextran polymer can be derivatized with a stearylamine lipid-soluble anchor via reductive amination using  $\text{Na}(\text{CN})\text{BH}_3$  and a solvent mixture, such as tetrahydrofuran and water in a 30:12 ratio at 60°C.

Liposomes modified with opsonization-inhibition moieties remain in the circulation much longer than unmodified liposomes. For this reason, such liposomes are sometimes called "stealth" liposomes. Stealth liposomes are known to accumulate in tissues fed by porous or "leaky" microvasculature. Thus, tissue characterized by such microvasculature defects, for example solid tumors, will efficiently accumulate these liposomes; see Gabizon, et al. (1988), Proc. Natl. Acad. Sci., USA, 18:6949-53. In addition, the reduced uptake by the RES lowers the toxicity of stealth liposomes by preventing significant accumulation of the liposomes in the liver and spleen. Thus, liposomes that are modified with opsonization-inhibition moieties are particularly suited to deliver the miRNA or miRNA encoding oligonucleotide to tumor cells.

The invention furthermore provides the sequences of tumor suppressor miRNAs, which allow identification of the target genes of these miRNAs, thereby enabling us to understand how the altered expression of these miRNAs affects cellular properties important for oncogenesis (including activation or repression in dysplastic tissues) and for the maintenance or progression of cancer. This, in turn, can be used to identify additional molecular targets for development of novel, targeted cancer treatments or chemoprevention.

The invention furthermore provides protocols for clinical application of miRNA detection as diagnostic and/or prognostic tools which would permit detecting these diagnostic miRNAs in order to screen for early stages of cancer. It is thus an object of the present invention to provide a method for detection, classification, diagnosis and prognosis of a disease comprising the steps of obtaining at least one sample from an individual and detecting the presence or absence of expression and/or the expression level of at least one miRNA described herein.

The following examples are presented in order to more fully illustrate some embodiments of the invention. They should in no way be construed as limiting the broad scope of the invention.

## EXAMPLES

### **Example 1: Expression of miR-10a, miR-27a, miR-16a and miR-155 in normal and malignant human hematopoietic cells**

MicroRNAs (miRNAs or miRs) are a recently identified class of epigenetic elements consisting of small noncoding RNAs that bind to the 3' untranslated region of mRNAs and down-regulate their translation to protein. miRNAs play critical roles in many different cellular processes including metabolism, apoptosis, differentiation, and development. To investigate the role of microRNA gene regulation in normal hematopoiesis and oncogenesis, microRNA expression profiling was performed on normal CD34<sup>+</sup> Hematopoietic Stem/Progenitor Cells (HSPCs), a large number of human leukemia/lymphoma cell lines and primary patient samples using a microRNA expression microarray.

MicroRNA expression microarray assays were performed as follows. CD34<sup>+</sup> HSPCs, human leukemia/lymphoma cell lines and primary patient sample cells were disrupted with TRIzol Reagent (15596-018; Invitrogen, Carlsbad, CA). Five micrograms of total RNA obtained from the TRIzol preparations were analyzed with miRNA chips (Liu *et al.* PNAS, 101:9740-9744 (2004), incorporated by reference in its entirety). Standard microarray methods were used for normalization and statistical analysis of the miRNA expression data. Statistical analyses were carried out in GeneSpring 7.0 (Silicon Genetics/Agilent Technologies, Palo Alto, CA) to determine the RFU value for a 95% confidence cutoff for miRNA expression. Significantly expressed miRNAs were identified by using the GeneSpring Cross-Gene Error Model (Georgantas *et al.* Cancer Research, 64:4434-4441 (2004), incorporated by reference in its entirety). For each set of samples, values from individual samples were normalized and averaged, and then expressed miRNAs were identified as those with a  $P < 0.05$  of expression over background (i.e., the weighted averaged values of all chip measurements).

CD34<sup>+</sup> HSPCs expressed *miR-10a*, *miR-27a*, *miR-16a*, *miR-155* at moderate to high levels. On the other hand, all of the hematopoietic cancer cell lines that were tested to date showed an absence of *miR-10a* and *miR-27a* expression, but highly variable expression of *miR-16a* and *miR-155*. Further, of three leukemia patient samples tested, *miR-10a* was absent in 2/3 and *miR-27a* was absent in all cases, while *miR-16a* was very highly expressed. The comparison of *miR-10a* and *miR-27a* expression to *miR-16a* expression in these samples is important, since *miR-16a* was the first miR to be identified as a possible tumor suppressor in leukemia (specifically in a subset of CLL cases). These results indicate that *miR-10a* and *miR-27a* are more generalized tumor suppressor

miRs in leukemia and lymphoma, and are therefore potentially better targets for the development of anti-cancer therapies.

**Example 2: Expression of miR-10a in leukemia cell lines suppresses cancer cell growth**

In order to test the hypothesis that *miR-10a* functions as a tumor suppressor, *pre-miR-10a* was transfected into TF-1a and K562 cells. TF-1a is a human acute myeloid leukemia (AML) cell line, while K562 is a human chronic myeloid leukemia (CML) blast crisis cell line. Neither of these cell lines exhibit significant *miR-10a* expression (Figure 2). Transfection of the cell lines was performed using Lipofectamine (Invitrogen) according to manufactures instructions.

TF-1a (Figure 3A) or K562 cells (Figure 3B) were transfected with Lipofectamine alone, or Lipofectamine and 0.25  $\mu$ M of artificial *pre-miR-10a*. Assessment of growth rate by MTT assay revealed that expression of *miR-10a* decreased the growth rate of both cell lines (Figure 3). This finding was confirmed when TF1-a cells were transfected with Lipofectamine alone, Lipofectamine plus *miR-10a*, or Lipofectamine plus *miR-10a* and anti-*miR-10a* locked nucleic acid (LNA). Anti-miRNA LNA suppresses the miRNA's ability to inhibit target gene expression. Consequently, cells transfected with both *miR-10a* and anti-*miR-10a* LNA grew at a rate similar to cells transfected with Lipofectamine alone (Figure 4).

**Example 3: Expression of miR-27a in leukemia cell lines suppresses cancer cell growth**

In order to test the hypothesis that *miR-27a* functions as a tumor suppressor, *miR-10a* was transfected into TF-1a and K562 cells. As described above, TF-1a is a human acute myeloid leukemia (AML) cell line, while K562 is a human chronic myeloid leukemia (CML) blast crisis cell line. Neither of these cell lines exhibit significant *miR-27a* expression (Figure 2). Transfection of the cell lines was performed using Lipofectamine (Invitrogen) according to manufactures instructions.

TF-1a (Figure 5A) or K562 cells (Figure 5B) were transfected with Lipofectamine alone, or Lipofectamine and artificial *miR-10a*. Assessment of growth rate by MTT assay revealed that expression of *miR-10a* decreased the growth rate of TF-1a cells by more than 60% at day 3 (Figure 5A), and decreased the growth rate K562 cells by more than 30% at day three (Figure 5B).

**Example 4: Expression of miR-27a makes human leukemia cells more sensitive to apoptosis induced by chemotherapeutic agents or growth factor withdrawal**

To test the potential of *miR-27a* to render cancer cells more susceptible to apoptosis, TF-1a cells (Figure 6A-B) and normal human CD34<sup>+</sup> cells (Figure 6B) were Lipofectamine-transfected with *miR-27a* or vehicle alone. Cells were then exposed to 0.1  $\mu$ M of the chemotherapy agent methotrexate for 24 hrs, and apoptosis was assayed using a FLICA assay for activated Caspase 3.

Though TF-1a cells are normally resistant to 0.1  $\mu$ M methotrexate-induced apoptosis (Figure 6A, left), when transduced with *miR-27a* they become susceptible to apoptosis (Figure 6A, right). After only 24 hrs of methotrexate treatment a sizable increase in the number of cells undergoing apoptosis was observed (Figure 6B). In contrast, when normal CD34<sup>+</sup> HSPCs were transfected with *miR-27a* and exposed to methotrexate, no significant increase in apoptosis was observed (Figure 6B). These results suggest that *miR-27a* can modulate leukemia cell susceptibility to chemotherapeutic agents without affecting that of normal HSPCs.

To confirm that expression of *miR-27a* renders cancer cells susceptible to a broad range of chemotherapeutic agents, TF-1a cells were lipofectamine-transfected with *miR-27a* or lipofectamine alone and exposed to chemotherapy vehicle mock (Figure 7A), 1  $\mu$ M Staurosporine (Figure 7B), or 4  $\mu$ M Camptothecin (Figure 7C) 48 hours following transfection. Cells were assayed for apoptosis induction using a FLCIA assay for activated Caspase 3.

Since growth factor withdrawal experiments revealed that maximal apoptosis sensitivity induction by *miR-27a* was around 48 hours post miR-transfection (Figure 8), subsequent chemotherapy experiments were performed at 48 hrs. Figure 7 shows that *miR-27a* transfection alone does not change apoptosis of leukemia cells (Figure 7A), but greatly increases the apoptotic response of leukemia cells to both staurosporine (Figure 7B, arrow indicates *miR-27a* transfected cells) and camptothecin (Figure 7C, arrow indicates *miR-27a* transfected cells). These results indicate that *miR-27a* modulates the apoptotic potential of cancers to chemotherapeutics, such as methotrexate, camptothecin, staurosporine.

To assess the effect of *miR-27a* expression on growth factor withdrawal induced apoptosis, TF-1 human myeloid leukemia cells, which are GM-CSF dependent, were transfected with artificial *miR-27a* and then 1 day later were washed and cultured in media lacking GM-CSF. After 24 hours of growth factor withdrawal, *miR-27a*-transfected cells showed a greatly decreased percentage of cells that appeared dying/dead by flow cytometric light scattering properties (Figure 8A) and an increase in the number of cells undergoing apoptosis (Figure 8B). This effect was even more pronounced 48 hours after GM-CSF withdrawal (Figure 8C-D, arrow indicates *miR-27a* transfected cells). In combination with the methotrexate data, these growth factor withdrawal data indicate that *miR-27a* may increase the sensitivity of leukemias to apoptosis, without an effect on normal CD34<sup>+</sup> HSPCs.

#### **Example 5: Expression of miR-27a enhances apoptosis in the Acute Lymphoblastic Leukemia cell line REH**

The REH cell line is a human Acute Lymphoblastic Leukemia (ALL) cell line was screened in the miRNA microarray analysis described in Example 1, which indicated that it has little or no

*miR-27a* expression (Figure 2). REH cells were transduced with *miR-27a* or control (FUGW) lentivector (LV) (Yu et al 2003, 2006; Alder et al 2008, each of which is incorporated by reference in their entirety). Successful transduction with either lentivector resulted in GFP expression. Seventy-two hours after transduction, cells were plated in methylcellulose media and grown for 7-12 days. GFP expression was used as a marker to select successfully transduced colonies by eye using fluorescence microscopy. Colonies of cells with a variety of intensities of GFP fluorescence were plucked from the plates and expanded. Four *miR-27a*-transduced and 3 control-transduced colonies were selected, denoted as control (FUGW) LV-transduced or *miR-27a* LV-transduced, and with arbitrary clone numbers. GFP expression by the selected colonies was confirmed by FACS (Figure 9). The *miR-27a* expressing colonies were matched to control colonies based on GFP expression (denoted in Figure 9 by asterisks), with one matched set having GFP levels at  $\sim 10^2$  fluorescence units, and one set having GFP expression at  $\sim 10^3$  fluorescence units. These matched sets of REH clones were used in subsequent experiments. In this and subsequent experiments, GFP- indicates “untransduced” cells and refers to cells that have not been exposed to LV.

