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(54) Title: MIRNAS DYSREGULATED IN EWING SARCOMA

(57) Abstract: The invention provides methods of diagnosing and treating cancer in a subject. The inventors have identified a series of dysregulated miRNAs that are indicative of Ewing Sarcoma and also therapeutic targets. In some embodiments, the invention further provides for the administration of a second cancer therapy to the subject.

DESCRIPTION

MIRNAS DYSREGULATED IN EWING SARCOMA

BACKGROUND OF THE INVENTION

This application claims benefit of priority to U.S. Provisional Application Serial No. 61/475,475, filed April 14, 2011, the entire contents of which are hereby incorporated by reference.

I. FIELD OF THE INVENTION

The present invention relates generally to the fields of oncology, molecular biology, and medicine. More particularly, the invention relates to use of certain miRNAs that are dysregulated in Ewing Sarcoma as both diagnostic and therapeutic targets.

II. DESCRIPTION OF RELATED ART

Ewing Sarcoma, the second most common cancer of bone and soft tissue in adolescents and young adults, is an aggressive malignancy with poor long-term outcome (Ludwig, 2008). The pathogenesis of Ewing Sarcoma is driven by EWS/Ets fusion oncoproteins, which are absolutely necessary and, in the appropriate context, may be sufficient for tumorigenesis (Arvand & Denny, 2001; Janknecht, 2005; Jedlicka, 2010; Riggi & Stamenkovic, 2007). Ets fusion oncoproteins, of which EWS/Fli1 is the most common, are highly expressed, gain-of-function regulators of gene expression that activate an oncogenic program in the cell of origin of the tumor, likely a mesenchymal progenitor cell (Riggi *et al.*, 2005; Tirode *et al.*, 2007). EWS/Ets fusions function as transcriptional regulators, with the Ets DNA-binding domain providing target specificity (Jedlicka, 2010; Riggi & Stamenkovic, 2007). Activation of downstream gene expression is achieved via a potent transcription activation domain within the EWS component of the fusion (Riggi & Stamenkovic, 2007). EWS/Ets silencing and rescue experiments identify more down-regulated than up-regulated target genes by EWS/Ets (Prieur *et al.*, 2004; Smith *et al.*, 2006). Mechanisms of transcriptional repression in Ewing Sarcoma include both EWS/Ets fusions themselves (Im *et al.*, 2000; Yang *et al.*, 2010), and downstream transcriptional regulators such as Nkx2.2, NR0B1 and EZH2 (Kinsey *et al.*, 2006; Richter *et al.*, 2009; Smith *et al.*, 2006).

A number of growth factor pathways are active in Ewing Sarcoma, with the Insulin-like Growth Factor (IGF) signaling pathway being of paramount importance (Jedlicka, 2010;

Kim *et al.*, 2009a). IGF-1 is a target of EWS/Ets oncoproteins and acts, via an autocrine loop, on the IGF-1 receptor (IGF-1R) (Cironi *et al.*, 2008; Herrero-Martin *et al.*, 2009; Scotlandi *et al.*, 1996; Yee *et al.*, 1990). EWS/Ets fusions also down-regulate expression of IGF binding proteins (Prieur *et al.*, 2004), thus further enhancing the activity of this autocrine loop. Ewing Sarcomas consistently express IGF-1R and exhibit activation of downstream Mitogen-activated Protein Kinase (MAPK) and Phosphatidyl Inositol 3 Kinase (PI3K) pathways (Benini *et al.*, 2004; Scotlandi *et al.*, 1996; Silvany *et al.*, 2000; Toretsky *et al.*, 1999). Monoclonal antibody and small molecule inhibitors of IGF-1R impair Ewing Sarcoma cell and tumor growth (Manara *et al.*, 2007; Martins *et al.*, 2006; Scotlandi *et al.*, 2005), and are currently in clinical trials as the chief adjunctive biological therapy for this aggressive cancer (Ludwig, 2008). Although clearly important, little is known at present about the details of the IGF signaling pathway in Ewing Sarcoma, including levels, regulation and mutational status of critical signaling components. MicroRNAs are important regulators of growth factor signaling (Inui *et al.*, 2010), but their role in IGF pathway control is at present largely uncharacterized.

MicroRNAs (miRs) are ecellular bioactive molecules with critical functions in the regulation of gene expression in normal biology and disease (Ghildiyal & Zamore, 2009). MiRs are short (20-30 nucleotide) RNA molecules that bind to protein-coding messenger RNA (mRNA) molecules, predominantly in the 3' untranslated region (UTR) (Ghildiyal & Zamore, 2009). This binding results in decreased synthesis of the coded protein, by a number of mechanisms including increased mRNA degradation and inhibition of translation (Ghildiyal & Zamore, 2009). In cancer, miRs have been shown to function as potent tumor suppressors or oncogenes (Sotiropoulou *et al.*, 2009; Visone & Croce, 2009). Importantly, miRs represent potentially powerful therapeutic agents and/or targets, a concept now borne out in a number of preclinical studies (Trang *et al.*, 2008; Wang & Wu, 2009; Weidhaas *et al.*, 2007). At present, very little is known about miRs in pediatric cancers and sarcomas. Further, despite their importance, little is currently known about the regulation of miR expression in normal physiology and disease. Interestingly, Ets transcription factors, which provide the DNA-binding component in Ewing Sarcoma oncogenic fusions, have been shown to regulate miR expression (Cowden Dahl *et al.*, 2009; Fujita *et al.*, 2008; Fukao *et al.*, 2007).

SUMMARY OF THE INVENTION

Thus, in accordance with the present invention, there is provided a method of diagnosing Ewing sarcoma in a subject comprising (a) obtaining a sample from said subject; and (b) assessing said sample for one or more miRNAs selected from the group consisting of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, miR-146a, miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a, miR-92b, miR-193b and miR-223; wherein a decreased level of one or more of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, or miR-146a, or an increased level of one or more of miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a miR-193b, miR-223 or miR-92b, as compared to a sample from normal subject, indicates that said subject has a Ewing sarcoma.

The sample may be a biopsy or resected tumor tissue. Assessing may comprise microarray hybridization. Two, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or all 14 of said miRNAs may be assessed. At least one of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, miR-193b, miR-223 or miR-146a and at least one of miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a or miR-92b may be assessed. All of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, miR-193b, miR-223 and miR-146a may be assessed, optionally with one or more of miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a or miR-92b. All of miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a and miR-92b may be assessed, optionally with one or more of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, miR-193b, miR-223 or miR-146a. All miRNAs but miR-17 may be assessed. All miRNAs but miR-17, miR-92a and miR-92b may be assessed, optionally with one or more of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, miR-193b, miR-223 or miR-146a. One or more of miR-25, miR-93, miR-106a, miR-92a, miR-20a or miR-92b may be assessed, optionally with one or more of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, miR-193b, miR-223 or miR-146a. One or more of miR-25, miR-93, miR-106a and miR-20a may be assessed, optionally with miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, miR-193b, miR-223or miR-146a. Either or both of miR-20a and miR-106a may be not assessed. miR-25 and miR-93 may be assessed, optionally with one or more of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, miR-193b, miR-223 or miR-146a. miR-100, miR-125b, and 29a may not be assessed. miR-22, miR-221, miR-27a, miR-193b, miR-223 and miR-146a may be assessed, optionally with one or more of miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a or miR-92b.

In another embodiment, there is provided a method of treating a subject with Ewing sarcoma comprising providing to said subject one or more miRNAs selected from the group consisting of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, miR-193b, miR-223 and/or miR-146a and/or an miRNA mimic thereof. Providing may comprises administration into a vein, artery, tumor or tumor vasculature. The one or more miRNAs may be formulated in a lipid vehicle. Providing may comprise administering to said subject an expression vector that expresses on or more of said miRNAs. The expression vector may be a viral expression vector. One, two, three or four of said miRNAs or mimics thereof may be provided. Each of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, miR-193b, miR-223 and miR-146a or mimics thereof may be provided. One or more of said miRNAs or mimics thereof may be provided more than once. Each of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, miR-193b, miR-223 and miR-146a, or mimics thereof, may be provided more than once. One, two or all three of miR-100, miR-125b, and 29a may not be provided to said subject.

In still another embodiment, there is provided a method of treating a subject with Ewing sarcoma comprising providing to said subject one or more antagomirs or inhibitory oligonucleotides for an miRNA selected from the group consisting of miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a and/or miR-92b. Providing may comprise administration into a vein, artery, tumor or tumor vasculature. The one or more antagomirs may be formulated in a lipid vehicle. Providing may comprise administering to said subject an expression vector that expresses on or more of said antagomirs. The expression vector may be a viral expression vector. One, two, three or four of said antagomirs may be provided. Antagomirs or inhibitory oligonucleotides for each of miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a and/or miR-92b may be provided. One or more of said antagomirs or inhibitory oliognucleotides may be provided more than once. Antagomirs or inhibitory oligonucleotides for each of miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a and/or miR-92b may be provided more than once. Antagomirs or inhibitory oligonucleotides for one, two, three or all four of miR-106a, miR-17, miR-92a and miR-92b nay not be provided to said subject. Antagomirs or inhibitory oligonucleotides for one, two, three, four or all five of miR-106a, miR-17, miR-92a, miR-20a and miR-92b may not be provided to said subject.

Also provided is a kit for using in accordance with any of the preceding embodiment. The kit may comprises an miR(s), a mimic(s), or an inhibitory oligonucleotide(s) including but not limited to onre or more antagomirs. The kit may also comprise probes for a plurality of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, miR-146a, miR-25, miR-93,

miR-106a, miR-17, miR-92a, miR-20a, miR-193b, miR-223 and miR-92b. The kit may provide miRs, mimics, antagomirs or probes for 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or all 14 of said miRNAs. The probes may be labeled. The kit may further comprise one or more buffers or diluents.

Any embodiment discussed with respect to one aspect of the invention applies to other aspects of the invention as well.

The embodiments in the Examples section are understood to be embodiments of the invention that are applicable to all aspects of the invention.

The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or."

Throughout this application, the term "about" is used to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.

Following long-standing patent law, the words "a" and "an," when used in conjunction with the word "comprising" in the claims or specification, denotes one or more, unless specifically noted.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE FIGURES

The following figures form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these figures in combination with the detailed description of specific embodiments presented herein.

FIGS. 1A-F. Identification of EWS/Fli1-regulated candidate tumor suppressor microRNAs in Ewing Sarcoma. (FIG. 1A) Lentiviral shRNAs targeting human Fli1 were obtained from Open Biosystems; the off-target control shRNA to EGFP has been described (Porter & DeGregori, 2008). Replication-incompetent infectious virus was prepared by transient transfection of 293FT cells, using the ExGen500 reagent (Fermentas), with shRNA construct, and packaging constructs expressing VSV-G, Gag, Pol, and Rev (Porter & DeGregori, 2008) per standard protocols (Invitrogen). A673 cells were infected with similar titers of virus and selected with Puromycin (3 mg/ml). Following 10 days of culture, cell pools were harvested for protein and RNA, with all groups at similar confluence (50-70%) at the time of harvest. Total cellular RNA was isolated using the TRIzol reagent (Invitrogen), per manufacturer instructions. Protein extract preparation, SDS-PAGE, immunoblotting and ECL detection were performed essentially as previously described (Jedlicka *et al.*, 2009). EWS/Fli1 protein levels were determined by immunoblotting with Fli1 antibody (BD Biosciences; 554266) and tubulin (Calbiochem; CP06) as loading control. The numerical values represent the EWS/Fli1 to Tubulin ratio as determined by densitometric quantitation, with the value in the control set to 1. (FIG. 1B) The expression of two established EWS/Fli1 targets (Nkx2.2 and NR0B1) was determined by qRT-PCR (normalized to actin); results represent mean and standard deviation of qRT-PCR performed on RNAs from triplicate cell culture plates. (FIG. 1C) The "EF2" and "luc" off-target control (Smith *et al.*, 2006) shRNA constructs were generated in pSUPERRetro-Puro (Oligoengine) using standard cloning techniques, and sequenceverified. Preparation of virus and infection of A673 cells were as in FIG. 1A, except that the packaging construct pCL-Ampho (Naviaux *et al.*, 1996) was used. EWS/Fli1 expression levels were determined as in FIG. 1A. (FIG. 1D) Global microRNA (miR) expression profiling was performed on triplicate RNA samples from cells stably expressing shEF1 and shEGFP by Dharmacon/ Thermo Fisher Scientific, using an Agilent-type custom array containing miRs in the Sanger 10.1 database. Relative

Intensity data for 6 experiments was subjected to statistical filtering, keeping miR probes called present with p-value less than or equal to 0.05 in at least 3 of the 6 experiments. The resulting data were inter-array scaled, and differential expression analysis was performed using textbook ANOVA with FDR (false discovery rate) multiple test correction. A list of miR probes significantly different (FDR less than or equal to 0.01) between the two treatment groups is shown in FIG. 5. The table shows miRs selected for further study, as described in the text. (FIG. 1E) Changes in miR levels observed by microarray were verified by qRT-PCR, using the miScript SYBRgreen qRT-PCR system (Qiagen) with U6 RNA as the endogenous control. Results are expressed as mean and standard deviation of fold miR increase relative to matched control (shEGFP for shEF1 and shEF2, and luc for EF2; *: not done). (FIG. 1F) RNA was isolated from triplicate culture plates of five different Ewing Sarcoma cell lines (A673, SK-N-MC, SK-ES-1, EWS502 and TC71), and low-passage human mesenchymal progenitor cells (hMPC) from two different sources (ScienCell (SC) and Lonza (L)). All cell lines were grown in DMEM supplemented with 10% fetal calf serum (FCS), under standard tissue culture conditions (miR expression levels in the hMPCs were similar when the cells were grown in proprietary progenitor cell media and DMEM/10%FCS). MiR levels were determined by qRT-PCR, normalized to U6 RNA. The average normalized value in A673 cells was set to 1. Results are expressed as mean and standard deviation.