Assessment of LV-transduced REH clones for viability and apoptosis levels indicated that *miR-27a* LV-transduced cells had decreased viability and increased apoptosis compared to control-LV transduced cells (Figure 10). Viability was determined by analysis of forward versus side scatter FACS plots. This revealed that *miR-27a* REH clone #3 was 12% less viable than its matched control LV-transduced clone (#9) (Figure 10A). Spontaneous apoptosis and cell death were determined by AnnexinV and 7-Amino-Actinomycin-D (7AAD) staining (Becton Dickinson, assay performed according to manufacturer’s instructions). The lower right quadrant of the FACS plots depicted in Figure 10B indicate AnnexinV<sup>+</sup>/7AAD<sup>-</sup> cells that are undergoing apoptosis (2% for control LV- and 17% for *miR-27a* LV-transduced cells), while the total number of AnnexinV<sup>+</sup> cells indicate the total level of cell death (11% for control LV- and 34% for *miR-27a* LV-transduced cells). The results of 3 independent apoptosis assays using the control #9 and *miR-27a* #3 clones are depicted in Figure 10C and indicate difference of 16% between REH cells transduced with a control lentivector and those transduced with a *miR-27a* lentivector. The p-value for *miR-27a* #3 versus control #9 is 0.006. Figure 10D represents the results of 2 separate cell death assays, and demonstrates that *miR-27a* overexpression (via LV-transduction) increases the rate of cell death.

To test whether transduction with a *miRNA-27a* lentivector results in increased susceptibility of REH cells to chemotherapy agents, cells from control LV- and *miR-27a* LV-transduced colonies were plated in 12-well plates at a concentration of  $0.5 \times 10^6$  cells/ml/well and treated either with vincristine in DMSO or DMSO alone (0  $\mu$ M vincristine) overnight. Cells were harvested and assayed for drug-induced apoptosis via AnnexinV and 7AAD staining. Figure 11A depicts FACS plots showing AnnexinV vs. 7AAD staining for control-LV clone #9. These results

indicate that culturing control-LV transduced cells in 1.0  $\mu$ M vincristine induced apoptosis by 23% compared to cells cultured without vincristine (indicated by the AnnexinV<sup>+</sup>/7AAD<sup>-</sup> quadrant). The FACS plots depicted in Figure 11B show AnnexinV vs. 7AAD for *miR-27a* clone #7. These REH cells, which express *miR-27a*, demonstrate increased vincristine-induced apoptosis at every concentration compared to REH cells transduced with control LV. Figures 11C and 11D depict the results of additional apoptosis and cell death experiments using control clone #9 and its matched *miR-27a* clone #7, and also demonstrate that *miR-27a* expressing REH cells undergo increased drug-induced apoptosis/death at lower doses of vincristine than control cells. These results were confirmed using matched control and *miR-27a* expressing clones with lower GFP intensity ( $10^2$ ) (Figures 11E-F). These experiments also indicated that the apoptosis induction by *miR-27a* is dose dependent.

**Example 6: Expression of miR-27a enhances apoptosis in the Acute Myeloid Leukemia cell line HL60**

The HL60 cell line is a human Acute Myeloid Leukemia (ALL) cell line screened in the miRNA microarray analysis described in Example 1, which indicated that it had little or no *miR-27a* expression (Figure 2). HL60 cells were transduced with *miR-27a* or control (FUGW) lentivector as described above. Successful transduction with either lentivector resulted in GFP expression. Seventy-two hours after transduction, cells were plated in methylcellulose media and grown for 7-12 days. GFP expression was used as a marker to select successfully transduced colonies by eye using fluorescence microscopy. Colonies of cells with a variety of intensities of GFP fluorescence were plucked from the plates and expanded. Lentivector-transduced colonies were selected, denoted as control (FUGW) LV-transduced or *miR-27a* LV-transduced, and with arbitrary clone numbers. GFP expression by the selected colonies was confirmed by FACS (Figure 12). In this and subsequent experiments, GFP<sup>-</sup> indicates “untransduced” cells and refers to cells that have not been exposed to LV.

Assessment of LV-transduced HL60 clones for viability and apoptosis levels indicated that *miR-27a* LV-transduced cells had decreased viability and increased apoptosis compared to control-LV transduced cells and GFP<sup>-</sup> cells (Figures 13 and 14). Viability was determined by analysis of forward versus side scatter FACS plots. This revealed that, at week 1 post-transduction *miR-27a* HL60 clones were no less viable than control clones (Figure 13A). At 2 weeks post-transduction, on the other hand, *miR-27a* H60 clones were significantly less viable than control clones (Figure 13B). This decrease in viability in the *miR-27a* HL60 clones is depicted in Figure 13C. Spontaneous apoptosis and cell death were determined by AnnexinV and 7-Amino-Actinomycin-D (7AAD) staining, as described above. The lower right quadrant of the FACS plots depicted in

Figure 14 indicate AnnexinV<sup>+</sup>/7AAD<sup>-</sup> cells undergoing apoptosis (5% for GFP<sup>-</sup> cells, 8% for control LV-transduced cells and 18-26% for *miR-27a* LV-transduced cells, Figure 14), while the total number of AnnexinV<sup>+</sup> cells indicate the total level of cell death (9% for GFP<sup>-</sup> cells, 13% for control LV-transduced cells and 26-32% for *miR-27a* LV-transduced cells, Figure 14). Similar experiments were performed in the presence of chemotherapy agents, staurosporine or camptothecin, which indicated that *miR-27a* expression also increased chemical-induced apoptosis in HL60 cells.

To test the effect of transduction with a *miRNA-27a* lentivector on the cell cycle progression of H60 cells, cells from selected GFP<sup>-</sup>, control and *miR-27a* expressing HL60 clones were fixed and stained with Propidium Iodide (PI, a fluorescent DNA intercalating agent; staining was performed according to the Becton Dickinson protocol) 24 hours after being placed in fresh RPMI media containing 10% serum. PI stained cells were analyzed by FACS. Histogram and Watson Pragmatic/Dean/Jett/Fox projections for GFP<sup>-</sup>, control and *miR-27a* clone #4 demonstrate an increased S phase population and decreased G<sub>1</sub> phase population in *miR-27a* clone #4 versus the two controls (Figure 15A). The plots for *miR-27a* #4 are representative of all *miR-27a* clones tested, which are quantitated in Figure 15B.

#### **Example 7: Expression of *miR-27a* enhances apoptosis in the Erythroleukemia cell line TF1**

The granulocyte-macrophage colony stimulating factor (GM-CFS) dependent cell line TF1 was another cell line screened in the *miR* microarray screen described in Example 1 that expressed little or no *miR-27a* compared to normal CD34<sup>+</sup> HSPCs. As described in Example 3, TF1 cells expressing *miR-27a* were more sensitive to staurosporine- and camptothecin-induced apoptosis assessed by percent active Caspase-3 (Vybrant FAM Caspase-3 and -7 assay kit, Invitrogen, performed according to manufacturer's instructions). Additionally, *miR-27a* expressing TF1 cells were more sensitive to apoptosis induced by growth factor withdrawal. This example presents a study of control LV- and *miR-27a* LV-transduced TF1 cells, which were generated and selected (as described above).

Assessment of LV-transduced TF1 clones for apoptosis level indicated that *miR-27a* LV-transduced cells had increased spontaneous apoptosis levels compared to control-LV transduced cells (Figure 16). As described above, spontaneous apoptosis was assessed via AnnexinV and 7AAD staining. The TF1 clone transduced with control LV had 9% of total cells that underwent spontaneous apoptosis (AnnexinV<sup>+</sup>/7AAD<sup>-</sup>) and 18% total cell death (AnnexinV<sup>+</sup>/7AAD<sup>-</sup> and AnnexinV<sup>+</sup>/7AAD<sup>+</sup>). *miR-27a* expressing clones, on the other hand, demonstrated an average of 22% spontaneous apoptosis (a 13% increase over control) and 37% total cell death (a 19% increase over control). All TF1 clones analyzed had similar amounts of *miR-27a* expression as assessed by

GFP intensity. The data presented in Figure 16A is representative of 2 separate experiments, and is summarized in Figure 16B.

**Example 8: Expression of miR-27a enhances apoptosis in the Chronic Myeloid Leukemia blast crisis cell line K562**

The K562 cell line is a human Chronic Myeloid Leukemia (CML) blast crisis cell line that was screened in the miRNA microarray analysis described in Example 1, which indicated that it had little or no *miR-27a* expression (Figure 2). This was confirmed by a second miRNA microarray analysis and Northern blot. K562 cells are known to be highly drug resistant (*e.g.* doses of the chemotherapeutic drug vincristine up to and including 0.5  $\mu$ M do not induce apoptosis above background levels). Transiently transfecting K562 cells with a *miR-27a* construct indicated a dose-dependent increase in vincristine-induced apoptosis.

K562 cells were transduced with *miR-27a* or control (FUGW) lentivector as described above. Successful transduction with either lentivector resulted in GFP expression. Seventy-two hours after transduction, cells were plated in methylcellulose media and grown for 8 days. GFP expression was used as a marker to select successfully transduced colonies by eye using fluorescence microscopy. Colonies of cells with a variety of intensities of GFP fluorescence were plucked from the plates and expanded. Lentivector-transduced colonies were selected, denoted as control (FUGW) LV-transduced or *miR-27a* LV-transduced, and with arbitrary clone numbers. GFP expression by the selected colonies was confirmed by FACS (Figure 17). In this experiment, GFP- indicates “untransduced” cells that were plucked from the same plate as the *miR-27a* LV-transduced cells.

Assessment of LV-transduced K562 clones for viability, apoptosis and cell death levels indicated that *miR-27a* LV-transduced cells had decreased viability and increased cell death and apoptosis compared to GFP<sup>+</sup> cells (Figures 18-20). Viability was determined by analysis of forward versus side scatter FACS plots. This revealed that, at week 1 post-transduction *miR-27a* K562 clones were marginally less viable than control clones (Figure 18). At 5 weeks post-transduction, on the other hand, *miR-27a* K562 clones were significantly less viable than control clones (Figure 18). Spontaneous cell death was determined by AnnexinV and 7-Amino-Actinomycin-D (7AAD) staining, as described above. The total number of AnnexinV<sup>+</sup> cells indicates the level of cell death (13% for GFP<sup>-</sup> cells and 21-36% for *miR-27a* LV-transduced cells, Figure 19). As described above, similar experiments were performed in the presence of the chemotherapy agent vincristine, which indicated that *miR-27a* expression also increased chemical-induced apoptosis in K562 cells. Spontaneous apoptosis and cell death were determined by AnnexinV and 7-Amino-Actinomycin-D (7AAD) staining, as described above. The lower right quadrant of the FACS plots depicted in

Figure 20 indicate AnnexinV<sup>+</sup>/7AAD<sup>-</sup> cells that are undergoing apoptosis (13% for GFP<sup>-</sup> cells, 11% for control LV-transduced cells and 17-31% for *miR-27a* LV-transduced cells, Figure 20). As described above, similar experiments were performed in the presence of the chemotherapy agent vincristine, which indicated that *miR-27a* expression also increased chemical-induced apoptosis in K562 cells.

To test the effect of transduction with a *miRNA-27a* lentivector on the cell cycle progression of K562 cells, cells from selected GFP<sup>-</sup> and *miR-27a* expressing K562 clones were fixed and stained with Propidium Iodide (PI) 24 hours after being placed in fresh RPMI media containing 10% serum, as described above. PI stained cells were analyzed by FACS. Histogram and Watson Pragmatic/Dean/Jett/Fox projections for GFP<sup>-</sup>, *miR-27a* clone #1, *miR-27a* clone #4 and *miR-27a* clone #6 demonstrate an increased S phase population and decreased G<sub>1</sub> phase population in *miR-27a* clones versus control (Figure 21A). These plots are representative of all *miR-27a* clones tested, which are quantitated in Figure 21B.

#### **Example 9: Binding of miR-27a to potential target genes**

In order to identify possible *miR-27a* target genes that may be involved in *miR-27a*'s function as a tumor suppressor, databases constructed using prediction algorithms (e.g. TargetScan, miRanda) were searched for genes with putative *miR-27a* target sequences in their 3'-untranslated region. Genes were selected from these search results based on the observed *miR-27a* overexpression effects described herein. Selected putative targets selected for further analysis include YWHAQ an anti-apoptotic protein, Polo-like kinase 2 (PLK2), which contains 2 highly conserved predicted miR-27a sites and is known to reduce taxol-induced apoptosis and multi-drug resistant transporter ABCC4. Luciferase expression assays were used to confirm that predicted miR-27a sites in the 3'UTR of YWHAQ, PLK2 and ABCC4 are able to bind to *miR-27a*.

To confirm *miR-27a* binding of YWHAQ, K562 cells were plated in 12-well plates at a concentration of  $0.5 \times 10^6$  cells/ml/well. The cells were co-transfected with either a control-luciferase expressing plasmid (pcDNA-luc) or a pcDNA-luc plasmid with the miR-27a site of the 3'UTR of YWHAQ cloned into it (YWHAQ-27), and a  $\beta$ -gal reporter plasmid for normalization. After 24 hr, cells were collected, lysed and assayed for luminescence (Luciferase assay system, Promega, according to manufacturer's protocol). Luciferase expression was lower by 33% in K562 cells transfected with the YWHAQ-27-luc reporter plasmid than in cells transfected with the control pcDNA-luc plasmid (Figure 22A). The decrease was even more pronounced in cells that were co-transfected with 100 nM of artificial *miR-27a* in addition to the YWHAQ-27-luc reporter plasmid (89% decrease), indicating that *miR-27a* does indeed bind to its predicted target site within the 3'UTR of YWHAQ. The results presented in Figure 22A are the average of three independent

experiments, standard deviations are indicated with error bars, and asterisks indicate a significant change compared to pcDNA-luc control reporter plasmid (p-value < 0.05).

To confirm *miR-27a* binding of PLK2, K562 cells were plated, co-transfected and analyzed as described above, but with the full-length 3'UTR of PLK2 cloned into the pcDNA-luc reporter plasmid (PLK2-full-luc). Similar to YWHAQ-27-luc, luciferase expression was decreased (59%) in K562 cells transfected with PLK2-full-luc compared to pcDNA-luc. Once again, co-transfection with 100 nM of artificial *miR-27a* yielded an even greater decrease in luciferase expression (96%) (Figure 22B). The experiment was performed 3 times, the standard deviation is presented as error bars, and the asterisks indicate a significant change compared to pcDNA-luc (p-value < 0.05).

To confirm *miR-27a* binding of ABCC4, K562 cells were plated, co-transfected and analyzed as described above, but with the full-length 3'UTR of ABCC4 cloned into the pcDNA-luc reporter plasmid (ABCC4-luc). This time, no significant difference in luciferase expression was observed between cells receiving the control pcDNA-luc reporter plasmid and the ABCC4-luc plasmid. However, upon co-transfection of 100 nM artificial *miR-27a*, there was a significant decrease (80%) in luciferase expression. This experiment was done in triplicate, values presented are an average of the 3 replicates with standard deviations presented as error bars. Significant differences compared to control pcDNA-luc is indicated with an asterisk (p-value = 0.0001). This result was further confirmed by co-transfecting K562 cells that express *miR-27a* with either pcDNA-luc, ABCC4-luc, or ABCC4-luc and anti-miR-27a-locked nucleic acid (LNA) inhibitor and a  $\beta$ -gal reporter plasmid for normalization. Cells were analyzed as described above. The *miR-27a* LV-transduced cells transfected with ABCC4-luc had 61% less luciferase expression than the same cells transfected with pcDNA-luc (Figure 22D). Additionally, it was confirmed that at least part of this observed decrease was specifically due to *miR-27a*, as the co-transfection of the *miR-27a* inhibiting LNA increased luciferase expression by 15%. This experiment was performed in triplicate, and values presented are an average of the 3 replicates with standard deviations as indicated as error bars. Significance for ABCC4-luc in comparison to pcDNA-luc (p-value = 0.0001) and significance for ABCC4 + LNA in comparison to the value for ABCC4 (p-value = 0.0003) are indicated by asterisks.

#### EQUIVALENTS

While specific embodiments of the subject invention have been discussed, the above specification is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification. The appended claims are not intended to claim all such embodiments and variations, and the full scope of the invention should be

determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

What is claimed is:

1. A method for inhibiting proliferation of a cancer cell comprising contacting the cancer cell with an effective amount of a nucleic acid molecule encoding a miRNA selected from the group consisting of *miR-10a*, *miR-27a*, and functional variants thereof.
2. The method according to claim 1 wherein the nucleic acid molecule is a miRNA.
3. The method according to claim 1 wherein the nucleic acid molecule is a pre-miRNA.
4. The method according to claim 1 wherein the nucleic acid molecule is a DNA molecule.
5. The method according to claim 1 wherein the cancer cell is transfected with the nucleic acid molecule.
6. The method according to claim 1 wherein the cancer cell is a solid tumor cell.
7. The method according to claim 1 wherein the cancer cell is a leukemia cell or a lymphoma cell.
8. A method for inducing apoptosis of a cancer cell comprising contacting the cancer cell with an effective amount of a nucleic acid molecule encoding a miRNA selected from the group consisting of *miR-10a*, *miR-27a*, and functional variants thereof.
9. The method according to claim 8 further comprising contacting the cancer cell with a chemotherapeutic agent.
10. The method according to claim 9 wherein the chemotherapeutic agent is selected from a group consisting of: altretamine, asparaginase, BCG, bleomycin sulfate, busulfan, camptothecin, carboplatin, carmusine, chlorambucil, cisplatin, cladribine, 2-chlorodeoxyadenosine, cyclophosphamide, cytarabine, dacarbazine imidazole carboxamide, dactinomycin, daunorubicin - dunomycin, dexamethosone, doxorubicin, etoposide, floxuridine, fluorouracil, fluoxymesterone, flutamide, fludarabine, goserelin, hydroxyurea, idarubicin HCL, ifosfamide, interferon alfa, interferon alfa 2a, interferon alfa 2b, interfereon alfa n3, irinotecan, leucovorin calcium, leuprolide, levamisole, lomustine, megestrol, melphalan, L-sarcosylin, melphalan hydrochloride, MESNA, mechlorethamine, methotrexate, mitomycin, mitoxantrone, mercaptopurine, paclitaxel, plicamycin, prednisone, procarbazine, streptozocin, tamoxifen, 6-thioguanine, thiotepa, topotecan, vinblastine, vincristine and vinorelbine tartrate.

11. The method according to claim 8 wherein the nucleic acid molecule is a miRNA.
12. The method according to claim 8 wherein the nucleic acid molecule is a pre-miRNA.
13. The method according to claim 8 wherein the nucleic acid molecule is a DNA molecule.
14. The method according to claim 8 wherein the cancer cell is transfected with the nucleic acid molecule.
15. The method according to claim 8 wherein the cancer cell is a solid tumor cell.
16. The method according to claim 8 wherein the cancer cell is a leukemia cell or a lymphoma cell.
17. A method for treating cancer in a subject comprising administering to the subject an effective amount of a nucleic acid molecule encoding a miRNA selected from the group consisting of *miR-10a*, *miR-27a*, and functional variants thereof.
18. The method according to claim 17 further comprising administering to the patient a chemotherapeutic agent.
19. The method according to claim 18 wherein the chemotherapeutic agent is selected from a group consisting of: altretamine, asparaginase, BCG, bleomycin sulfate, busulfan, camptothecin, carboplatin, carmustine, chlorambucil, cisplatin, cladribine, 2-chlorodeoxyadenosine, cyclophosphamide, cytarabine, dacarbazine imidazole carboxamide, dactinomycin, daunorubicin - daunomycin, dexamethasone, doxorubicin, etoposide, floxuridine, fluorouracil, fluoxymesterone, flutamide, fludarabine, goserelin, hydroxyurea, idarubicin HCL, ifosfamide, interferon alfa, interferon alfa 2a, interferon alfa 2b, interferon alfa n3, irinotecan, leucovorin calcium, leuprolide, levamisole, lomustine, megestrol, melphalan, L-sarcosine, melphalan hydrochloride, MESNA, mechlorethamine, methotrexate, mitomycin, mitoxantrone, mercaptopurine, paclitaxel, plicamycin, prednisone, procarbazine, streptozocin, tamoxifen, 6-thioguanine, thiotepa, topotecan, vinblastine, vincristine and vinorelbine tartrate.
20. The method according to claim 17 wherein the nucleic acid molecule is a miRNA.
21. The method according to claim 17 wherein the nucleic acid molecule is a pre-miRNA.
22. The method according to claim 17 wherein the nucleic acid molecule is a DNA molecule.
23. The method according to claim 17 wherein the cancer is a solid tumor.

24. The method according to claim 17 wherein the cancer is a leukemia or a lymphoma.
25. A pharmaceutical composition comprising an isolated nucleic acid molecule encoding a miRNA selected from the group consisting of *miR-10a*, *miR-27a*, and functional variants thereof.
26. The pharmaceutical composition according to claim 25 further comprising a chemotherapeutic agent.
27. The pharmaceutical composition according to claim 26 wherein the chemotherapeutic agent is selected from a group consisting of: altretamine, asparaginase, BCG, bleomycin sulfate, busulfan, camptothecin, carboplatin, carmusine, chlorambucil, cisplatin, cladribine, 2-chlorodeoxyadenosine, cyclophosphamide, cytarabine, dacarbazine imidazole carboxamide, dactinomycin, daunorubicin - daunomycin, dexamethosone, doxorubicin, etoposide, floxuridine, fluorouracil, fluoxymesterone, flutamide, fludarabine, goserelin, hydroxyurea, idarubicin HCL, ifosfamide, interferon alfa, interferon alfa 2a, interferon alfa 2b, interferon alfa n3, irinotecan, leucovorin calcium, leuprolide, levamisole, lomustine, megestrol, melphalan, L-sarcosyl, melphalan hydrochloride, MESNA, mechlorethamine, methotrexate, mitomycin, mitoxantrone, mercaptopurine, paclitaxel, plicamycin, prednisone, procarbazine, streptozocin, tamoxifen, 6-thioguanine, thiotepa, topotecan, vinblastine, vincristine and vinorelbine tartrate.
28. The pharmaceutical composition according to claim 25 further comprising a pharmaceutically-acceptable carrier.
29. The pharmaceutical composition according to claim 25 wherein the nucleic acid molecule is a miRNA.
30. The pharmaceutical composition according to claim 25 wherein the nucleic acid molecule is a pre-miRNA.
31. The pharmaceutical composition according to claim 25 wherein the nucleic acid molecule is a DNA molecule.
32. A kit comprising an isolated nucleic acid molecule encoding a miRNA selected from the group consisting of *miR-10a*, *miR-27a*, and functional variants thereof.
33. The kit according to claim 32 further comprising a chemotherapeutic agent.
34. The kit according to claim 33 wherein the chemotherapeutic agent is selected from a group consisting of: altretamine, asparaginase, BCG, bleomycin sulfate, busulfan, camptothecin,

carboplatin, carmusine, chlorambucil, cisplatin, cladribine, 2-chlorodeoxyadenosine, cyclophosphamide, cytarabine, dacarbazine imidazole carboxamide, dactinomycin, daunorubicin - dunomycin, dexamethosone, doxorubicin, etoposide, floxuridine, fluorouracil, fluoxymesterone, flutamide, fludarabine, goserelin, hydroxyurea, idarubicin HCL, ifosfamide, interferon alfa, interferon alfa 2a, interferon alfa 2b, interfereon alfa n3, irinotecan, leucovorin calcium, leuprolide, levamisole, lomustine, megestrol, melphalan, L-sarcosylin, melphalan hydrochloride, MESNA, mechlorethamine, methotrexate, mitomycin, mitoxantrone, mercaptopurine, paclitaxel, plicamycin, prednisone, procarbazine, streptozocin, tamoxifen, 6-thioguanine, thiotepa, topotecan, vinblastine, vincristine and vinorelbine tartrate.