FIGS. 2A-F. EWS/Fli1 represses microRNAs by a transcriptional mechanism. (FIG. 2A) PrimiR levels were determined by qRT-PCR, with U6 RNA as the endogenous control, using primers outside the pre-miR hairpin sequence, but within the known/predicted primary transcript. The ratio of expression in A673 cells with stably silenced EWS/Fli1 (shEF1) to control (shEGFP) is shown (error bars represent standard deviation). (FIG. 2B) The type 1 EWS/Fli1 oncogenic fusion was RT-PCR-cloned from SK-N-MC Ewing Sarcoma cells into the pCMV6 expression vector (Origene), and subsequently subcloned (along with the C-terminal Myc/DDK epitope tag) into the pCDH-CMV-MCS-EF1-Puro expression vector (System Biosciences); all products were sequence-verified. Infectious lentivirus, prepared for the pCDH-EWS/Fli1 expression construct and control (empty vector) as in FIG. 1A above, was used to infect 293FT cells. Following selection with Puromycin (2 μg/ml), protein extract preparation and EWS/Fli1 immunoblotting were performed as in Fig. 1A to confirm expression of a protein product of the predicted size. (FIGS. 2C and 2D) Quantification of pri-miR and miR levels in RNA isolated from EWS/Fli1-expressing 293FT cells and controls was performed as in

FIGS. 1E and 2A. Results are plotted as mean and standard deviation of two independent experiments, each performed in triplicate (except for the pri-miR-27a data, which represent one experiment performed in triplicate). (FIG. 2E) Candidate EWS/Fli1 DNA-binding sites in the miR promoters were identified by searching predicted miR promoter sequence (roughly -2 Kb to +0.5 Kb relative to the transcription start site (Corcoran *et al.*, 2009; Eyholzer *et al.,* 2010; Ozsolak *et al.*, 2008)) with the Fli1 position weight matrix ACA/CGGAA/T (Wei *et al.*, 2010), using the ConSite transcription factor binding site prediction algorithm (asp.ii.uib.no:8090/cgibin/CONSITE/consite). Note that miRs 221 and 222 share a common promoter, while the miR-125b promoter has not been defined and may be shared with miR-100, as in lower organisms (Sokol *et al.*, 2008). (FIG. 2F) Chromatin immunoprecipitation (ChIP) analysis of EWS/Fli1 binding to the miR-100 promoter. A673 cells (9×10^7) cells were collected by centrifugation and resuspended in medium containing 1% formaldehyde for crosslinking. Following rocking at room temperature for 10 minutes, cross-linking was terminated by the addition of 0.125 M glycine and rocking for an additional 5 minutes. ChIP was then performed using antibody to Fli1 (Santa Cruz Biotechnology, sc-356-X) or IgG (Cell Signaling, 2729S) as control, as described (Schmidt *et al.*, 2008), except that sonication was done at 25% power, 25 cycles, 15 seconds on, 1 minute off. The negative control (Neg) is an average of the ChIP fold-enrichment at the Albumin and Bcl promoters, subsequently set to 1; ChIP of the NR0B1 promoter is the positive control (Gangwal *et al.*, 2008). The positions of the qPCR amplicons (~250-400 bp each) relative to the miR-100 transcription start site are indicated. Results are represented as mean and standard error of the mean of 2 independent ChIP experiments, each performed in duplicate.

FIGS. 3A-F. Inhibition of expression of IGF pathway targets by EWS/Fli1-repressed microRNAs. (FIG. 3A) A673 cells were transiently transfected with negative control mimic or miR-27a mimic (20 nM; Applied Biosystems), using the Lipofectamine 2000 reagent, and cultured in growth medium with 10% serum. The medium was changed 2 days after transfection, and IGF-1 levels in the medium were quantified by ELISA (R+D Systems) per manufacturer protocol an additional 2 days later. To determine IGF production per cell, IGF-1 levels in growth medium alone were subtracted, and the resulting value was normalized to cell number. Results are expressed as mean and standard error of the mean of three independent experiments; $p = 0.0002$. (FIGS. 3B and 3C) A673 cells were infected with control or pCDH-miR-100-Puro lentivirus, using the same methods as in FIG. 1A. The pCDH-miR-100-Puro construct was generated by

subcloning miR-100 precursor sequence from the pCDH-miR-100-GFP construct into the pCDH-Puro construct (both from System Biosciences), using standard techniques. Following Puromycin selection (2 μg/ml), IGF-1R and mTOR expression levels were determined by Western blotting of whole cell extracts with specific antibodies (Cell Signaling; 3027 and 2972, respectively), and tubulin as loading control. Figures to the right of the immunoblots show quantification of expression levels by densitometric scanning; data are represented as mean and standard deviation of at least two independent experiments. (FIG. 3D) A673 cells were transiently transfected with negative control or miR-125b mimic as in FIG. 3A. RSK1 expression levels were analyzed as in FIG. 3B, using specific antibody (Cell Signaling; 9333). (FIG. 3E) Conserved miR sites in the 3'UTRs of IGF-1R, RSK1 and IGF-1, as predicted by TargetScan. Vertical lines indicate predicted miR-UTR pairing; miR seed sequence is in bold. Mutated 3'UTR sequence is shown below each UTR-miR pair, with substituted bases in italics (SEQ ID NOS:18-26). (FIG. 3F) 3'UTR sequences containing wild-type (wt) and mutated (mut, as shown in FIG. 3E) miR sites were cloned downstream of the *Renilla* luciferase sequence of the siCHECK dual luciferase reporter (Promega) and verified by sequencing. A673 cells were transiently cotransfected with the indicated reporter construct (wt or mut) and negative control or miR mimic, using the Lipofectamine 2000 reagent as in FIG. 3A. *Renilla* luciferase activity, normalized to the firefly luciferase internal control, was determined 24 h later. Data are represented as mean and standard error of the mean of two independent experiments, each performed in triplicate; activity in the control groups is set to 1 (*$p<0.01$).

FIGS. 4A-E. Inhibition of Ewing Sarcoma cell growth by EWS/Fli1-repressed microRNAs. (FIG. 4A) A673 cells were transiently transfected with 20 nM of the indicated miR mimics or negative control mimic, as in FIGS. 3A-F. One day following transfection, cells were counted, replated at 3×10^4 cells per well in a 24-well plate, and cultured in growth medium with 10% serum. On the indicated days, cells were fixed with 10% formalin, washed with PBS, and stained with 0.1% crystal violet. Following extensive washing with water, cells were lysed with 10% acetic acid and the lysates quantified spectrophotometrically (OD_{570nm}). Results are expressed as mean and standard deviation of 2-3 independent experiments, each performed in triplicate; the relative cell number in the control group is set to 1. The p-values for differences between control and miR overexpressing cells on day 7 are: 0.04 (miR-29a); 0.02 (miR-27a); <0.01 (miRs 100, 125b, 22 and 221). (FIG. 4B) Fold miR overexpression in the growth experiments in

FIG. 4A, as determined by qRT-PCR normalized to U6 RNA. (FIG. 4C) A673 cells were stably transduced with control or pCDH-miR-100-Puro lentivirus as in FIG. 3B. Following antibiotic selection, miR-100 expression levels were determined by qRT-PCR normalized to U6 RNA. (FIGS. 4D and 4E) 1 x 10^4 control or miR-100 stable overexpressor cells were grown in 0.4% agar and growth medium with 20% serum. Colonies were stained with Nitroblue Tetrazolium (NBT) ~2-3 weeks later, and quantified using ImageJ software. Results are shown as mean and standard deviation of triplicate cultures (D; $p = 0.01$) and representative image (FIG. 4E). The experiment was repeated three independent times with similar results.

FIG. 5. MicroRNAs modulated upon EWS/Fli1 silencing. MicroRNAs significantly upregulated or downregulated in cells with strong silencing of EWS/Fli1 (A673/shEF1) compared to controls (A673/shEGFP), by microarray profiling, performed and analyzed as described in FIGS. 1A-F legend.

FIG. 6. Model of regulation of IGF signaling pathway by EWS/Fli1-repressed microRNAs. The EWS/Fli1 oncoprotein represses a group of microRNAs by direct and/or indirect mechanisms, acting at the level of transcription, as well as possibly post-transcriptional processing. Some of these microRNAs normally downregulate the levels of pro-oncogenic proteins in the IGF signaling pathway, including IGF-1, IGF-1R, mTOR and RSK1; regulation of IGF-1 by miR-27a may be by indirect mechanisms. Through these and other mechanisms, these microRNAs normally inhibit oncogenesis. When repressed by EWS/Fli1, this inhibition is relieved, resulting in enhanced oncogenesis.

FIGS. 7A-C. Modest overexpression of miR-146a is inhibitory to Ewing Sarcoma tumorigenesis. A673 Ewing Sarcoma cells were stably transduced with a lentiviral vector expressing a miR-146a precursor or a control (empty) vector. (FIG. 7A) Relative miR-146a overexpression was determined in cultured cells and in tumors arising from these same cells. (FIG. 7B) Nude mice were injected in the flank with 1 x 10^6 miR-146a overexpressing or control cells, and monitored for tumor formation. (FIG. 7C) NOD-SCID mice were injected in the flank with 1 x 10^5 miR-146a overexpressing or control cells, and monitored for tumor formation by caliper measurements. Once a tumor reached the maximum size permissible by institutional protocol, all animals in the experiment were euthanized, tumors were harvested, and the tumor weight was determined.

FIG. 8. MiRs 193b and 223 each potently inhibit Ewing Sarcoma cell growth in a clonogenic assay. A673 Ewing Sarcoma cells were transiently transfected with 20 nM of the indicated miR mimic or negative control mimic (both from Applied Biosystems),

using the Lipofectamine 2000 reagent. One day later, cells were plated at 500 per well. The cells were cultured in standard culture medium, and colonies were stained 14 days later. Colonies were quantified using a Nikon digital image analysis system (NIS-Elements). Results represent the mean and SEM of 4 independent experiments, each performed in triplicate. The statistical significance of differences between control and miR mimic were determined using the student's t-test.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

MicroRNAs (miRNAs) are small RNA molecules, 19-25 nucleotides in length. miRNAs are not translated, instead they serve as regulators of mRNA expression (Ouellet *et al.*, 2006). For the most part, miRNAs bind to complementary regions in target mRNA 3'UTRs and either cause mRNA degradation or prevent its translation (Engels and Hutvagner, 2006). Generally, one observes a decrease of the target at the protein level; however, there is some emerging evidence that miRNAs can also cause upregulation of their targets. Individual miRNAs have been found to be expressed in cell-specific manner, at specific developmental stages, as well as differentially expressed in disease states (Yi *et al.*, 2008; Sempere *et al.*, 2007). Importantly, miRNAs have been implicated as playing roles as oncogenes and tumor suppressors (Cho, 2007; Cowland *et al.*, 2007). While there are miRNAs that are found to be overexpressed in cancers, many appear to be lost and they tend to localize to fragile sites (Calin *et al.*, 2004).

The IGF signaling pathway plays a central role in Ewing Sarcoma oncogenesis, as well as a variety of other cancers, including other pediatric solid tumors (Jedlicka, 2010; Kim *et al.*, 2009a). Key components of the pathway, namely IGF-1 (Cironi *et al.*, 2008) and IGFBP3 (Prieur *et al.*, 2004), have previously been shown to be targeted directly by the transcriptional activity of EWS/Fli1. The studies presented here uncover a novel mechanism whereby EWS/Fli1 regulates the expression of pro-oncogenic IGF signaling pathway components indirectly via miRs (FIG. 6). miRs have recently been identified as important regulators of signaling pathway activity in other systems (Inui *et al.*, 2010). To the inventors' knowledge, these findings of a pivotal fusion oncoprotein regulating multiple miRs, which then target multiple components of a single signaling pathway, represent a novel mechanism of oncogenesis.

Such a mechanism has the potential for substantial potency. Although individual miR/target effects tend to be relatively modest, as seen in these studies, the additive effect of multiple miR/target interactions converging on a single pathway can have profound consequences. The inventors propose that this mechanism makes an important contribution to the high activity of the IGF autocrine loop in Ewing Sarcoma. With the exception of mTOR regulation by miR-100, previously demonstrated in other contexts (Nagaraja *et al.*, 2010; Wang *et al.*, 2008a), these studies identify a number of novel and important miR/target interactions. IGF-1 expression is regulated by the EWS/Fli1 oncoprotein, as previously demonstrated by both EWS/Fli1 silencing studies in Ewing Sarcoma cells (Herrero-Martin *et*

al., 2009; Mateo-Lozano *et al.*, 2006), and EWS/Fli1 expression studies in mesenchymal progenitor cells (Cironi *et al.*, 2008).

Analyses of the IGF-1 promoter suggest a direct transcriptional component for this regulation (Cironi *et al.*, 2008). The studies described here uncover an additional regulatory mechanism involving miR-27a. The precise molecular mechanism by which miR-27a controls IGF-1 levels remains to be determined, as miR-27a does not appear to act through the predicted, conserved IGF-1 3'UTR site. Possibilities include miR regulation through other sites in the mRNA sequence, and indirect mechanisms such as miR regulation of molecules involved in controlling IGF-1 synthesis, secretion and stability. IGF-1R is also positively regulated by EWS/Fli1 at the protein level (Mateo-Lozano *et al.*, 2006), and these studies identify repression of miR-100 as one mechanism of this regulation. miR-125b has multiple predicted oncogenic targets in the MAPK arm of the IGF pathway, one of which (RSK1) is demonstrated in the instant studies. Examination of published mRNA global profiling data moreover suggests that EWS/Fli1-repressed miRs may play a role in the regulation of other target genes in Ewing Sarcoma, including GSTM4.

The phenotypic effects of most miRs show a dependence on cellular context. The present studies involving a primitive malignancy of mesenchymal origin expand the scope of the contextual landscape of miR functions in cancer. Specifically, the inventors present evidence for tumor suppressive functions for miRs 100, 125b, 22, 221, 27a, 29a and 146a in Ewing Sarcoma. Other studies of miR-100 in cancer have also found tumor suppressive effects (Cairo *et al.*, 2010; Henson *et al.*, 2009; Nagaraja *et al.*, 2010; Sun *et al.*, 2011), suggesting that this may be the prevalent function of miR-100 in cancer. Similarly, miR-22 appears to have a prevalent tumor suppressive function in cancer (Li *et al.* 2010; Nagaraja *et al.*, 2010; Patel *et al.*, 2010; Ting *et al.*, 2010; Xiong *et al.*, 2010; Xiong *et al.,* 2010; Zhang *et al.*, 2010), although it promotes prostate tumorigenesis (Poliseno *et al.*, 2010). MiR-125b has tumor suppressive (Guan *et al.,* 2010; Henson *et al.*, 2009; Huang *et al.,* 2011; Liang *et al.* 2010; Scott *et al.*, 2007; Shi *et al.,* 2010) or tumor promoting (Gefen *et al* 2010.; Shi *et al.,* 2011; Xia *et al.*, 2009) functions, depending on context. Studies of miR-221/222 in epithelial cancers (Garofalo *et al.*, 2009; Mercatelli *et al.*, 2008; Wong *et al.*, 2010), melanoma (Felicetti *et al.*, 2008) and glioma (Zhang *et al.*, 2009; Zhang *et al.*, 2010; Zhang *et al.*, 2010) have identified pro-oncogenic functions. Interestingly, however, one study of prostate cancer showed diminution of expression with tumor progression (Spahn *et al.*, 2010). Further, relevant to the findings here in a primitive malignancy of progenitor cell origin, miR-221/222 inhibits the proliferation of embryonic stem cells (Mayoral *et al.*, 2009). Studies of miR-27a

in carcinomas (cancers of epithelia) have identified oncogenic functions (Chintharlapalli *et al.*, 2009; Liu *et al.*, 2009; Mertens-Talcott *et al.*, 2007), although miR-27a can also enhance apoptosis (Chhabra *et al.*, 2009). Lastly, miR-29a also has context-dependent oncogenic (Gebeshuber *et al.*, 2009; Han *et al.,* 2010) or tumor suppressive (Muniyappa *et al.*, 2009) functions. Interestingly, the related miR-29b/c cluster is tumor-suppressive in rhabdomyosarcoma (Wang *et al.*, 2008b), another primitive pediatric malignancy. Possible reasons for the striking context-dependence of miR functions are many, including the broad repertoire of individual miR targets, miR and target relative expression levels, and coexpression of other regulators. Thus, the importance of any given miR/target interaction is likely dependent on the gene regulatory landscape of the cell. The contextual component of miR effects underscores the importance of understanding miR effects in specific cancers, especially those with unrelated histogenesis.

These findings indicate that transcriptional repression, including possibly direct mechanisms, is at least one means by which EWS/Fli1 regulates the expression of miRs. Transcriptional repression appears to be a prevalent mechanism of target gene regulation by EWS/Fli1 in Ewing Sarcoma, and there is evidence for both direct and indirect mechanisms (Jedlicka, 2010). Direct mechanisms, in particular, are poorly understood. It will be of interest to further dissect the precise mechanisms by which EWS/Fli1 regulates miR expression at the transcriptional, as well as possibly post-transcriptional (Gregory *et al.*, 2004), level. Thus, these studies identify a novel pro-oncogenic mechanism in Ewing Sarcoma, involving targeting of the IGF signaling pathway by EWS/Fli1-regulated microRNAs. MicroRNAs have been proposed, and in a number of preclinical studies demonstrated (Cairo *et al.*, 2010; Corsten *et al.*, 2007; Kota *et al.*, 2009; Swarbrick *et al.,* 2010; Trang *et al.,* 2010; Wiggins *et al.*, 2010; Zhou *et al.*, 2010), to possess therapeutic potential. Although a more detailed understanding of this pathway is needed, its identification provides not only new diagnostic methods, but also innovative therapeutic targeting strategies in Ewing Sarcoma and other cancers with activated IGF, as well as related receptor tyrosine kinase signaling. These and other aspects of the invention are described in further detail below.

I. Ewing Sarcoma

A. Background

Ewing sarcoma is a malignant round-cell tumor. It is a rare disease in which cancer cells are found in the bone or in soft tissue. The most common areas in which it occurs are the

pelvis, the femur, the humerus, the ribs and clavicle. Because a common genetic locus is responsible for a large percentage of Ewing sarcoma and primitive neuroectodermal tumors, these are sometimes grouped together in a category known as the Ewing family of tumors. The diseases are, however, considered to be different: peripheral primitive neuroectodermal tumours are generally not associated with bones, while Ewing sarcomas are most commonly related to bone. Ewing sarcoma occurs most frequently in male teenagers, with a male/female ratio of 1.6:1.

Although usually classified as a bone tumor, Ewing sarcoma can have characteristics of both mesodermal and ectodermal origin, making it difficult to classify. Genetic exchange between chromosomes can cause cells to become cancerous. Ewing sarcoma is the result of a translocation between chromosomes 11 and 22, which fuses the EWS gene of chromosome 22 to the FLI1 gene of chromosome 11. EWS/FLI functions as the master regulator. Other translocations are at t(21;22) and t(7;22). The cancer cells are positive for CD99 and negative for CD45.

Five-year survival for localized disease is 70% to 80% when treated with chemotherapy. Long term survival for metastatic disease can be less than 10% but some sources state it is 25-30%.

B. Symptoms

Ewing sarcoma is more common in males and usually presents in childhood or early adulthood, with a peak between 10 and 20 years of age. It can occur anywhere in the body, but most commonly in the pelvis and proximal long tubular bones, especially around the growth plates. The diaphyses of the femur are the most common sites, followed by the tibia and the humerus. Thirty percent are overtly metastatic at presentation. As such, patients usually experience extreme bone pain.

The disease most commonly afflicts the long bones of the body - the arms and the legs - and general symptoms such as pain and swelling are experienced in these areas. Ewing sarcoma symptoms include pain at the tumor site, swelling around the site of the tumor (skin may or may not be red), fever, unintended weight loss, fatigue and loss of appetite. When a tumor affects the spine, symptoms like loss of bladder/bowel control or paralysis may occur. Tumors can also affect nerve pathways, causing feelings of numbness and tingling. Fractures can also occur as a result of weakening of the bone.

C. Epidemiology

The frequency in the United States depends on the patient's age, with a rate of 0.3 case per 1,000,000 children in those younger than 3 years of age to as high as 4.6 cases per 1,000,000 in adolescents aged 15–19 years. Internationally the annual incidence rate averages less than 2 cases per 1,000,000 children. In the United Kingdom an average of six children per year are diagnosed, mainly males in early stages of puberty. Due to the prevalence of diagnosis during teenage years, there may possibly be a link between the onset of puberty and the early stages of this disease, although no research is currently being conducted to confirm this theory.

D. Diagnosis and Staging

On conventional radiographs, the most common osseous presentation is a permeative lytic lesion with periosteal reaction. The classic description of lamellated or "onion skin" type periosteal reaction is often associated with this lesion. Plain films add valuable information in the initial evaluation or screening. The wide zone of transition (*e.g.*, permeative) is the most useful plain film characteristic in differentiation of benign versus aggressive or malignant lytic lesions.

MRI should be routinely used in the work-up of malignant tumours. MRI will show the full bony and soft tissue extent and relate the tumour to other nearby anatomic structures (*e.g.*, vessels). Gadolinium contrast is not necessary as it does not give additional information over noncontrast studies, though some current researchers argue that dynamic, contrast enhanced MRI may help determine the amount of necrosis within the tumour, thus help in determining response to treatment prior to surgery.

CT can also be used to define the extraosseous extent of the tumour, especially in the skull, spine, ribs and pelvis. Both CT and MRI can be used to follow response to radiation and/or chemotherapy. Bone scintigraphy can also be used to follow tumour response to therapy.

In the group of malignant small round cell tumors which include Ewing's sarcoma, bone lymphoma and small cell osteosarcoma, the cortex may appear almost normal radiographically, while there is permeative growth throughout the Haversian channels. These tumours may be accompanied by a large soft tissue mass while there is almost no visible bone destruction. The radiographs frequently do not shown any signs of cortical destruction.

Other entities that may have a similar clinical presentation include osteomyelitis, osteosarcoma (especially telangiectatic osteosarcoma) and eosinophilic granuloma. Soft

tissue neoplasms such as malignant fibrous histiocytoma that erode into adjacent bone may also have a similar appearance.

Staging attempts to distinguish patients with localized from those with metastatic disease. Most commonly, metastases occur in the chest, bone and/or bone marrow. Less common sites include the central nervous system and lymph nodes.

E. Treatment

Because almost all patients with apparently localized disease at diagnosis have occult metastatic disease, multidrug chemotherapy (often including ifosfamide and etoposide) as well as local disease control with surgery and/or radiation is indicated in the treatment of all patients.

Treatment often consists of neo-adjuvant chemotherapy generally followed by a limb salvage or an amputation and may also include radiotherapy. Complete excision at the time of biopsy may be performed if malignancy is confirmed at the time it is examined. Treatment lengths vary depending on location and stage of the disease at diagnosis. Radical chemotherapy may be as short as 6 treatments at 3 week cycles, however most patients will undergo chemotherapy for 6–12 months and radiation therapy for 5–8 weeks.

Antisense oligodeoxynucleotides have been proposed as possible treatment by down-regulating the expression of the oncogenic fusion protein associated with the development of Ewing sarcoma resulting from the EWS-ETS gene translocation. In addition, the synthetic retinoid derivative fenretinide (4-hydroxy(phenyl)retinamide) has been reported to induce high levels of cell death in Ewing sarcoma cell lines *in vitro* and to delay growth of Ewing sarcoma xenografts *in vivo* mouse models.

II. miRNAs

A. Background

In 2001, several groups used a novel cloning method to isolate and identify a large group of "microRNAs" (miRNAs) from *C. elegans*, *Drosophila*, and humans (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001). Several hundreds of miRNAs have been identified in plants and animals—including humans—which do not appear to have endogenous siRNAs. Thus, while similar to siRNAs, miRNAs are nonetheless distinct.

miRNAs thus far observed have been approximately 21-22 nucleotides in length and they arise from longer precursors, which are transcribed from non-protein-encoding genes. *See* review of Carrington *et al.* (2003). The precursors form structures that fold back on each

other in self-complementary regions; they are then processed by the nuclease Dicer in animals or DCL1 in plants. miRNA molecules interrupt translation through precise or imprecise base-pairing with their targets.

miRNAs are transcribed by RNA polymerase II and can be derived from individual miRNA genes, from introns of protein coding genes, or from poly-cistronic transcripts that often encode multiple, closely related miRNAs. Pre-miRNAs, generally several thousand bases long are processed in the nucleus by the RNase Drosha into 70- to 100-nt hairpin-shaped precursors. Following transport to the cytoplasm, the hairpin is further processed by Dicer to produce a double-stranded miRNA. The mature miRNA strand is then incorporated into the RNA-induced silencing complex (RISC), where it associates with its target mRNAs by base-pair complementarity. In the relatively rare cases in which a miRNA base pairs perfectly with an mRNA target, it promotes mRNA degradation. More commonly, miRNAs form imperfect heteroduplexes with target mRNAs, affecting either mRNA stability or inhibiting mRNA translation.

The 5' portion of a miRNA spanning bases 2-8, termed the 'seed' region, is especially important for target recognition (Krenz and Robbins, 2004; Kiriazis and Krania, 2000). The sequence of the seed, together with phylogenetic conservation of the target sequence, forms the basis for many current target prediction models. Although increasingly sophisticated computational approaches to predict miRNAs and their targets are becoming available, target prediction remains a major challenge and requires experimental validation. Ascribing the functions of miRNAs to the regulation of specific mRNA targets is further complicated by the ability of individual miRNAs to base pair with hundreds of potential high and low affinity mRNA targets and by the targeting of multiple miRNAs to individual mRNAs.

The first miRNAs were identified as regulators of developmental timing in *C. elegans,* suggesting that miRNAs, in general, might play decisive regulatory roles in transitions between different developmental states by switching off specific targets (Fatkin *et al.*, 2000; Lowes *et al.*, 1997). However, subsequent studies suggest that miRNAs, rather than functioning as on-off "switches," more commonly function to modulate or fine-tune cell phenotypes by repressing expression of proteins that are inappropriate for a particular cell type, or by adjusting protein dosage. miRNAs have also been proposed to provide robustness to cellular phenotypes by eliminating extreme fluctuations in gene expression (Miyata *et al.*, 2000).