35. The kit according to claim 32 wherein the nucleic acid molecule is a miRNA.
36. The kit according to claim 32 wherein the nucleic acid molecule is a pre-miRNA.
37. The kit according to claim 32 wherein the nucleic acid molecule is a DNA molecule.

**Figure 1**

***miR-10a*** (SEQ ID NO: 1):

UACCCUGUAGAUCCGAAUUUGUG

***miR\*-10a*** (SEQ ID NO: 2):

CAAAUUCGUAUCUAGGGGAAUA

***pre-miR-10a*** (SEQ ID NO: 3):

UCUGUCUGUCUUCUGUAUAUACCCUGUAGAUCCGAAUUUGUGUAAGGAAUU  
UUGUGGUCACAAAUUCGUAUCUAGGGGAAUAUGUAGUUGACAUAACACUC  
CGCUCU

***miR-27a*** (SEQ ID NO: 4):

UUCACAGUGGCUAAGUUCCGC

***miR\*-27a*** (SEQ ID NO: 5):

AGGGCUUAGCUGCUUGUGAGCA

***pre-miR-27a*** (SEQ ID NO: 6):

CUGAGGAGCAGGGCUUAGCUGCUUGUGAGCAGGGUCCACACCAAGUCGUGUU  
CACAGUGGCUAAGUUCCGCCCCCAG

**Figure 2**

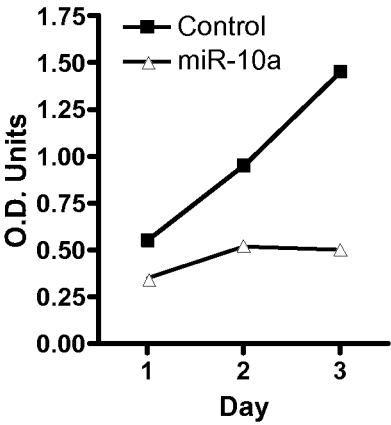
| <b>Cells</b>               | <b><i>miR-10a</i></b> | <b><i>miR-27a</i></b> | <b><i>miR-16a</i></b> | <b><i>miR-155</i></b> |
|----------------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| None (Chip Background)     | 47,568                | 47,568                | 47,568                | 47,568                |
| CD34+ HSPCs                | 123,147               | 113,259               | 123,147               | 355,623               |
| Hal-01 (ALL) cell line     | <b>34,647</b>         | <b>43,458</b>         | <b>68,774</b>         | 222,753               |
| HL-60 (AML) cell line      | <b>32,536</b>         | <b>46,834</b>         | 253,984               | 427,614               |
| K562 (CML) cell line       | 47,118                | <b>38,009</b>         | 105,060               | 620,020               |
| KG1 (AML) cell line        | <b>54,633</b>         | <b>68,475</b>         | 224,084               | 805,300               |
| MV4-1 (AML) cell line      | <b>42,004</b>         | <b>48,150</b>         | <b>69,321</b>         | 740,009               |
| REH (ALL) cell line        | <b>37,467</b>         | <b>47,337</b>         | <b>60,526</b>         | 158,352               |
| TF-1 (AML) cell line       | <b>38,123</b>         | <b>34,913</b>         | <b>23,459</b>         | <b>32,336</b>         |
| TF-1a (AML) cell line      | <b>50,080</b>         | <b>44,667</b>         | <b>48,579</b>         | <b>45,106</b>         |
| CRL2632 (ALL) cell line    | <b>33,040</b>         | <b>40,575</b>         | 233,550               | 94,500                |
| H292 (ALL) cell line       | <b>34,510</b>         | <b>25,125</b>         | 298,875               | 338,250               |
| KMH2 (ALL) cell line       | <b>23,512</b>         | <b>58,462</b>         | 166,500               | 220,350               |
| KMS12 (ALL) cell line      | <b>36,000</b>         | <b>36,862</b>         | 237,300               | <b>63,788</b>         |
| L484 (ALL) cell line       | <b>39,637</b>         | <b>55,463</b>         | 231,600               | 4,792,050             |
| RPMI8226 (ALL) cell line   | <b>36,712</b>         | <b>35,063</b>         | 366,675               | 108,600               |
| U266 (ALL) cell line       | <b>32,655</b>         | <b>33,375</b>         | 309,525               | <b>51,975</b>         |
| ALL case #1 patient sample | 106,820               | <b>47,817</b>         | 765,940               | 3,502,100             |
| ALL case #2 patient sample | <b>68,880</b>         | <b>35,700</b>         | 205,240               | 966,840               |
| ALL case #3 patient sample | <b>72,940</b>         | <b>36,505</b>         | 241,500               | 1,444,240             |

Any value under **80,000** is considered negative based on chip statistics and on measurements such as PCR, northern blot, etc. Negative values are shown in **Bold**.

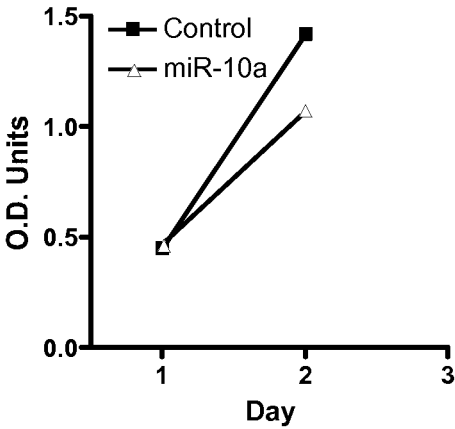
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Figure 3

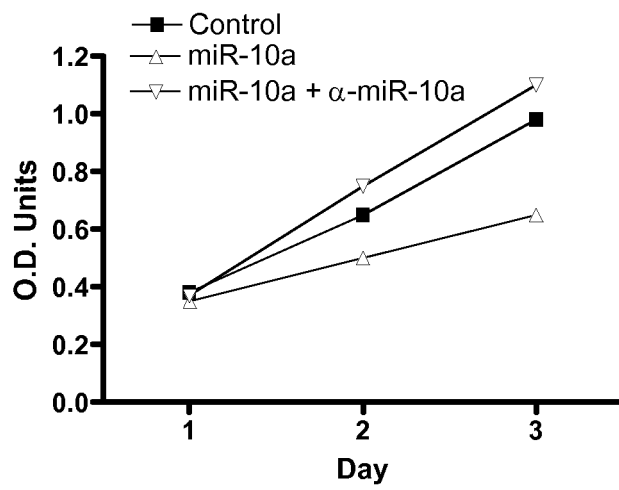
A



B



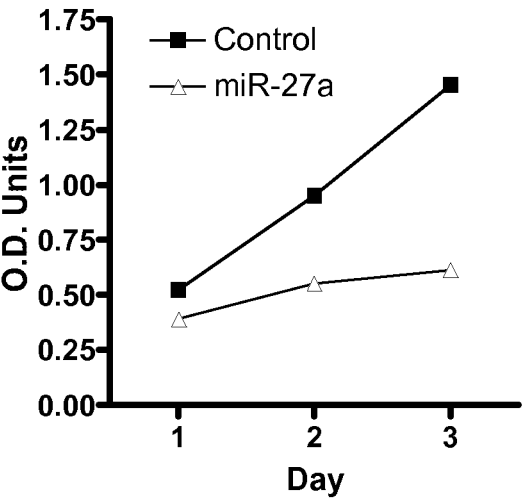
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**Figure 4**

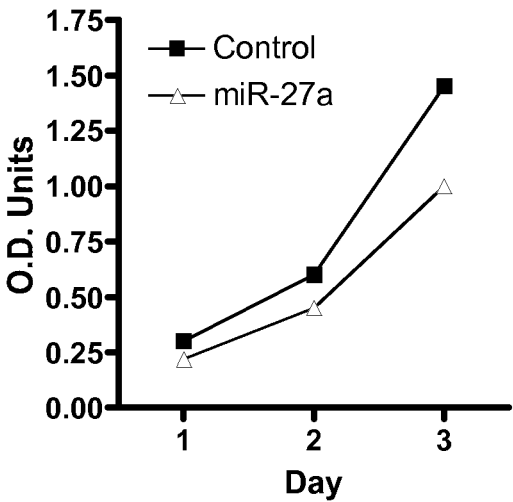
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Figure 5

A



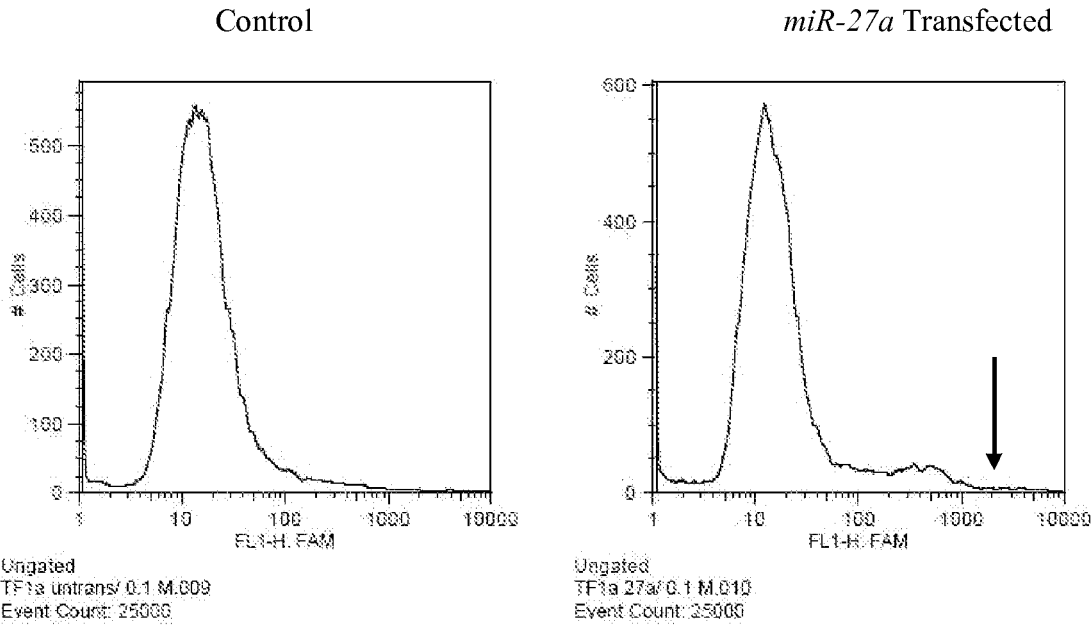
B



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Figure 6

A.



B.