Research on microRNAs is increasing as scientists are beginning to appreciate the broad role that these molecules play in the regulation of eukaryotic gene expression. The two

best understood miRNAs, *lin-4* and *let-7*, regulate developmental timing in *C. elegans* by regulating the translation of a family of key mRNAs (reviewed in Pasquinelli, 2002). Several hundred miRNAs have been identified in *C. elegans*, *Drosophila*, mouse, and humans. As would be expected for molecules that regulate gene expression, miRNA levels have been shown to vary between tissues and developmental states. In addition, one study shows a strong correlation between reduced expression of two miRNAs and chronic lymphocytic leukemia, providing a possible link between miRNAs and cancer (Calin, 2002). Although the field is still young, there is speculation that miRNAs could be as important as transcription factors in regulating gene expression in higher eukaryotes.

There are a few examples of miRNAs that play critical roles in cell differentiation, early development, and cellular processes like apoptosis and fat metabolism. *lin-4* and *let-7* both regulate passage from one larval state to another during *C. elegans* development (Ambros, 2003). *mir-14* and *bantam* are *drosophila* miRNAs that regulate cell death, apparently by regulating the expression of genes involved in apoptosis (Brennecke *et al.*, 2003, Xu *et al.*, 2003). MiR14 has also been implicated in fat metabolism (Xu *et al.*, 2003). *Lsy-6* and *miR-273* are *C. elegans* miRNAs that regulate asymmetry in chemosensory neurons (Chang *et al.*, 2004). Another animal miRNA that regulates cell differentiation is miR-181, which guides hematopoietic cell differentiation (Chen *et al.,* 2004). These molecules represent the full range of animal miRNAs with known functions. Enhanced understanding of the functions of miRNAs will undoubtedly reveal regulatory networks that contribute to normal development, differentiation, inter- and intra-cellular communication, cell cycle, angiogenesis, apoptosis, and many other cellular processes. Given their important roles in many biological functions, it is likely that miRNAs will offer important points for therapeutic intervention or diagnostic analysis.

Characterizing the functions of biomolecules like miRNAs often involves introducing the molecules into cells or removing the molecules from cells and measuring the result. If introducing a miRNA into cells results in apoptosis, then the miRNA undoubtedly participates in an apoptotic pathway. Methods for introducing and removing miRNAs from cells have been described. Two recent publications describe antisense molecules that can be used to inhibit the activity of specific miRNAs (Meister *et al.*, 2004; Hutvagner *et al.*, 2004). Another publication describes the use of plasmids that are transcribed by endogenous RNA polymerases and yield specific miRNAs when transfected into cells (Zeng *et al.*, 2002). These two reagent sets have been used to evaluate single miRNAs.

B. Synthesis and Alternative Nucleic Acid Chemistries

Oligonucleotides like miRNAs are generally chemically synthesized using nucleoside phosphoramidites. A phosphoramidite is a derivative of natural or synthetic nucleoside with protection groups added to its reactive exocyclic amine and hydroxy groups. The naturally occuring nucleotides (nucleoside-3'-phosphates) are insufficiently reactive to afford the synthetic preparation of oligonucleotides. A dramatically more reactive (2-cyanoethyl) N,N-diisopropyl phosphoramidite group is therefore attached to the 3'-hydroxy group of a nucleoside to form nucleoside phosphoramidite. The protection groups prevent unwanted side reactions or facilitate the formation of the desired product during synthesis. The 5'-hydroxyl group is protected by DMT (dimethoxytrityl) group, the phosphite group by a diisopropylamino (iPr2N) group and a 2-cyanoethyl (OCH_2CH_2CN) group. The nucleic bases also have protecting groups on the exocyclic amine groups (benzoyl, acetyl, isobutyryl, or many other groups). In RNA synthesis, the 2' group is protected with a TBDMS (*t*-butyldimethylsilyl) group or with a TOM (*t*-butyldimethylsilyloxymethyl) group. With the completion of the synthesis process, all the protection groups are removed.

Whereas enzymes synthesize DNA in a 5' to 3' direction, chemical DNA synthesis is done backwards in a 3' to 5' reaction. Based on the desired nucleotide sequence of the product, the phosphoramidites of nucleosides A, C, G, and T are added sequentially to react with the growing chain in a repeating cycle until the sequence is complete. In each cycle, the product's 5'-hydroxy group is deprotected and a new base is added for extension. In solid-phase synthesis, the oligonucleotide being assembled is bound, via its 3'-terminal hydroxy group, to a solid support material on which all reactions take place. The 3' group of the first base is immobilized via a linker onto a solid support (most often, controlled pore glass particles or macroporouspolystyrene beads). This allows for easy addition and removal of reactants. In each cycle, several solutions containing reagents required for the elongation of the oligonucleotide chain by one nucleotide residue are sequentually pumped through the column from an attached reagent delivery system and removed by washing with an inert solvent.

Antagomirs can be synthesized to include a modification that imparts a desired characteristic. For example, the modification can improve stability, hybridization thermodynamics with a target nucleic acid, targeting to a particular tissue or cell-type, or cell permeability, *e.g.*, by an endocytosis-dependent or -independent mechanism. Modifications can also increase sequence specificity, and consequently decrease off-site targeting. In one

embodiment, the antagomir includes a non-nucleotide moiety, *e.g.*, a cholesterol moiety. The non-nucleotide moiety can be attached to the 3' or 5' end of the oligonucleotide agent.

A wide variety of well-known, alternative oligonucleotide chemistries may be used (see, *e.g.*, U.S. Patent Publications 2007/0213292, 2008/0032945, 2007/0287831, *etc.*), particularly single-stranded complementary oligonucleotides comprising 2' methoxyethyl, 2'-fluoro, and morpholino bases (see *e.g.*, Summerton and Weller, 1997). The oligonucleotide may include a 2'-modified nucleotide, *e.g.*, a 2'-deoxy, 2'-deoxy-2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), 2'-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE), or 2'-O--N-methylacetamido (2'-O--NMA). Also contemplated are locked nucleic acid (LNA) and peptide nucleic acids (PNA).

Peptide nucleic acids (PNAs) are nonionic DNA mimics that have outstanding potential for recognizing duplex DNA (Kaihatsu *et al.*, 2004; Nielsen *et al.*, 1991). PNAs can be readily synthesized and bind to complementary sequences by standard Watson-Crick base-pairing (Egholm *et al.*, 1993), allowing them to target any sequence within the genome without the need for complex synthetic protocols or design considerations. Strand invasion of duplex DNA by PNAs is not hindered by phosphate-phosphate repulsion and is both rapid and stable (Kaihatsu *et al.*, 2004; Nielsen *et al.*, 1991). Applications for strand invasion by PNAs include creation of artificial primosomes (Demidov *et al.*, 2001), inhibition of transcription (Larsen and Nielsen, 1996), activation of transcription (Mollegaard *et al.*, 1994), and directed mutagenesis (Faruqi *et al.*, 1998). PNAs would provide a general and potent strategy for probing the structure and function of chromosomal DNA in living systems if their remarkable strand invasion abilities could be efficiently applied inside cells.

Strand invasion by PNAs in cell-free systems is most potent at sequences that are partially single-stranded (Bentin and Nielsen, 1996; Zhang *et al.*, 2000). Assembly of RNA polymerase and transcription factors into the pre-initiation complex on DNA induces the formation of a structure known as the open complex that contains several bases of single-stranded DNA (Holstege *et al.*, 1997; Kahl *et al.*, 2000). The exceptional ability of PNAs to recognize duplex DNA allows them to intercept the open complex of an actively transcribed gene without a requirement for preincubation. The open complex is formed during transcription of all genes and PNAs can be synthesized to target any transcription initiation site. Therefore, antigene PNAs that target an open complex at a promoter region within chromosomal DNA would have the potential to be general tools for controlling transcription initiation inside cells.

A locked nucleic acid (LNA), often referred to as inaccessible RNA, is a modified RNA nucleotide (Elmén *et al.*, 2008). The ribose moiety of an LNA nucleotide is modified with an extra bridge connecting the 2' and 4' carbons. The bridge "locks" the ribose in the 3'-endo structural conformation, which is often found in the A-form of DNA or RNA. LNA nucleotides can be mixed with DNA or RNA bases in the oligonucleotide whenever desired. Such oligomers are commercially available. The locked ribose conformation enhances base stacking and backbone pre-organization. This significantly increases the thermal stability (melting temperature) of oligonucleotides (Kaur *et al.*, 2006). LNA bases may be included in a DNA backbone, by they can also be in a backbone of LNA, 2'-O-methyl RNA, 2'-methoxyethyl RNA, or 2'-fluoro RNA. These molecules may utilize either a phosphodiester or phosphorothioate backbone.

Other oligonucleotide modifications can be made to produce oligonucleotides. For example, stability against nuclease degradation has been achieved by introducing a phosphorothioate (P=S) backbone linkage at the 3′ end for exonuclease resistance and 2′ modifications (2'-OMe, 2'-F and related) for endonuclease resistance (WO 2005115481; Li *et al.*, 2005; Choung *et al.*, 2006). A motif having entirely of 2′-*O*-methyl and 2′-fluoro nucleotides has shown enhanced plasma stability and increased *in vitro* potency (Allerson *et al.*, 2005). The incorporation of 2'-*O*-Me and 2'-*O*-MOE does not have a notable effect on activity (Prakash *et al.*, 2005).

Sequences containing a 4'-thioribose modification have been shown to have a stability 600 times greater than that of natural RNA (Hoshika *et al*, 2004). Crystal structure studies reveal that 4'-thioriboses adopt conformations very similar to the C3'-endo pucker observed for unmodified sugars in the native duplex (Haeberli *et al.*, 2005). Stretches of 4'-thio-RNA were well tolerated in both the guide and nonguide strands. However, optimization of both the number and the placement of 4'-thioribonucleosides is necessary for maximal potency.

In the boranophosphate linkage, a non-bridging phosphodiester oxygen is replaced by an isoelectronic borane (BH3-) moiety. Boranophosphate siRNAs have been synthesized by enzymatic routes using T7 RNA polymerase and a boranophosphate ribonucleoside triphosphate in the transcription reaction. Boranophosphate siRNAs are more active than native siRNAs if the center of the guide strand is not modified, and they may be at least ten times more nuclease resistant than unmodified siRNAs (Hall *et al.*, 2004; Hall *et al.*, 2006).

Certain terminal conjugates have been reported to improve or direct cellular uptake. For example, NAAs conjugated with cholesterol improve *in vitro* and *in vivo* cell permeation

in liver cells (Rand *et al.*, 2005). Soutschek *et al.* (2004) have reported on the use of chemically-stabilized and cholesterol-conjugated siRNAs have markedly improved pharmacological properties *in vitro* and *in vivo*. Chemically-stabilized siRNAs with partial phosphorothioate backbone and 2'-O-methyl sugar modifications on the sense and antisense strands (discussed above) showed significantly enhanced resistance towards degradation by exo- and endonucleases in serum and in tissue homogenates, and the conjugation of cholesterol to the 3' end of the sense strand of an oligonucleotides by means of a pyrrolidine linker does not result in a significant loss of gene-silencing activity in cell culture. These study demonstrates that cholesterol conjugation significantly improves *in vivo* pharmacological properties of oligonucleotides.

U.S. Patent Publication 2008/0015162, incorporated herein by reference, provide additional examples of nucleic acid analogs useful in the present invention. The following excerpts are derived from that document and are exemplary in nature only.

In certain embodiments, oligomeric compounds comprise one or more modified monomers, including 2'-modified sugars, such as BNA's and monomers (*e.g.*, nucleosides and nucleotides) with 2'-substituents such as allyl, amino, azido, thio, O-allyl, O--C_1-C_{10} alkyl, --OCF_3, O--$(CH_2)_2$--O--CH_3, 2'-O$(CH_2)_2SCH_3$, O--$(CH_2)_2$--O--N$(R_m)(R_n)$, or O--CH_2--C(=O)--N$(R_m)(R_n)$, where each R_m and R_n is, independently, H or substituted or unsubstituted C_1-C_{10} alkyl.

In certain embodiments, the oligomeric compounds including, but no limited to short oligomers of the present invention, comprise one or more high affinity monomers provided that the oligomeric compound does not comprise a nucleotide comprising a 2'-O$(CH_2)_n$H, wherein n is one to six. In certain embodiments, the oligomeric compounds including, but no limited to short oligomers of the present invention, comprise one or more high affinity monomer provided that the oligomeric compound does not comprise a nucleotide comprising a 2'-OCH_3 or a 2'-O$(CH_2)_2OCH_3$. In certain embodiments, the oligomeric compounds comprise one or more high affinity monomers provided that the oligomeric compound does not comprise a α-L-methyleneoxy (4'-CH_2--O-2') BNA and/or a β-D-methyleneoxy (4'-CH_2--O-2') BNA.

Certain BNAs have been prepared and disclosed in the patent literature as well as in scientific literature (Singh *et al.*, 1998; Koshkin *et al.*, 1998; Wahlestedt *et al.*, 2000; Kumar *et al.,* 1998; WO 94/14226; WO 2005/021570; Singh *et al,* 1998; examples of issued US patents and published applications that disclose BNAs include, for example, U.S. Patents

7,053,207; 6,268,490; 6,770,748; 6,794,499; 7,034,133; and 6,525,191; and U.S. Patent Publication Nos. 2004/0171570; 2004/0219565; 2004/0014959; 2003/0207841; 2004/0143114; and 2003/0082807.