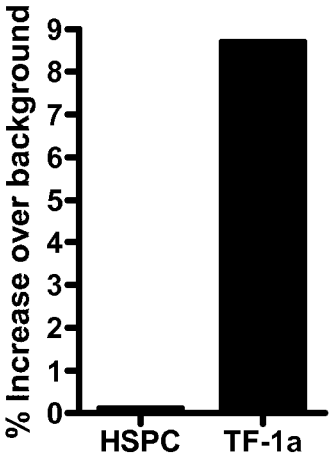
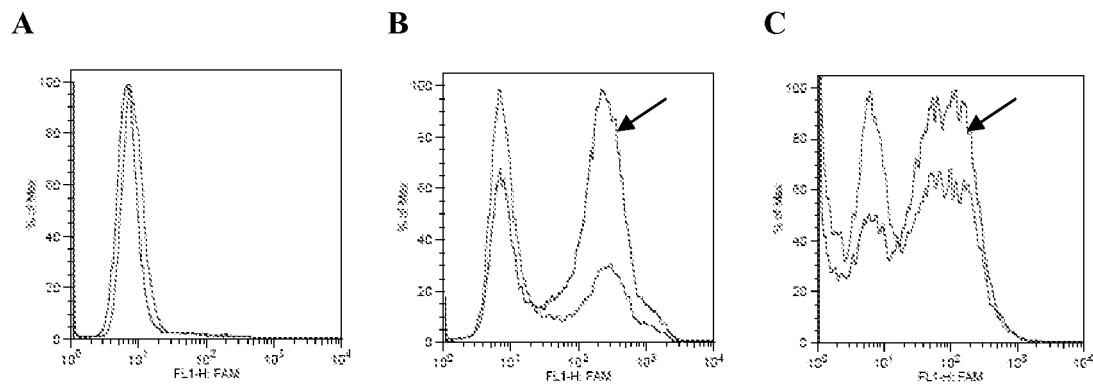
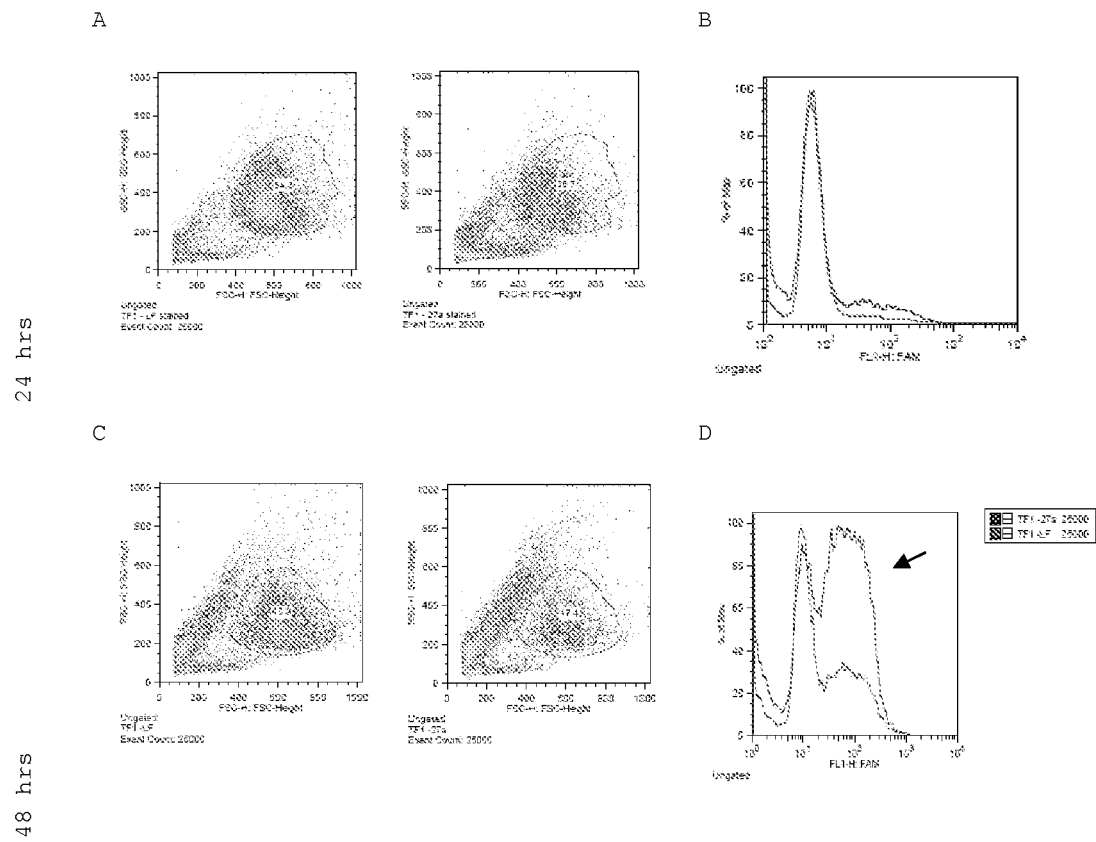


Figure 7



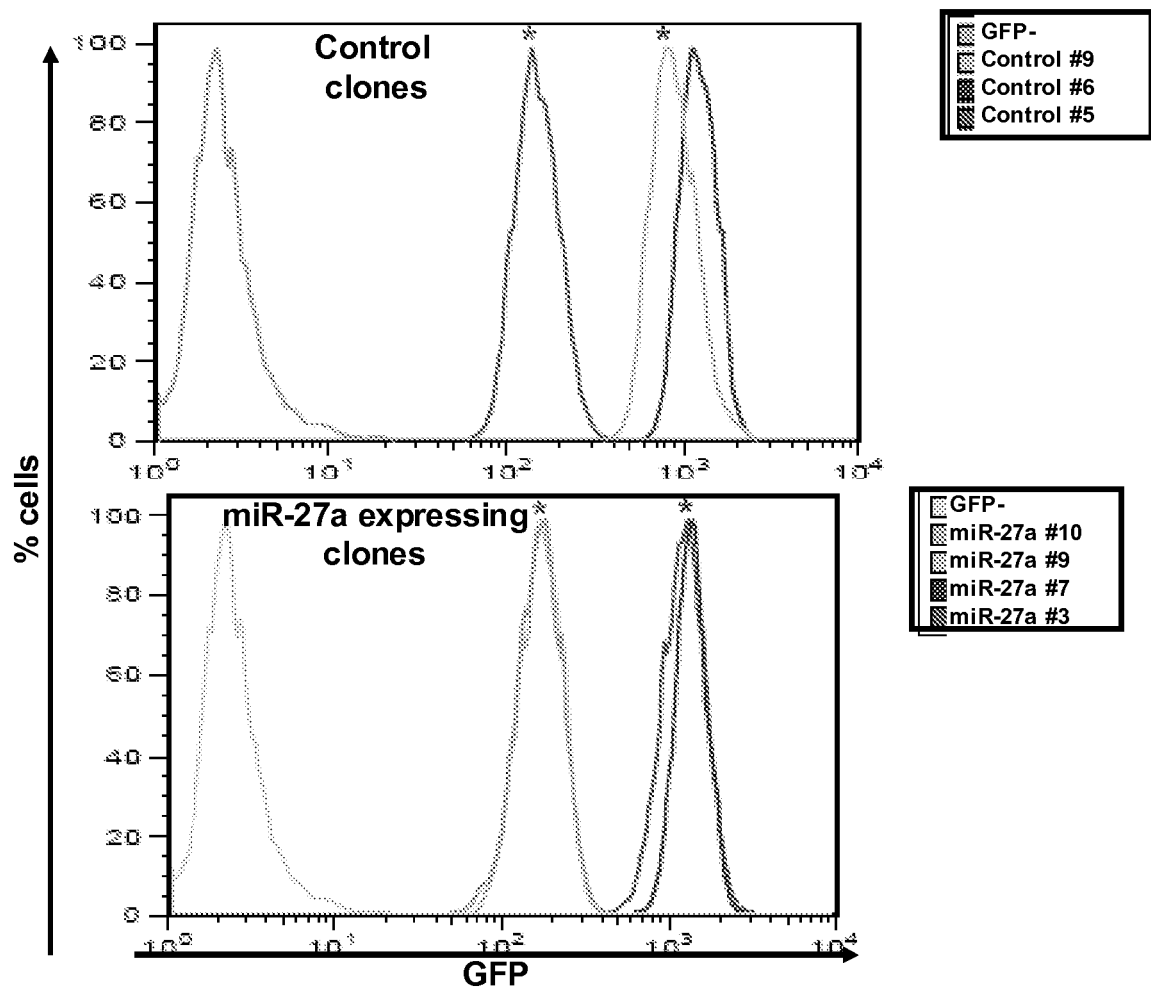
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Figure 8



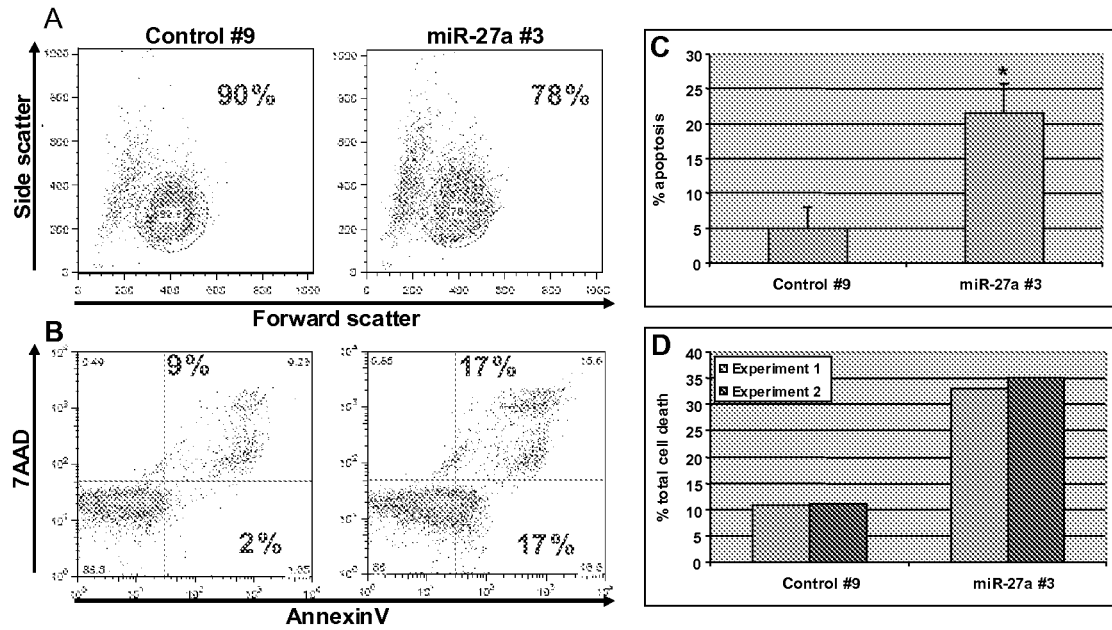
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Figure 9



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Figure 10



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Figure 11

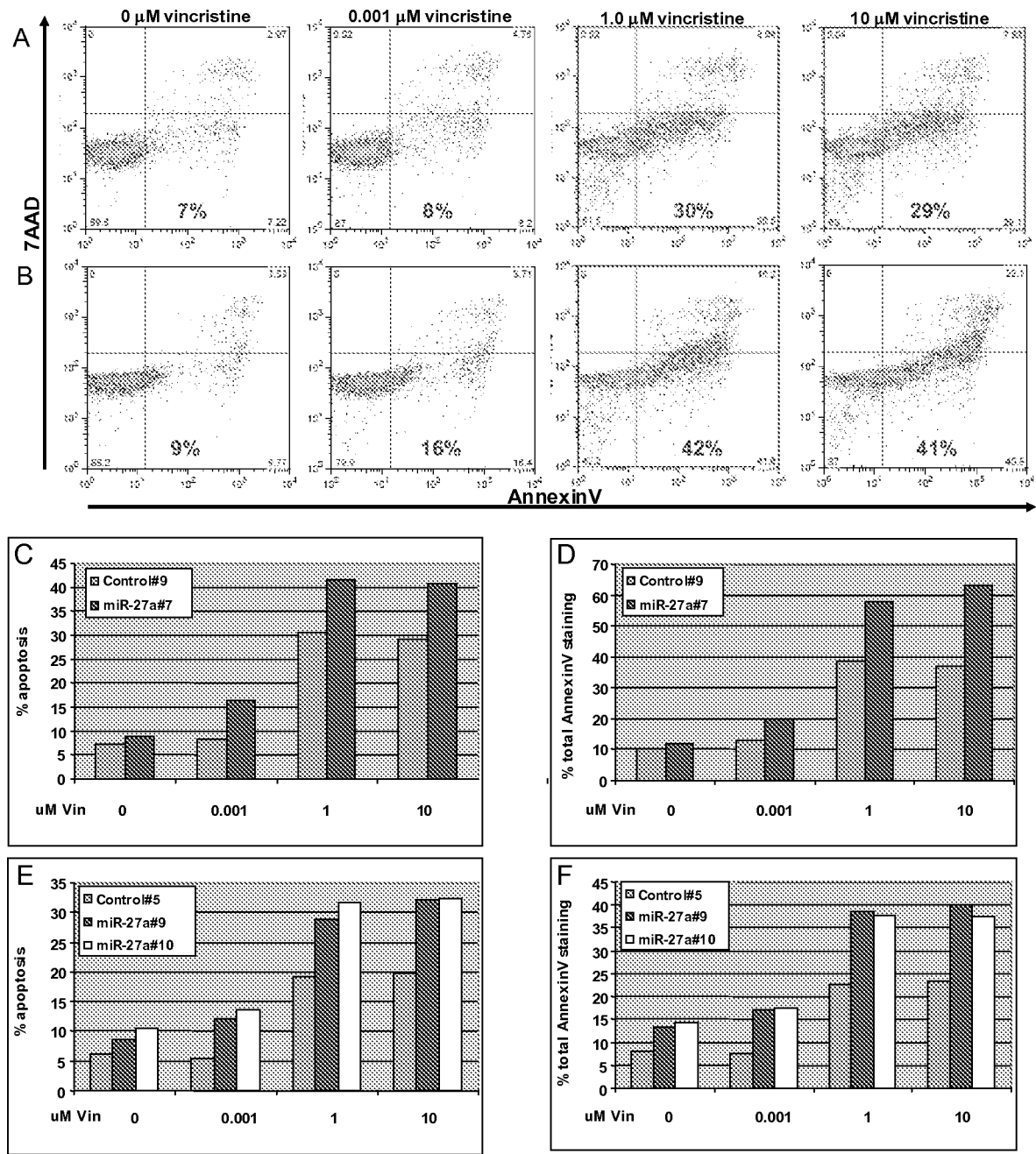
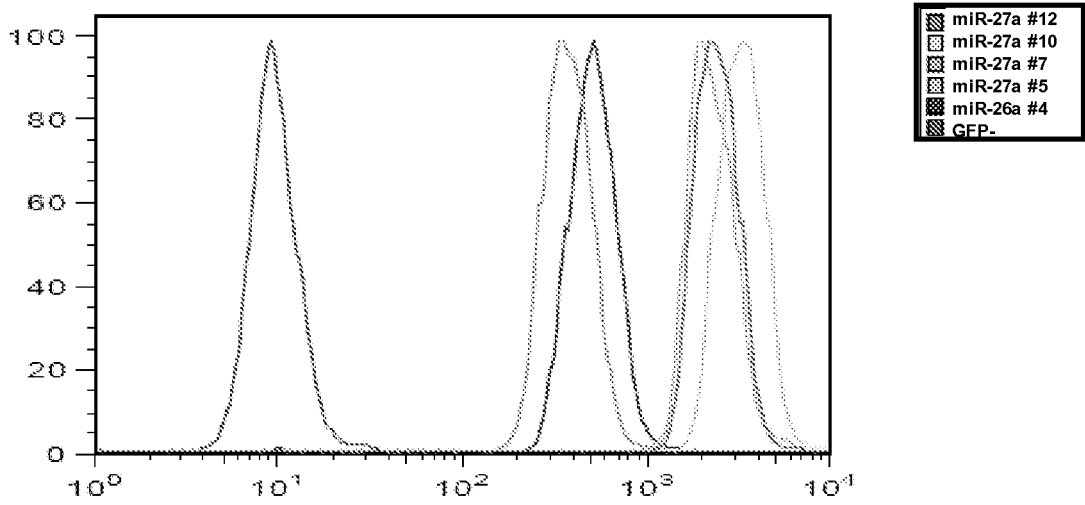


Figure 12



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Figure 13

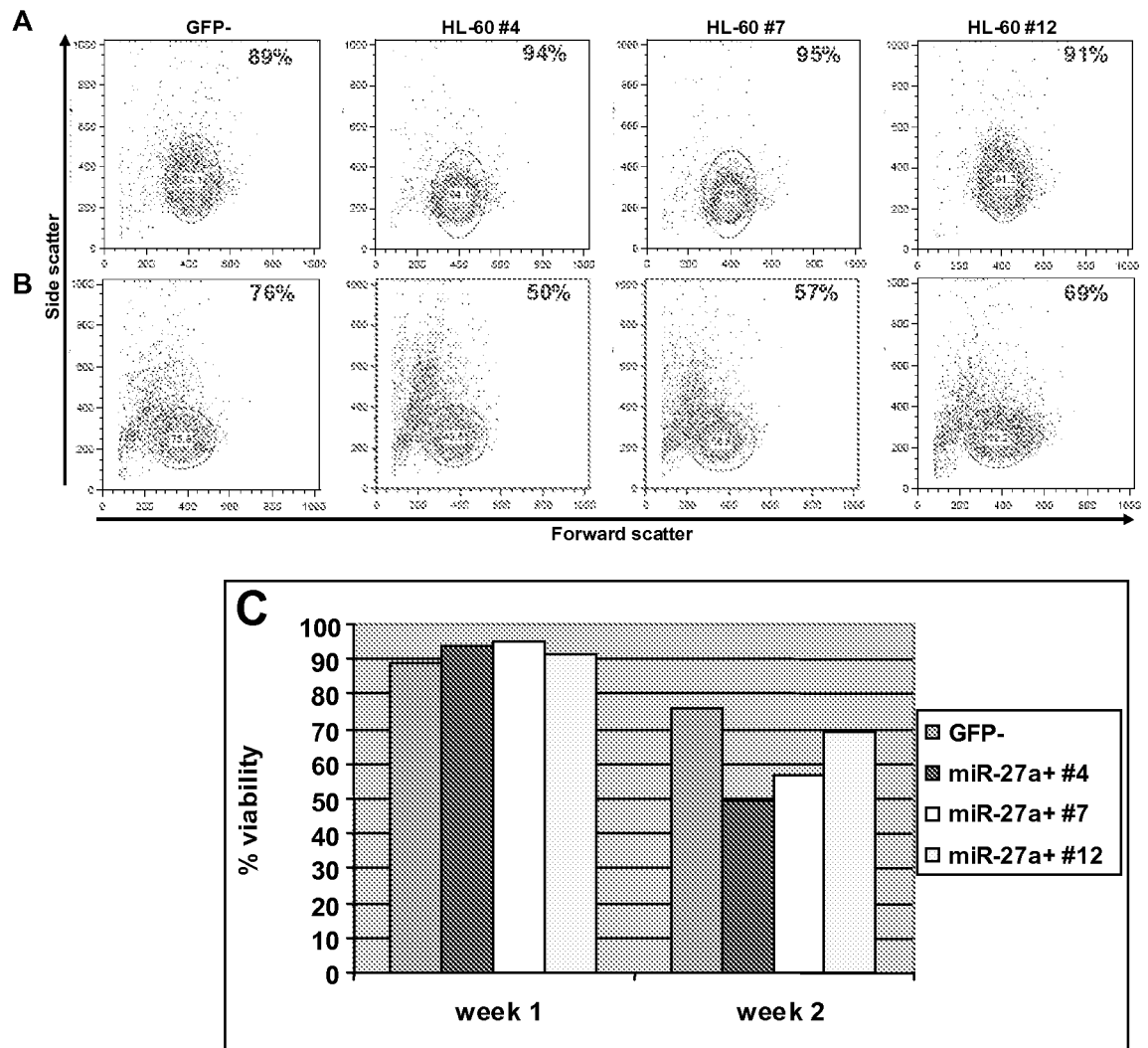
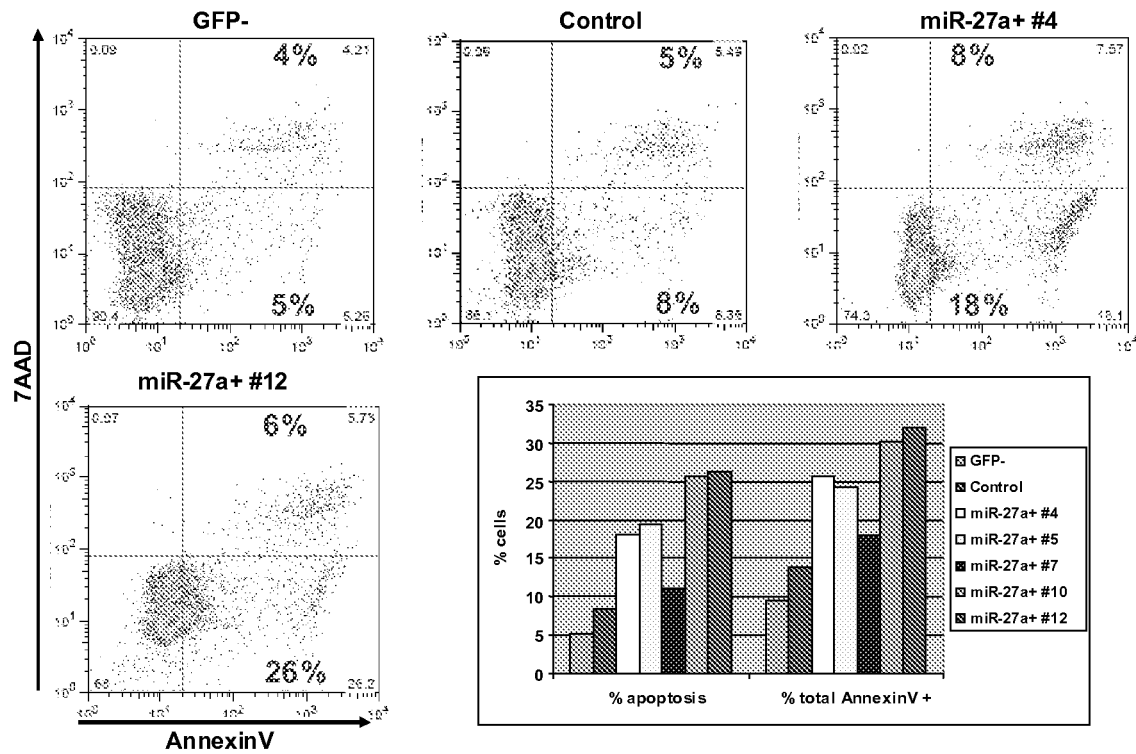