Also provided herein are BNAs in which the 2'-hydroxyl group of the ribosyl sugar ring is linked to the 4' carbon atom of the sugar ring thereby forming a methyleneoxy (4'-CH_2--O-2') linkage to form the bicyclic sugar moiety (reviewed in Elayadi *et al.,* 2001; Braasch *et al.,* 2001; see also U.S. Patents 6,268,490 and 6,670,461). The linkage can be a methylene (--CH_2--) group bridging the 2' oxygen atom and the 4' carbon atom, for which the term methyleneoxy (4'-CH_2--O-2') BNA is used for the bicyclic moiety; in the case of an ethylene group in this position, the term ethyleneoxy (4'-CH_2CH_2--O-2') BNA is used (Singh *et al.*, 1998; Morita *et al.,* 2003). Methyleneoxy (4'-CH_2--O-2') BNA and other bicyclic sugar analogs display very high duplex thermal stabilities with complementary DNA and RNA (Tm = +3 to +10°C), stability towards 3'-exonucleolytic degradation and good solubility properties. Potent and nontoxic antisense oligonucleotides comprising BNAs have been described (Wahlestedt *et al.,* 2000).

An isomer of methyleneoxy (4'-CH_2--O-2') BNA that has also been discussed is α-L-methyleneoxy (4'-CH_2--O-2') BNA which has been shown to have superior stability against a 3'-exonuclease. The α-L-methyleneoxy (4'-CH_2--O-2') BNA's were incorporated into antisense gapmers and chimeras that showed potent antisense activity (Frieden *et al.,* 2003).

The synthesis and preparation of the methyleneoxy (4'-CH_2--O-2') BNA monomers adenine, cytosine, guanine, 5-methyl-cytosine, thymine and uracil, along with their oligomerization, and nucleic acid recognition properties have been described (Koshkin *et al.,* 1998). BNAs and preparation thereof are also described in WO 98/39352 and WO 99/14226.

Analogs of methyleneoxy (4'-CH_2--O-2') BNA, phosphorothioate-methyleneoxy (4'-CH_2--O-2') BNA and 2'-thio-BNAs, have also been prepared (Kumar *et al.,* 1998). Preparation of locked nucleoside analogs comprising oligodeoxyribonucleotide duplexes as substrates for nucleic acid polymerases has also been described (Wengel *et al.*, WO 99/14226). Furthermore, synthesis of 2'-amino-BNA, a novel comformationally restricted high-affinity oligonucleotide analog has been described in the art (Singh *et al.,* 1998). In addition, 2'-amino- and 2'-methylamino-BNA's have been prepared and the thermal stability of their duplexes with complementary RNA and DNA strands has been previously reported.

Modified sugar moieties are well known and can be used to alter, typically increase, the affinity of oligomers for targets and/or increase nuclease resistance. A representative list

of modified sugars includes, but is not limited to, bicyclic modified sugars (BNA's), including methyleneoxy (4'-CH_2--O-2') BNA and ethyleneoxy (4'-$(CH_2)_2$--O-2' bridge) BNA; substituted sugars, especially 2'-substituted sugars having a 2'-F, 2'-OCH_3 or a 2'-$O(CH_2)_2$--OCH_3 substituent group; and 4'-thio modified sugars. Sugars can also be replaced with sugar mimetic groups among others. Methods for the preparations of modified sugars are well known to those skilled in the art. Some representative patents and publications that teach the preparation of such modified sugars include, but are not limited to, U.S. Patents 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; 5,700,920; 6,531,584; and 6,600,032; and WO 2005/121371.

The naturally-occurring base portion of a nucleoside is typically a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. For those nucleosides that include a pentofuranosyl sugar, a phosphate group can be linked to the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, those phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. Within oligonucleotides, the phosphate groups are commonly referred to as forming the internucleotide backbone of the oligonucleotide. The naturally occurring linkage or backbone of RNA and of DNA is a 3' to 5' phosphodiester linkage.

In addition to "unmodified" or "natural" nucleobases such as the purine nucleobases adenine (A) and guanine (G), and the pyrimidine nucleobases thymine (T), cytosine (C) and uracil (U), many modified nucleobases or nucleobase mimetics known to those skilled in the art are amenable with the compounds described herein. In certain embodiments, a modified nucleobase is a nucleobase that is fairly similar in structure to the parent nucleobase, such as for example a 7-deaza purine, a 5-methyl cytosine, or a G-clamp. In certain embodiments, nucleobase mimetic include more complicated structures, such as for example a tricyclic phenoxazine nucleobase mimetic. Methods for preparation of the above noted modified nucleobases are well known to those skilled in the art.

Described herein are linking groups that link monomers (including, but not limited to, modified and unmodified nucleosides and nucleotides) together, thereby forming an oligomeric compound. The two main classes of linking groups are defined by the presence or absence of a phosphorus atom. Representative phosphorus containing linkages include, but are not limited to, phosphodiesters (P=O), phosphotriesters, methylphosphonates, phosphoramidate, and phosphorothioates (P=S). Representative non-phosphorus containing

linking groups include, but are not limited to, methylenemethylimino (--CH_2--N(CH_3)--O--CH_2--), thiodiester (--O--C(O)--S--), thionocarbamate (--O--C(O)(NH)--S--); siloxane (--O--$Si(H)_2$--O--); and N,N'-dimethylhydrazine (--CH_2--N(CH_3)--N(CH_3)--). Oligomeric compounds having non-phosphorus linking groups are referred to as oligonucleosides. Modified linkages, compared to natural phosphodiester linkages, can be used to alter, typically increase, nuclease resistance of the oligomeric compound. In certain embodiments, linkages having a chiral atom can be prepared a racemic mixtures, as separate enantiomers. Representative chiral linkages include, but are not limited to, alkylphosphonates and phosphorothioates. Methods of preparation of phosphorous-containing and non-phosphorous-containing linkages are well known to those skilled in the art.

C. Antagomirs and Other Antagonists

Antagonism of miRNA function may be achieved by "antagomirs." Initially described by Krützfeldt and colleagues (Krützfeldt *et al.*, 2005), antagomirs are single-stranded, chemically-modified ribonucleotides that are at least partially complementary to the miRNA sequence. Antagomirs may comprise one or more modified nucleotides, such as 2'-O-methyl-sugar modifications. In some embodiments, antagomirs comprise only modified nucleotides. Antagomirs may also comprise one or more phosphorothioate linkages resulting in a partial or full phosphorothioate backbone. To facilitate *in vivo* delivery and stability, the antagomir may be linked to a cholesterol moiety at its 3' end. Antagomirs suitable for inhibiting miRNAs may be about 15 to about 50 nucleotides in length, more preferably about 18 to about 30 nucleotides in length, and most preferably about 20 to about 25 nucleotides in length. "Partially complementary" refers to a sequence that is at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% complementary to a target polynucleotide sequence. The antagomirs may be at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% complementary to a mature miRNA sequence. In some embodiments, the antagomir may be substantially complementary to a mature miRNA sequence, that is at least about 95%, 96%, 97%, 98%, or 99% complementary to a target polynucleotide sequence. In other embodiments, the antagomirs are 100% complementary to the mature miRNA sequence.

Inhibition of miRNA function may also be achieved by administering antisense oligonucleotides. The antisense oligonucleotides may be ribonucleotides or deoxyribonucleotides. In particular, the antisense oligonucleotides may have at least one chemical modification. Antisense oligonucleotides may be comprised of one or more "locked nucleic acids." "Locked nucleic acids" (LNAs) are modified ribonucleotides that contain an

extra bridge between the 2' and 4' carbons of the ribose sugar moiety resulting in a "locked" conformation that confers enhanced thermal stability to oligonucleotides containing the LNAs. Alternatively, the antisense oligonucleotides may comprise peptide nucleic acids (PNAs), which contain a peptide-based backbone rather than a sugar-phosphate backbone. Other chemical modifications that the antisense oligonucleotides may contain include, but are not limited to, sugar modifications, such as 2'-O-alkyl (*e.g.*, 2'-O-methyl, 2'-O-methoxyethyl), 2'-fluoro, and 4' thio modifications, and backbone modifications, such as one or more phosphorothioate, morpholino, or phosphonocarboxylate linkages (see, for example, U.S. Patents 6,693,187 and 7,067,641, which are herein incorporated by reference in their entireties). In some embodiments, suitable antisense oligonucleotides are 2'-O-methoxyethyl "gapmers" which contain 2'-O-methoxyethyl-modified ribonucleotides on both 5' and 3' ends with at least ten deoxyribonucleotides in the center. These "gapmers" are capable of triggering RNase H-dependent degradation mechanisms of RNA targets. Other modifications of antisense oligonucleotides to enhance stability and improve efficacy, such as those described in U.S. Patent 6,838,283, which is herein incorporated by reference in its entirety, are known in the art and are suitable for use in the methods of the invention. Particular antisense oligonucleotides useful for inhibiting the activity of microRNAs are about 19 to about 25 nucleotides in length. Antisense oligonucleotides may comprise a sequence that is at least partially complementary to a mature miRNA sequence, *e.g.*, at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% complementary to a mature miRNA sequence. In some embodiments, the antisense oligonucleotide may be substantially complementary to a mature miRNA sequence, that is at least about 95%, 96%, 97%, 98%, or 99% complementary to a target polynucleotide sequence. In one embodiment, the antisense oligonucleotide comprises a sequence that is 100% complementary to a mature miRNA sequence.

Another approach for inhibiting the function of miRs is administering an inhibitory RNA molecule having at least partial sequence identity to the mature miR sequence. The inhibitory RNA molecule may be a double-stranded, small interfering RNA (siRNA) or a short hairpin RNA molecule (shRNA) comprising a stem-loop structure. The double-stranded regions of the inhibitory RNA molecule may comprise a sequence that is at least partially identical, *e.g.*, about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical, to the mature miRNA sequence. In some embodiments, the double-stranded regions of the inhibitory RNA comprise a sequence that is at least substantially identical to the mature miRNA sequence. "Substantially identical" refers to a sequence that is at least about 95%, 96%, 97%, 98%, or 99% identical to a target polynucleotide sequence. In other embodiments,

the double-stranded regions of the inhibitory RNA molecule may contain 100% identity to the target miRNA sequence.

In other embodiments of the invention, inhibitors of miRs may be inhibitory RNA molecules, such as ribozymes, siRNAs, or shRNAs. In one embodiment, an inhibitor is an inhibitory RNA molecule comprising a double-stranded region, wherein the double-stranded region comprises a sequence having 100% identity to the mature miR sequence. In some embodiments, inhibitors are inhibitory RNA molecules which comprise a double-stranded region, wherein said double-stranded region comprises a sequence of at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to the mature miR sequence.

D. Delivery

A variety of methods may be used to deliver oligonucleotides, including miRs, antagomirs and mimics, into a target cell. For cells *in vitro* embodiments, delivery can often be accomplished by direct injection into cells, and delivery can often be enhanced using hydrophobic or cationic carriers. Alternatively, the cells can be permeabilized with a permeabilization and then contacted with the oligonucleotide. The antagomir can be administered to the subject either as a naked oligonucleotide agent, in conjunction with a delivery reagent, or as a recombinant plasmid or viral vector which expresses the oligonucleotide agent.

For cells *in situ*, several applicable delivery methods are well-established, *e.g.*, Elmen *et al.* (2008), Akinc *et al.* (2008); Esau *et al.* (2006), Krützfeldt *et al.* (2005). In particular, cationic lipids (see *e.g.*, Hassani *et al.*, 2004) and polymers such as polyethylenimine (see *e.g.*, Urban-Klein, 2005) have been used to facilitate oligonucleotide delivery. Compositions consisting essentially of the oligomer (*i.e.,* the oligomer in a carrier solution without any other active ingredients) can be directly injected into the host (see *e.g.*, Tyler *et al.*, 1999; McMahon *et al.*, 2002). *In vivo* applications of duplex RNAs are reviewed in Paroo and Corey (2004).

When microinjection is not an option, delivery can be enhanced in some cases by using Lipofectamine™ (Invitrogen, Carlsbad, CA). PNA oligomers can be introduced into cells *in vitro* by complexing them with partially complementary DNA oligonucleotides and cationic lipid. The lipid promotes internalization of the DNA, while the PNA enters as cargo and is subsequently released. Peptides such as penetratin, transportan, Tat peptide, nuclear localization signal (NLS), and others, can be attached to the oligomer to promote cellular

uptake (see *e.g.*, Kaihatsu *et al.*, 2003; Kaihatsu *et al.*, 2004). Alternatively, the cells can be permeabilized with a permeabilization agent such as lysolecithin, and then contacted with the oligomer.

Alternatively, certain single-stranded oligonucleotide agents featured in the instant invention can be expressed within cells from eukaryotic promoters (*e.g.*, Izant and Weintraub, 1985; McGarry and Lindquist, 1986; Scanlon *et al.*, 1991; Kashani-Sabet *et al.*, 1992; Propulic *et al.*, 1992; Weerasinghe *et al.*, 1991; Ojwang *et al.*, 1992; Chen *et al.*, 1992; Sarver *et al.*, 1990; Thompson *et al.*, 1995). Those skilled in the art realize that any nucleic acid can be expressed in eukaryotic cells from the appropriate DNA/RNA vector. The activity of such nucleic acids can be augmented by their release from the primary transcript by a enzymatic nucleic acid (PCT WO 93/23569; PCT WO 94/02595; Ohkawa *et al.*, 1992; Taira *et al.*, 1991; Ventura *et al.*, 1993; Chowrira *et al.*, 1994).