Figure 14



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Figure 15

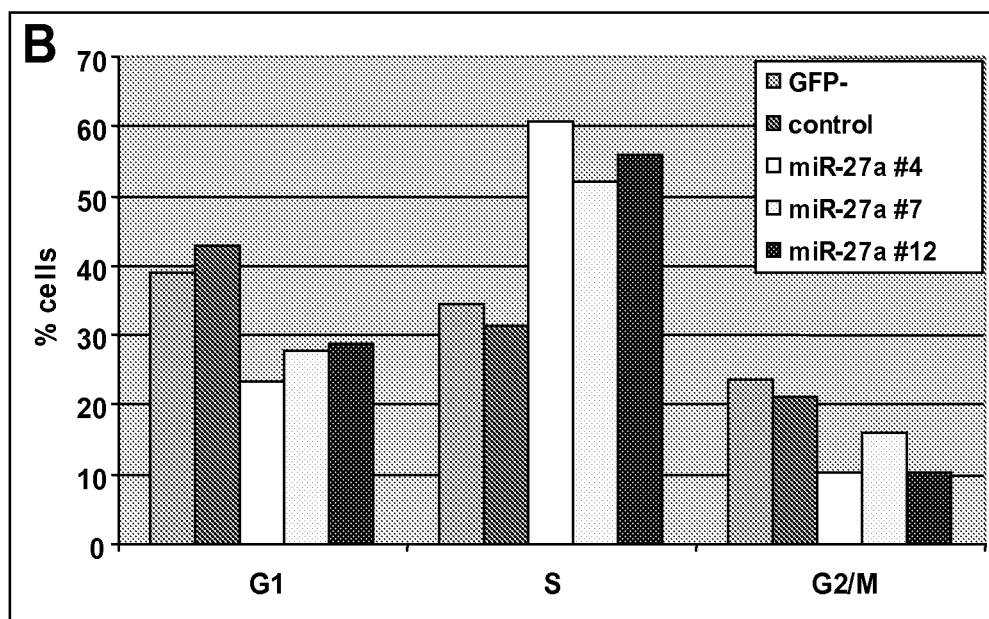
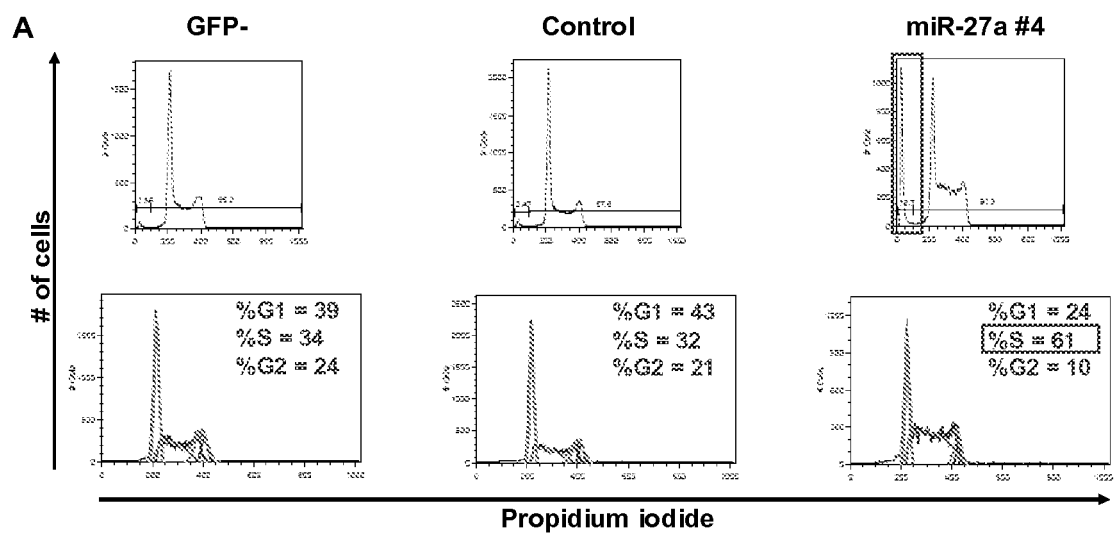


Figure 16

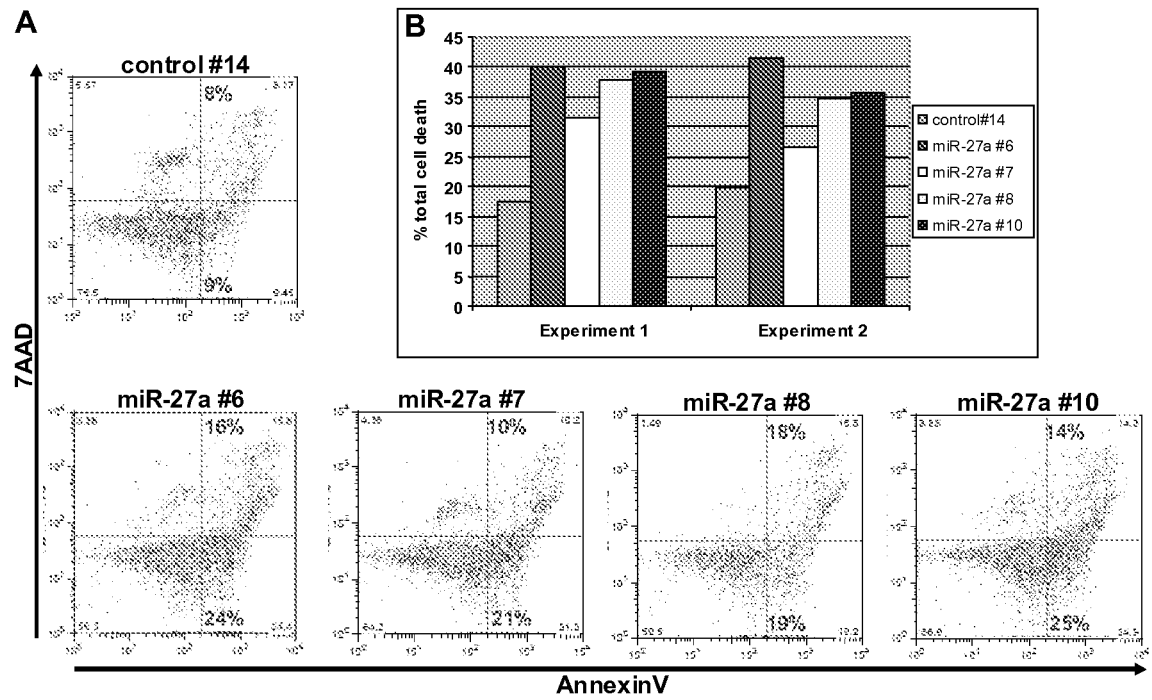
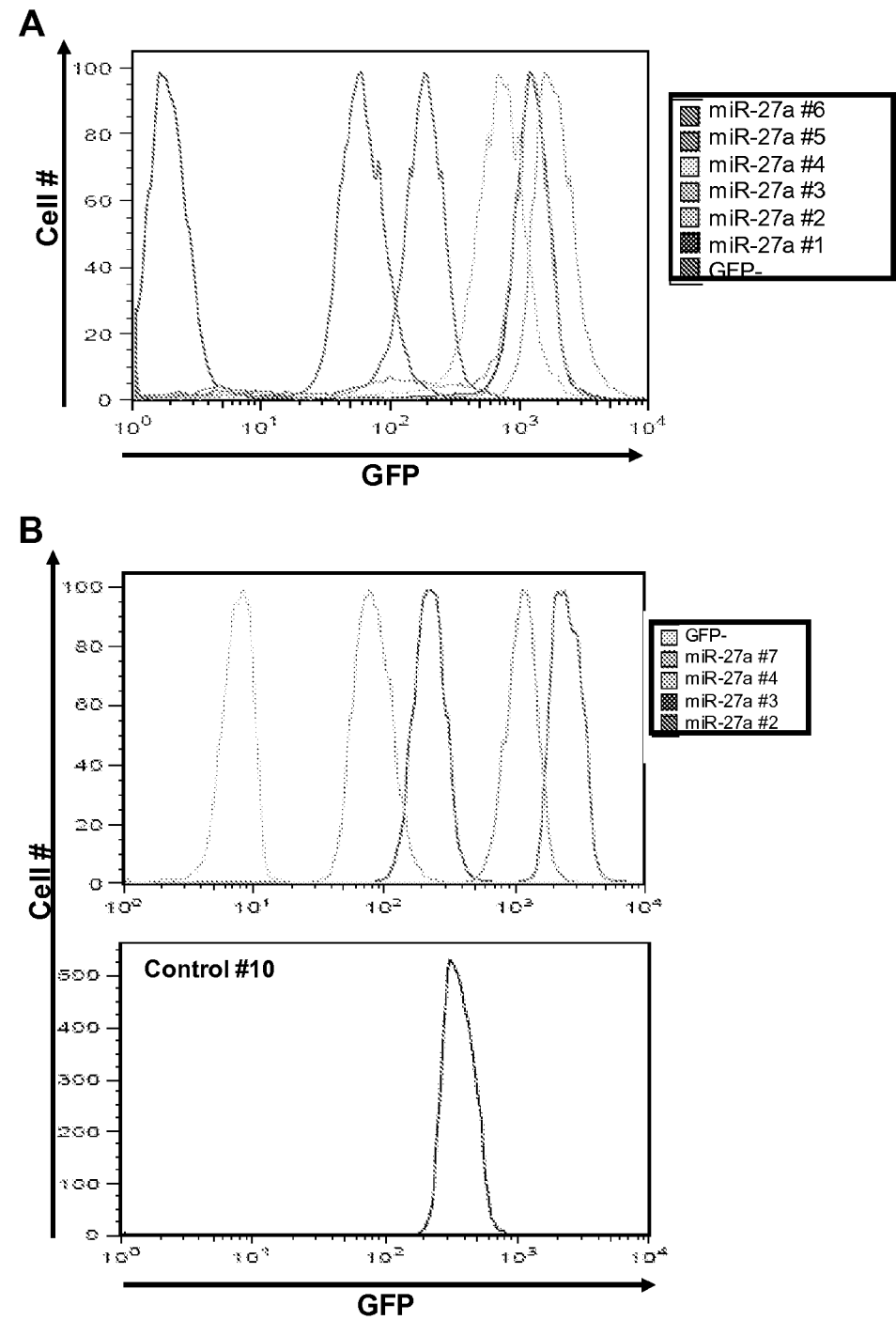


Figure 17



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Figure 18

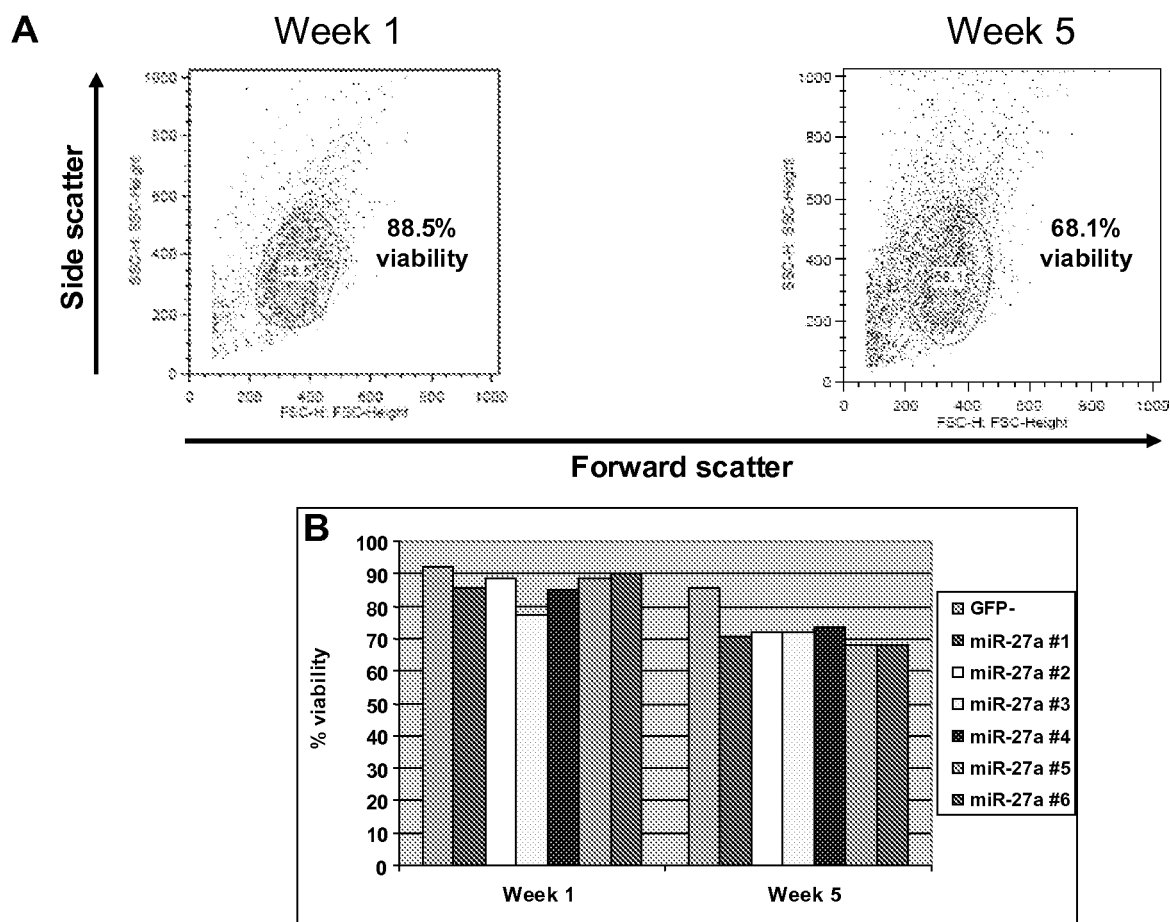


Figure 19

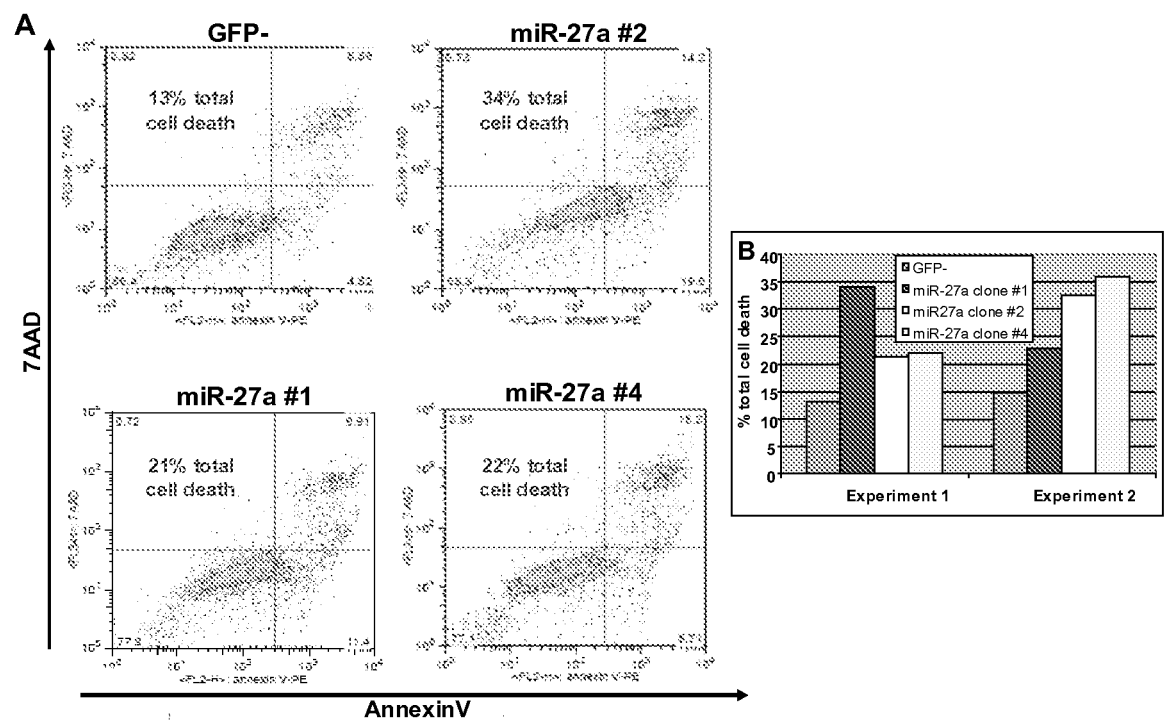
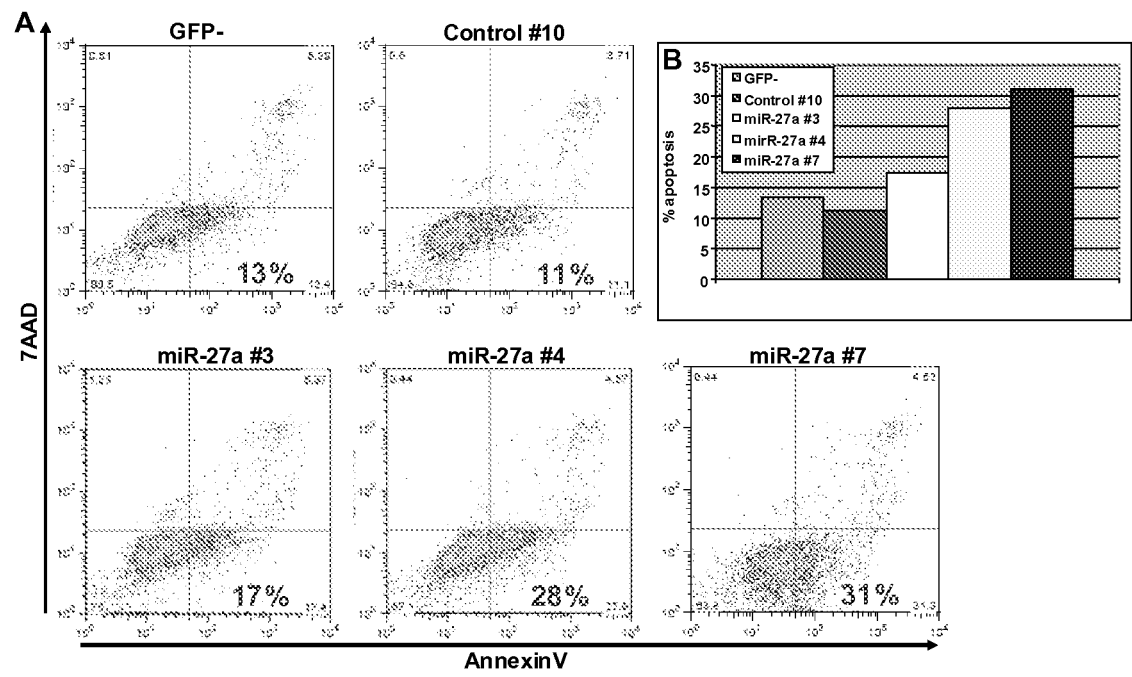


Figure 20



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Figure 21

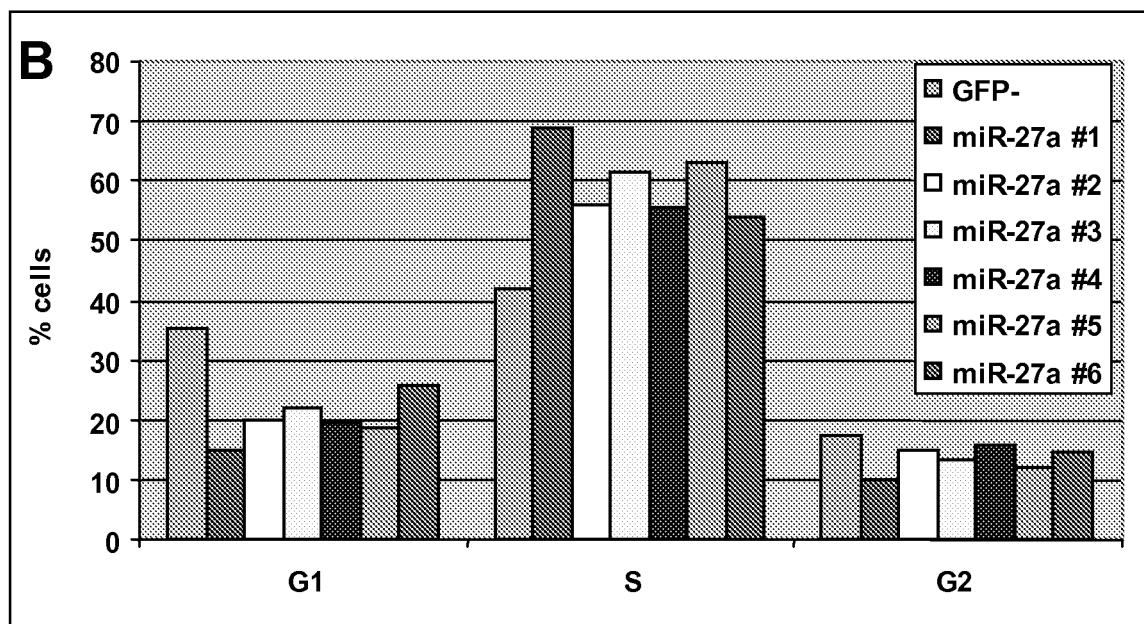
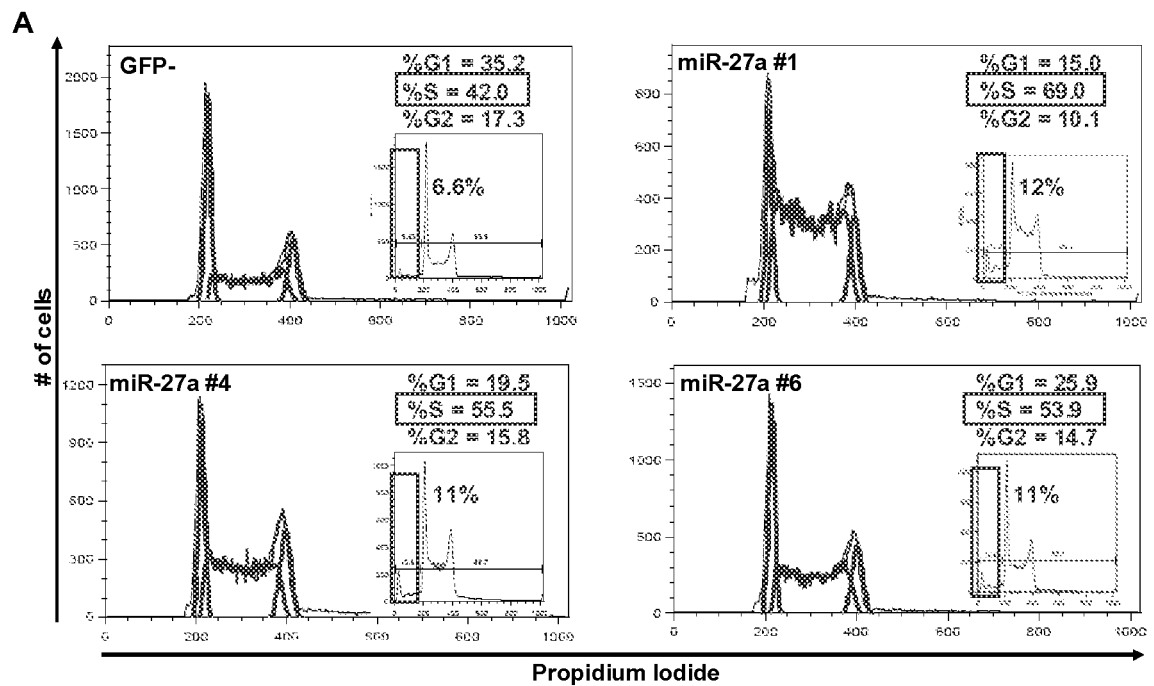


Figure 22