The recombinant vectors can be DNA plasmids or viral vectors. Oligonucleotide agent-expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. In another embodiment, pol III based constructs are used to express nucleic acid molecules of the invention (see for example Morris *et al.*, 2004; U.S. Patents 5,902,880 and 6,146,886). The recombinant vectors capable of expressing the oligonucleotide agents can be delivered as described above, and can persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of nucleic acid molecules. Such vectors can be repeatedly administered as necessary. Once expressed, the antagomir interacts with the target RNA (*e.g.*, miRNA or pre-miRNA) and inhibits miRNA activity. In a particular embodiment, the antagomir forms a duplex with the target miRNA, which prevents the miRNA from binding to its target mRNA, which results in increased translation of the target mRNA. Delivery of oligonucleotide agent-expressing vectors can be systemic, such as by intravenous or intra-muscular administration, by administration to target cells ex-planted from a subject followed by reintroduction into the subject, or by any other means that would allow for introduction into the desired target cell (see Couture *et al.*, 1996).

Methods for the delivery of nucleic acid molecules are also described in Akhtar *et al.* (1992), Akhtar (1995), Maurer *et al.* (1999), Hofland and Huang (1999), Lee *et al.* (2000), all of which are incorporated herein by reference. U.S. Patent 6,395,713 and PCT WO 94/02595 and WO 00/53722 further describe general methods for delivery of nucleic acid molecules.

III. NUCLEIC ACID VECTORS

As mentioned above, miRNAs of the present invention may be delivered and produced via a recombinant vector. The term "vector" is used to refer to a carrier nucleic acid molecule into which a nucleic acid sequence can be inserted for introduction into a cell where it can be replicated. A nucleic acid sequence can be "exogenous," which means that it is foreign to the cell into which the vector is being introduced or that the sequence is homologous to a sequence in the cell but in a position within the host cell nucleic acid in which the sequence is ordinarily not found.

Vectors include plasmids, cosmids, viruses (bacteriophage, animal viruses, and plant viruses), and artificial chromosomes (*e.g.,* YACs). One of skill in the art would be well equipped to construct a vector through standard recombinant techniques (Sambrook *et al.*, 1989; Ausubel *et al.,* 1996, both incorporated herein by reference). A vector may encode non-modified polypeptide sequences such as a tag or targetting molecule. Useful vectors encoding such fusion proteins include pIN vectors (Inouye *et al.*, 1985), vectors encoding a stretch of histidines, and pGEX vectors, for use in generating glutathione S-transferase (GST) soluble fusion proteins for later purification and separation or cleavage. A targetting molecule is one that directs the modified polypeptide to a particular organ, tissue, cell, or other location in a subject's body.

Expression Vectors or Expression Cassettes

The term "expression vector" refers to a vector containing a nucleic acid sequence coding for at least part of a gene product capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. In other cases, such as with miRNAs, these sequences are not translated. Expression vectors can contain a variety of "control sequences," which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operably linked coding sequence in a particular host organism. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well and are described *infra.*

Promoters and Enhancers

A "promoter" is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at

which regulatory proteins and molecules may bind such as RNA polymerase and other transcription factors. The phrases "operatively positioned," "operatively linked," "under control," and "under transcriptional control" mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or expression of that sequence. A promoter may or may not be used in conjunction with an "enhancer," which refers to a cis-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

A promoter may be one naturally associated with a gene or sequence, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as "endogenous." Similarly, an enhancer may be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other prokaryotic, viral, or eukaryotic cell, and promoters or enhancers not "naturally occurring," *i.e.,* containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR™, in connection with the compositions disclosed herein (see U.S. Patent 4,683,202, U.S. Patent 5,928,906, each incorporated herein by reference). Furthermore, it is contemplated the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well.

Naturally, it may be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the cell type, organelle, and organism chosen for expression. Those of skill in the art of molecular biology generally know the use of promoters, enhancers, and cell type combinations for protein expression, for example, see Sambrook *et al.* (1989), incorporated herein by reference. The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. The promoter may be

heterologous or endogenous. The identity of tissue-specific promoters or elements, as well as assays to characterize their activity, is well known to those of skill in the art.

Multiple Cloning Sites

Vectors can include a multiple cloning site (MCS), which is a nucleic acid region that contains multiple restriction enzyme sites, any of which can be used in conjunction with standard recombinant technology to digest the vector. (See Carbonelli *et al.,* 1999, Levenson *et al.,* 1998, and Cocea, 1997, incorporated herein by reference.) "Restriction enzyme digestion" refers to catalytic cleavage of a nucleic acid molecule with an enzyme that functions only at specific locations in a nucleic acid molecule. Many of these restriction enzymes are commercially available. Use of such enzymes is widely understood by those of skill in the art. Frequently, a vector is linearized or fragmented using a restriction enzyme that cuts within the MCS to enable exogenous sequences to be ligated to the vector. "Ligation" refers to the process of forming phosphodiester bonds between two nucleic acid fragments, which may or may not be contiguous with each other. Techniques involving restriction enzymes and ligation reactions are well known to those of skill in the art of recombinant technology.

Termination Signals

The vectors or constructs of the present invention will generally comprise at least one termination signal. A "termination signal" or "terminator" is comprised of the DNA sequences involved in specific termination of an RNA transcript by an RNA polymerase. Thus, in certain embodiments a termination signal that ends the production of an RNA transcript is contemplated. A terminator may be necessary *in vivo* to achieve desirable message levels.

In eukaryotic systems, the terminator region may also comprise specific DNA sequences that permit site-specific cleavage of the new transcript so as to expose a polyadenylation site. This signals a specialized endogenous polymerase to add a stretch of about 200 A residues (polyA) to the 3' end of the transcript. RNA molecules modified with this polyA tail appear to more stable and are translated more efficiently. Thus, in other embodiments involving eukaryotes, it is preferred that that terminator comprises a signal for the cleavage of the RNA, and it is more preferred that the terminator signal promotes polyadenylation of the message. The terminator and/or polyadenylation site elements can

serve to enhance message levels and/or to minimize read through from the cassette into other sequences.

Terminators contemplated for use in the invention include any known terminator of transcription described herein or known to one of ordinary skill in the art, including but not limited to, for example, the termination sequences of genes, such as for example the bovine growth hormone terminator or viral termination sequences, such as for example the SV40 terminator. In certain embodiments, the termination signal may be a lack of transcribable or translatable sequence, such as due to a sequence truncation.

Origins of Replication

In order to propagate a vector in a host cell, it may contain one or more origins of replication sites (often termed "ori"), which is a specific nucleic acid sequence at which replication is initiated. Alternatively an autonomously replicating sequence (ARS) can be employed if the host cell is yeast.

Expression Systems

Numerous expression systems exist that comprise at least a part or all of the compositions discussed above. Prokaryote- and/or eukaryote-based systems can be employed for use with the present invention to produce nucleic acid sequences, or their cognate polypeptides, proteins and peptides. Many such systems are commercially and widely available.

The insect cell/baculovirus system can produce a high level of protein expression of a heterologous nucleic acid segment, such as described in U.S. Patent No. 5,871,986, 4,879,236, both herein incorporated by reference, and which can be bought, for example, under the name MAXBAC® 2.0 from INVITROGEN® and BACPACK™ BACULOVIRUS EXPRESSION SYSTEM FROM CLONTECH®.

In addition to the disclosed expression systems of the invention, other examples of expression systems include STRATAGENE®'s COMPLETE CONTROL™ Inducible Mammalian Expression System, which involves a synthetic ecdysone-inducible receptor, or its pET Expression System, an *E. coli* expression system. Another example of an inducible expression system is available from INVITROGEN®, which carries the T-REX™ (tetracycline-regulated expression) System, an inducible mammalian expression system that uses the full-length CMV promoter. INVITROGEN® also provides a yeast expression system called the

Pichia methanolica Expression System, which is designed for high-level production of recombinant proteins in the methylotrophic yeast *Pichia methanolica*. One of skill in the art would know how to express a vector, such as an expression construct, to produce a nucleic acid sequence or its cognate polypeptide, protein, or peptide.

Viral Vectors

There are a number of ways in which expression vectors may be introduced into cells. In certain embodiments of the invention, the expression vector comprises a virus or engineered vector derived from a viral genome. The ability of certain viruses to enter cells via receptor-mediated endocytosis, to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells (Ridgeway, 1988; Nicolas and Rubinstein, 1988; Baichwal and Sugden, 1986; Temin, 1986). The first viruses used as gene vectors were DNA viruses including the papovaviruses (simian virus 40, bovine papilloma virus, and polyoma) (Ridgeway, 1988; Baichwal and Sugden, 1986) and adenoviruses (Ridgeway, 1988; Baichwal and Sugden, 1986). These have a relatively low capacity for foreign DNA sequences and have a restricted host spectrum. Furthermore, their oncogenic potential and cytopathic effects in permissive cells raise safety concerns. They can accommodate only up to 8 kb of foreign genetic material but can be readily introduced in a variety of cell lines and laboratory animals (Nicolas and Rubenstein, 1988; Temin, 1986).

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells; they can also be used as vectors. Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.,* 1988) adeno-associated virus (AAV) (Ridgeway, 1988; Baichwal and Sugden, 1986; Hermonat and Muzycska, 1984) and herpesviruses may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.,* 1988; Horwich *et al.,* 1990).

IV. DETECTION METHODS

In some embodiments, it may prove useful to assess the expression of miRNAs in a cell from a subject having or suspected of having Ewing Sarcoma. Any method of detection known to one of skill in the art falls within the general scope of the present invention.

Nucleic acids can used be as probes or primers for embodiments involving nucleic acid hybridization. As such, they may be used to assess miRNA expression. Commerically available systems, such as Qiagen's miScript System™ are available for detection of miRNAs. Various aspects of nucleic acid detection as discussed below.

A. Hybridization

The use of a probe or primer of between 13 and 100 nucleotides, preferably between 17 and 100 nucleotides in length, or in some aspects of the invention up to 1-2 kilobases or more in length, allows the formation of a duplex molecule that is both stable and selective. Molecules having complementary sequences over contiguous stretches greater than 20 bases in length are generally preferred, to increase stability and/or selectivity of the hybrid molecules obtained. One will generally prefer to design nucleic acid molecules for hybridization having one or more complementary sequences of 20 to 30 nucleotides, or even longer where desired. Such fragments may be readily prepared, for example, by directly synthesizing the fragment by chemical means or by introducing selected sequences into recombinant vectors for recombinant production.

Accordingly, the nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of DNAs and/or RNAs or to provide primers for amplification of DNA or RNA from samples. Depending on the application envisioned, one would desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of the probe or primers for the target sequence.

For applications requiring high selectivity, one will typically desire to employ relatively high stringency conditions to form the hybrids. For example, relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.10 M NaCl at temperatures of about 50°C to about 70°C. Such high stringency conditions tolerate little, if any, mismatch between the probe or primers and the template or target strand and would be particularly suitable for isolating specific genes or for detecting specific mRNA transcripts. It is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide.

For certain applications it is appreciated that lower stringency conditions are preferred. Under these conditions, hybridization may occur even though the sequences of the hybridizing strands are not perfectly complementary, but are mismatched at one or more positions. Conditions may be rendered less stringent by increasing salt concentration and/or decreasing temperature. For example, a medium stringency condition could be provided by about 0.1 to 0.25 M NaCl at temperatures of about 37°C to about 55°C, while a low stringency condition could be provided by about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C to about 55°C. Hybridization conditions can be readily manipulated depending on the desired results.

In other embodiments, hybridization may be achieved under conditions of, for example, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1.0 mM dithiothreitol, at temperatures between approximately 20°C to about 37°C. Other hybridization conditions utilized could include approximately 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, at temperatures ranging from approximately 40°C to about 72°C.

In certain embodiments, it will be advantageous to employ nucleic acids of defined sequences of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of being detected. In particular embodiments, one may desire to employ a fluorescent label or an enzyme tag such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmentally undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known that can be employed to provide a detection means that is visibly or spectrophotometrically detectable, to identify specific hybridization with complementary nucleic acid containing samples.

In general, it is envisioned that the probes or primers described herein will be useful as reagents in solution hybridization, as in PCR™, for detection of expression of corresponding genes, as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to hybridization with selected probes under desired conditions. The conditions selected will depend on the particular circumstances (depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe, *etc.*). Optimization of hybridization conditions for the particular application of interest is well known to those of skill in the art.

After washing of the hybridized molecules to remove non-specifically bound probe molecules, hybridization is detected, and/or quantified, by determining the amount of bound label. Representative solid phase hybridization methods are disclosed in U.S. Patents 5,843,663, 5,900,481 and 5,919,626. Other methods of hybridization that may be used in the practice of the present invention are disclosed in U.S. Patents 5,849,481, 5,849,486 and 5,851,772 and U.S. Patent Publication 2008/0009439. The relevant portions of these and other references identified in this section of the Specification are incorporated herein by reference.

B. *In situ* Hybridization

In situ hybridization (ISH) is a type of hybridization that uses a labeled complementary DNA or RNA strand (*i.e.*, probe) to localize a specific DNA or RNA sequence in a portion or section of tissue (*in situ*), or, if the tissue is small enough (*e.g.* plant seeds, Drosophila embryos), in the entire tissue (whole mount ISH). This is distinct from immunohistochemistry, which localizes proteins in tissue sections. Fluorescent DNA ISH (FISH) can, for example, be used in medical diagnostics to assess chromosomal integrity. RNA ISH (hybridization histochemistry) is used to measure and localize mRNAs and other transcripts within tissue sections or whole mounts.

For hybridization histochemistry, sample cells and tissues are usually treated to fix the target transcripts in place and to increase access of the probe. As noted above, the probe is either a labeled complementary DNA or, now most commonly, a complementary RNA (riboprobe). The probe hybridizes to the target sequence at elevated temperature, and then the excess probe is washed away (after prior hydrolysis using RNase in the case of unhybridized, excess RNA probe). Solution parameters such as temperature, salt and/or detergent concentration can be manipulated to remove any non-identical interactions (*i.e.*, only exact sequence matches will remain bound). Then, the probe that was labeled with either radio-, fluorescent- or antigen-labeled bases (*e.g.*, digoxigenin) is localized and quantitated in the tissue using either autoradiography, fluorescence microscopy or immunohistochemistry, respectively. ISH can also use two or more probes, labeled with radioactivity or the other non-radioactive labels, to simultaneously detect two or more transcripts.

C. Amplification of Nucleic Acids

Nucleic acids used as a template for amplification may be isolated from cells, tissues or other samples according to standard methodologies (Sambrook *et al.*, 2001). In certain embodiments, analysis is performed on whole cell or tissue homogenates or biological fluid samples without substantial purification of the template nucleic acid. The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to first convert the RNA to a complementary DNA.

The term "primer," as used herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty and/or thirty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded and/or single-stranded form, although the single-stranded form is preferred.

Pairs of primers designed to selectively hybridize to nucleic acids corresponding to any sequence corresponding to a nucleic acid sequence are contacted with the template nucleic acid under conditions that permit selective hybridization. Depending upon the desired application, high stringency hybridization conditions may be selected that will only allow hybridization to sequences that are completely complementary to the primers. In other embodiments, hybridization may occur under reduced stringency to allow for amplification of nucleic acids containing one or more mismatches with the primer sequences. Once hybridized, the template-primer complex is contacted with one or more enzymes that facilitate template-dependent nucleic acid synthesis. Multiple rounds of amplification, also referred to as "cycles," are conducted until a sufficient amount of amplification product is produced.

The amplification product may be detected or quantified. In certain applications, the detection may be performed by visual means. Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of incorporated radiolabel or fluorescent label or even via a system using electrical and/or thermal impulse signals (Bellus, 1994).

A number of template dependent processes are available to amplify the oligonucleotide sequences present in a given template sample. One of the best known amplification methods is the polymerase chain reaction (referred to as PCR™) which is described in detail in U.S. Patents 4,683,195, 4,683,202 and 4,800,159, and in Innis *et al.* (1988), each of which is incorporated herein by reference in their entirety.

A reverse transcriptase PCR™ amplification procedure may be performed to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known (see Sambrook *et al.*, 2001). Alternative methods for reverse transcription utilize thermostable DNA polymerases. These methods are described in WO 90/07641. Polymerase chain reaction methodologies are well known in the art. Representative methods of RT-PCR are described in U.S. Patent 5,882,864.

Reverse transcription (RT) of RNA to cDNA followed by quantitative PCR (RT-PCR) can be used to determine the relative concentrations of specific miRNA species isolated from a cell. By determining that the concentration of a specific mRNA species varies, it is shown that the gene encoding the specific mRNA species is differentially expressed. If a graph is plotted in which the cycle number is on the X axis and the log of the concentration of the amplified target DNA is on the Y axis, a curved line of characteristic shape is formed by connecting the plotted points. Beginning with the first cycle, the slope of the line is positive and constant. This is said to be the linear portion of the curve. After a reagent becomes limiting, the slope of the line begins to decrease and eventually becomes zero. At this point the concentration of the amplified target DNA becomes asymptotic to some fixed value. This is said to be the plateau portion of the curve.

The concentration of the target DNA in the linear portion of the PCR amplification is directly proportional to the starting concentration of the target before the reaction began. By determining the concentration of the amplified products of the target DNA in PCR reactions that have completed the same number of cycles and are in their linear ranges, it is possible to determine the relative concentrations of the specific target sequence in the original DNA mixture. If the DNA mixtures are cDNAs synthesized from RNAs isolated from different tissues or cells, the relative abundances of the specific mRNA from which the target sequence was derived can be determined for the respective tissues or cells. This direct proportionality between the concentration of the PCR products and the relative mRNA abundances is only true in the linear range of the PCR reaction.

The final concentration of the target DNA in the plateau portion of the curve is determined by the availability of reagents in the reaction mix and is independent of the original concentration of target DNA. Therefore, the first condition that must be met before the relative abundances of a mRNA species can be determined by RT-PCR for a collection of RNA populations is that the concentrations of the amplified PCR products must be sampled when the PCR reactions are in the linear portion of their curves.

A second condition for an RT-PCR experiment is to determine the relative abundances of a particular mRNA species. Typically, relative concentrations of the amplifiable cDNAs are normalized to some independent standard. The goal of an RT-PCR experiment is to determine the abundance of a particular mRNA species relative to the average abundance of all mRNA species in the sample.

Most protocols for competitive PCR utilize internal PCR standards that are approximately as abundant as the target. These strategies are effective if the products of the PCR amplifications are sampled during their linear phases. If the products are sampled when the reactions are approaching the plateau phase, then the less abundant product becomes relatively over represented. Comparisons of relative abundances made for many different RNA samples, such as is the case when examining RNA samples for differential expression, become distorted in such a way as to make differences in relative abundances of RNAs appear less than they actually are. This is not a significant problem if the internal standard is much more abundant than the target. If the internal standard is more abundant than the target, then direct linear comparisons can be made between RNA samples.

RT-PCR can be performed as a relative quantitative RT-PCR with an internal standard in which the internal standard is an amplifiable cDNA fragment that is larger than the target cDNA fragment and in which the abundance of the mRNA encoding the internal standard is roughly 5-100-fold higher than the mRNA encoding the target. This assay measures relative abundance, not absolute abundance of the respective mRNA species.

Another method for amplification is ligase chain reaction ("LCR"), disclosed in European Application No. 320 308, incorporated herein by reference in its entirety. U.S. Patent 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence. A method based on PCR™ and oligonucleotide ligase assy (OLA), disclosed in U.S. Patent 5,912,148, may also be used.

Alternative methods for amplification of target nucleic acid sequences that may be used in the practice of the present invention are disclosed in U.S. Patents 5,843,650, 5,846,709, 5,846,783, 5,849,546, 5,849,497, 5,849,547, 5,858,652, 5,866,366, 5,916,776, 5,922,574, 5,928,905, 5,928,906, 5,932,451, 5,935,825, 5,939,291 and 5,942,391, GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety.

Qbeta Replicase, described in PCT Application No. PCT/US87/00880, may also be used as an amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to that of a target is added to a sample in the presence of

an RNA polymerase. The polymerase will copy the replicative sequence which may then be detected.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[alpha-thio]-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention (Walker *et al.*, 1992). Strand Displacement Amplification (SDA), disclosed in U.S. Patent 5,916,779, is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR (Kwoh *et al.*, 1989; PCT Application WO 88/10315, incorporated herein by reference in their entirety). European Application No. 329 822 disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention.

PCT Application WO 89/06700 (incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter region/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "RACE" and "one-sided PCR" (Frohman, 1990; Ohara *et al.*, 1989).

Following any amplification, it may be desirable to separate the amplification product from the template and/or the excess primer. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods (Sambrook *et al.*, 2001). Separated amplification products may be cut out and eluted from the gel for further manipulation. Using low melting point agarose gels, the separated band may be removed by heating the gel, followed by extraction of the nucleic acid.

Separation of nucleic acids may also be effected by chromatographic techniques known in art. There are many kinds of chromatography which may be used in the practice of the present invention, including adsorption, partition, ion-exchange, hydroxylapatite, molecular sieve, reverse-phase, column, paper, thin-layer, and gas chromatography as well as HPLC.

In certain embodiments, the amplification products are visualized. A typical visualization method involves staining of a gel with ethidium bromide and visualization of bands under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the separated amplification products can be exposed to x-ray film or visualized under the appropriate excitatory spectra.

In one embodiment, following separation of amplification products, a labeled nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, or another binding partner carrying a detectable moiety.

In particular embodiments, detection is by Southern blotting and hybridization with a labeled probe. The techniques involved in Southern blotting are well known to those of skill in the art (see Sambrook *et al.*, 2001). One example of the foregoing is described in U.S. Patent 5,279,721, incorporated by reference herein, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

Various nucleic acid detection methods known in the art are disclosed in U.S. Patents 5,840,873, 5,843,640, 5,843,651, 5,846,708, 5,846,717, 5,846,726, 5,846,729, 5,849,487, 5,853,990, 5,853,992, 5,853,993, 5,856,092, 5,861,244, 5,863,732, 5,863,753, 5,866,331, 5,905,024, 5,910,407, 5,912,124, 5,912,145, 5,919,630, 5,925,517, 5,928,862, 5,928,869, 5,929,227, 5,932,413 and 5,935,791, each of which is incorporated herein by reference.

D. Chip Technologies and Arrays

Specifically contemplated by the present inventors are chip-based DNA technologies such as those described by Hacia *et al.* (1996) and Shoemaker *et al.* (1996). Briefly, these techniques involve quantitative methods for analyzing large numbers of genes rapidly and accurately. By tagging genes with oligonucleotides or using fixed probe arrays, one can employ chip technology to segregate target molecules as high density arrays and screen these molecules on the basis of hybridization (see also, Pease *et al.*, 1994; and Fodor *et al*, 1991). It is contemplated that this technology may be used in conjunction with evaluating the expression level of an miRNA with respect to diagnostic, as well as preventative and treatment methods of the invention.

The present invention may involve the use of arrays or data generated from an array. Data may be readily available. Moreover, an array may be prepared in order to generate data that may then be used in correlation studies.

An array generally refers to ordered macroarrays or microarrays of nucleic acid molecules (probes) that are fully or nearly complementary or identical to a plurality of mRNA molecules or cDNA molecules and that are positioned on a support material in a spatially separated organization. Macroarrays are typically sheets of nitrocellulose or nylon upon which probes have been spotted. Microarrays position the nucleic acid probes more densely such that up to 10,000 nucleic acid molecules can be fit into a region typically 1 to 4 square centimeters. Microarrays can be fabricated by spotting nucleic acid molecules, *e.g.*, genes, oligonucleotides, *etc.*, onto substrates or fabricating oligonucleotide sequences *in situ* on a substrate. Spotted or fabricated nucleic acid molecules can be applied in a high density matrix pattern of up to about 30 non-identical nucleic acid molecules per square centimeter or higher, *e.g.* up to about 100 or even 1000 per square centimeter. Microarrays typically use coated glass as the solid support, in contrast to the nitrocellulose-based material of filter arrays. By having an ordered array of complementing nucleic acid samples, the position of each sample can be tracked and linked to the original sample. A variety of different array devices in which a plurality of distinct nucleic acid probes are stably associated with the surface of a solid support are known to those of skill in the art. Useful substrates for arrays include nylon, glass and silicon Such arrays may vary in a number of different ways, including average probe length, sequence or types of probes, nature of bond between the probe and the array surface, *e.g.* covalent or non-covalent, and the like. The labeling and screening methods of the present invention and the arrays are not limited in its utility with respect to any parameter except that the probes detect expression levels; consequently, methods and compositions may be used with a variety of different types of genes.

Representative methods and apparatus for preparing a microarray have been described, for example, in U.S. Patent Nos. 5,143,854; 5,202,231; 5,242,974; 5,288,644; 5,324,633; 5,384,261; 5,405,783; 5,412,087; 5,424,186; 5,429,807; 5,432,049; 5,436,327; 5,445,934; 5,468,613; 5,470,710; 5,472,672; 5,492,806; 5,525,464; 5,503,980; 5,510,270; 5,525,464; 5,527,681; 5,529,756; 5,532,128; 5,545,531; 5,547,839; 5,554,501; 5,556,752; 5,561,071; 5,571,639; 5,580,726; 5,580,732; 5,593,839; 5,599,695; 5,599,672; 5,610;287; 5,624,711; 5,631,134; 5,639,603; 5,654,413; 5,658,734; 5,661,028; 5,665,547; 5,667,972; 5,695,940; 5,700,637; 5,744,305; 5,800,992; 5,807,522; 5,830,645; 5,837,196; 5,871,928; 5,847,219; 5,876,932; 5,919,626; 6,004,755; 6,087,102; 6,368,799; 6,383,749; 6,617,112;

6,638,717; 6,720,138, as well as WO 93/17126; WO 95/11995; WO 95/21265; WO 95/21944; WO 95/35505; WO 96/31622; WO 97/10365; WO 97/27317; WO 99/35505; WO 09923256; WO 09936760; WO0138580; WO 0168255; WO 03020898; WO 03040410; WO 03053586; WO 03087297; WO 03091426; WO03100012; WO 04020085; WO 04027093; EP 373 203; EP 785 280; EP 799 897 and UK 8 803 000; the disclosures of which are all herein incorporated by reference.

It is contemplated that the arrays can be high density arrays, such that they contain 100 or more different probes. It is contemplated that they may contain 1000, 16,000, 65,000, 250,000 or 1,000,000 or more different probes. The probes can be directed to targets in one or more different organisms. The oligonucleotide probes range from 5 to 50, 5 to 45, 10 to 40, or 15 to 40 nucleotides in length in some embodiments. In certain embodiments, the oligonucleotide probes are 20 to 25 nucleotides in length.

The location and sequence of each different probe sequence in the array are generally known. Moreover, the large number of different probes can occupy a relatively small area providing a high density array having a probe density of generally greater than about 60, 100, 600, 1000, 5,000, 10,000, 40,000, 100,000, or 400,000 different oligonucleotide probes per cm^2. The surface area of the array can be about or less than about 1, 1.6, 2, 3, 4, 5, 6, 7, 8, 9, or 10 cm^2.

Moreover, a person of ordinary skill in the art could readily analyze data generated using an array. Such protocols are disclosed above, and include information found in WO 9743450; WO 03023058; WO 03022421; WO 03029485; WO 03067217; WO 03066906; WO 03076928; WO 03093810; WO 03100448A1, all of which are specifically incorporated by reference.

V. METHODS OF THERAPY

In some embodiments, the invention provides compositions and methods for the treatment of Ewing Sarcoma. In one embodiment, the invention provides a method of treating Ewing Sarcoma comprising administering to a patient an effective amount of a one or more miRNAs selected from miR-193b, miR-34a, miR-148a, miR-301 and miR-15b. This treatment may be further combined with additional cancer treatments. One of skill in the art will be aware of many treatments that may be combined with the methods of the present invention, some but not all of which are described below.

In many contexts, it is not necessary that the tumor cell be killed or induced to undergo normal cell death or "apoptosis." Rather, to accomplish a meaningful treatment, all that is required is that the tumor growth be slowed to some degree. It may be that the tumor growth is completely blocked, however, or that some tumor regression is achieved. Clinical terminology such as "remission" and "reduction of tumor" burden also are contemplated given their normal usage.

A. miRNAs, Mimics and Inhibitory Oligonucleotides

One therapy approach is the provision, to a subject, of an miRNA, a mimic, an and inhibitory oligonucleotide, such as an antagomir. The miRNA, mimic, or inhibitory oligonucleotide is generally produced by an automated synthesizer (see above), although it may also be produced recombinantly. Formulations for delivery of the miRNA, mimic, or inhibitory oligonucleotide are selected based on the route of administration and purpose including, but not limited to, liposomal formulations and classic pharmaceutical preparations (discussed below).

Non-limiting examples of agents suitable for formulation include P-glycoprotein inhibitors (such as PluronicP85), which can enhance entry of drugs into the CNS (Jolliet-Riant and Tillement, 1999), biodegradable polymers, such as poly(DL-lactide-coglycolide) microspheres for sustained release delivery. Other non-limiting examples of delivery strategies for miRNAs include material described in Boado *et al.* (1998), Tyler *et al.* (1999a;b); Pardridge *et al.* (1995); Boado (1995); Aldrian-Herrada *et al.* (1998).

The invention also features the use of a composition that includes surface-modified liposomes containing poly(ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes). These formulations offer a method for increasing the accumulation of drugs in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug (Lasic *et al.*, 1995; Ishiwata *et al.*, 1995).

Such liposomes have been shown to accumulate selectively in tumors, presumably by extravasation and capture in the neovascularized target tissues (Lasic *et al.*, 1995; Oku *et al.*, 1995). The long-circulating liposomes enhance the pharmacokinetics and pharmacodynamics of DNA and RNA, particularly compared to conventional cationic liposomes which are known to accumulate in tissues of the MPS (Liu *et al.*, 1995; PCT Publication No. WO

96/10391; PCT Publication No. WO 96/10390; PCT Publication No. WO 96/10392). Long-circulating liposomes are also likely to protect drugs from nuclease degradation to a greater extent compared to cationic liposomes, based on their ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen.

B. Genetic Delivery

The inventors also contemplate the use of expression constructs encoding miRNA, mimic, or inhibitory oligonucleotide. The construction and structure of viral vectors is discussed above. Administration protocols would generally involve intratumoral, local or regional (to the tumor) administration, as well as systemic administration in appropriate clinical situations.

C. Formulations and Routes for Administration to Patients

In some embodiments, the invention provides a method of treating cancer comprising providing to a patient an effective amount of an miRNA, mimic, or inhibitory oligonucleotide. Where clinical applications are contemplated, it will be necessary to prepare pharmaceutical compositions in a form appropriate for the intended application. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

One will generally desire to employ appropriate salts and buffers to render delivery vectors stable and allow for uptake by target cells. Buffers also will be employed when recombinant cells are introduced into a patient. Aqueous compositions of the present invention comprise an effective amount of the vector to cells, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. 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. The use of such media and agents for pharmaceutically active substances is well know in the art. Except insofar as any conventional media or agent is incompatible with the vectors or cells of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The active compositions of the present invention may include classic pharmaceutical preparations. Administration of these compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration may be by intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions. Of particular interest is direct intratumoral administration, perfusion of a tumor, or administration local or regional to a tumor, for example, in the local or regional vasculature or lymphatic system, or in a resected tumor bed (*e.g.*, post-operative catheter). For practically any tumor, systemic delivery also is contemplated. This will prove especially important for attacking microscopic or metastatic cancer.

The active compounds may also be administered as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial an antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated

above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

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. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The compositions of the present invention may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The actual dosage amount of a composition of the present invention administered to a patient or subject can be determined by physical and physiological factors such as body weight, severity of condition, the type of disease being treated, previous or concurrent therapeutic interventions, idiopathy of the patient and on the route of administration. The practitioner responsible for administration will, in any event, determine the concentration of active ingredient(s) in a composition and appropriate dose(s) for the individual subject.

"Treatment" and "treating" refer to administration or application of a therapeutic agent to a subject or performance of a procedure or modality on a subject for the purpose of obtaining a therapeutic benefit of a disease or health-related condition.

The term "therapeutic benefit" or "therapeutically effective" as used throughout this application refers to anything that promotes or enhances the well-being of the subject with

respect to the medical treatment of this condition. This includes, but is not limited to, a reduction in the frequency or severity of the signs or symptoms of a disease.

A "disease" can be any pathological condition of a body part, an organ, or a system resulting from any cause, such as infection, genetic defect, and/or environmental stress.

"Prevention" and "preventing" are used according to their ordinary and plain meaning to mean "acting before" or such an act. In the context of a particular disease, those terms refer to administration or application of an agent, drug, or remedy to a subject or performance of a procedure or modality on a subject for the purpose of blocking the onset of a disease or health-related condition.

The subject can be a subject who is known or suspected of being free of a particular disease or health-related condition at the time the relevant preventive agent is administered. The subject, for example, can be a subject with no known disease or health-related condition (*i.e.*, a healthy subject).

In additional embodiments of the invention, methods include identifying a patient in need of treatment. A patient may be identified, for example, based on taking a patient history or based on findings on clinical examination.

D. Ewing Sarcoma Combination Treatments

In some embodiments, the method further comprises treating a patient with Ewing Sarcoma with a conventional cancer treatment. One goal of current cancer research is to find ways to improve the efficacy of chemo- and radiotherapy, such as by combining traditional therapies with other anti-cancer treatments. In the context of the present invention, it is contemplated that this treatment could be, but is not limited to, chemotherapeutic, radiation, a polypeptide inducer of apoptosis or other therapeutic intervention. It also is conceivable that more than one administration of the treatment will be desired.

1. Chemotherapy

A wide variety of chemotherapeutic agents may be used in accordance with the present invention. The term "chemotherapy" refers to the use of drugs to treat cancer. A "chemotherapeutic agent" is used to connote a compound or composition that is administered in the treatment of cancer. These agents or drugs are categorized by their mode of activity within a cell, for example, whether and at what stage they affect the cell cycle. Alternatively, an agent may be characterized based on its ability to directly cross-link DNA, to intercalate

into DNA, or to induce chromosomal and mitotic aberrations by affecting nucleic acid synthesis. Most chemotherapeutic agents fall into the following categories: alkylating agents, antimetabolites, antitumor antibiotics, mitotic inhibitors, and nitrosoureas.

Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide 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 ranimnustine; antibiotics such as the enediyne antibiotics (*e.g.,* calicheamicin, especially calicheamicin gammaI and calicheamicin omegaI1; 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, carabicin, carminomycin, 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, nogalarnycin, 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; sizofiran; 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; difluorometlhylornithine (DMFO); retinoids such as retinoic acid; capecitabine; cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, busulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide (VP16), tamoxifen, raloxifene, estrogen receptor binding agents, taxol, paclitaxel, docetaxel, gemcitabien, navelbine, farnesyl-protein tansferase inhibitors, transplatinum, 5-fluorouracil, vincristin, vinblastin and methotrexate and pharmaceutically acceptable salts, acids or derivatives of any of the above.

2. Radiotherapy

Radiotherapy, also called radiation therapy, is the treatment of cancer and other diseases with ionizing radiation. Ionizing radiation deposits energy that injures or destroys cells in the area being treated by damaging their genetic material, making it impossible for these cells to continue to grow. Although radiation damages both cancer cells and normal cells, the latter are able to repair themselves and function properly.

Radiation therapy used according to the present invention may include, but is not limited to, the use of γ-rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV-irradiation. It is most likely that all of these factors effect a broad range of damage on DNA, on the precursors of DNA, on the replication and repair of DNA, and on the assembly

and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 wk), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

Radiotherapy may comprise the use of radiolabeled antibodies to deliver doses of radiation directly to the cancer site (radioimmunotherapy). Antibodies are highly specific proteins that are made by the body in response to the presence of antigens (substances recognized as foreign by the immune system). Some tumor cells contain specific antigens that trigger the production of tumor-specific antibodies. Large quantities of these antibodies can be made in the laboratory and attached to radioactive substances (a process known as radiolabeling). Once injected into the body, the antibodies actively seek out the cancer cells, which are destroyed by the cell-killing (cytotoxic) action of the radiation. This approach can minimize the risk of radiation damage to healthy cells.

Conformal radiotherapy uses the same radiotherapy machine, a linear accelerator, as the normal radiotherapy treatment but metal blocks are placed in the path of the x-ray beam to alter its shape to match that of the cancer. This ensures that a higher radiation dose is given to the tumor. Healthy surrounding cells and nearby structures receive a lower dose of radiation, so the possibility of side effects is reduced. A device called a multi-leaf collimator has been developed and can be used as an alternative to the metal blocks. The multi-leaf collimator consists of a number of metal sheets which are fixed to the linear accelerator. Each layer can be adjusted so that the radiotherapy beams can be shaped to the treatment area without the need for metal blocks. Precise positioning of the radiotherapy machine is very important for conformal radiotherapy treatment and a special scanning machine may be used to check the position of your internal organs at the beginning of each treatment.

High-resolution intensity modulated radiotherapy also uses a multi-leaf collimator. During this treatment the layers of the multi-leaf collimator are moved while the treatment is being given. This method is likely to achieve even more precise shaping of the treatment beams and allows the dose of radiotherapy to be constant over the whole treatment area.

Although research studies have shown that conformal radiotherapy and intensity modulated radiotherapy may reduce the side effects of radiotherapy treatment, it is possible that shaping the treatment area so precisely could stop microscopic cancer cells just outside the treatment area being destroyed. This means that the risk of the cancer coming back in the future may be higher with these specialized radiotherapy techniques.

Scientists also are looking for ways to increase the effectiveness of radiation therapy. Two types of investigational drugs are being studied for their effect on cells undergoing radiation. Radiosensitizers make the tumor cells more likely to be damaged, and radioprotectors protect normal tissues from the effects of radiation. Hyperthermia, the use of heat, is also being studied for its effectiveness in sensitizing tissue to radiation.

3. Immunotherapy

In the context of cancer treatment, immunotherapeutics, generally, rely on the use of immune effector cells and molecules to target and destroy cancer cells. Trastuzumab (Herceptin™) is such an example. The immune effector may be, for example, an antibody specific for some marker on the surface of a tumor cell. The antibody alone may serve as an effector of therapy or it may recruit other cells to actually affect cell killing. The antibody also may be conjugated to a drug or toxin (chemotherapeutic, radionuclide, ricin A chain, cholera toxin, pertussis toxin, *etc.*) and serve merely as a targeting agent. Alternatively, the effector may be a lymphocyte carrying a surface molecule that interacts, either directly or indirectly, with a tumor cell target. Various effector cells include cytotoxic T cells and NK cells. The combination of therapeutic modalities, *i.e.*, direct cytotoxic activity and inhibition or reduction of ErbB2 would provide therapeutic benefit in the treatment of ErbB2 overexpressing cancers.

Another immunotherapy could also be used as part of a combined therapy with gen silencing therapy discussed above. In one aspect of immunotherapy, the tumor cell must bear some marker that is amenable to targeting, *i.e.*, is not present on the majority of other cells. Many tumor markers exist and any of these may be suitable for targeting in the context of the present invention. Common tumor markers include carcinoembryonic antigen, prostate specific antigen, urinary tumor associated antigen, fetal antigen, tyrosinase (p97), gp68, TAG-72, HMFG, Sialyl Lewis Antigen, MucA, MucB, PLAP, estrogen receptor, laminin receptor, erb B and p155. An alternative aspect of immunotherapy is to combine anticancer effects with immune stimulatory effects. Immune stimulating molecules also exist including: cytokines such as IL-2, IL-4, IL-12, GM-CSF, γ-IFN, chemokines such as MIP-1, MCP-1, IL-8 and growth factors such as FLT3 ligand. Combining immune stimulating molecules, either as proteins or using gene delivery in combination with a tumor suppressor has been shown to enhance anti-tumor effects (Ju *et al.*, 2000). Moreover, antibodies against any of these compounds can be used to target the anti-cancer agents discussed herein.

Examples of immunotherapies currently under investigation or in use are immune adjuvants *e.g.*, *Mycobacterium bovis*, *Plasmodium falciparum*, dinitrochlorobenzene and aromatic compounds (U.S. Patents 5,801,005 and 5,739,169; Hui and Hashimoto, 1998; Christodoulides *et al.*, 1998), cytokine therapy, *e.g.*, interferons α, β, and γ; IL-1, GM-CSF and TNF (Bukowski *et al.*, 1998; Davidson *et al.*, 1998; Hellstrand *et al.*, 1998) gene therapy, *e.g.*, TNF, IL-1, IL-2, p53 (Qin *et al.*, 1998; Austin-Ward and Villaseca, 1998; U.S. Patents 5,830,880 and 5,846,945) and monoclonal antibodies, *e.g.*, anti-ganglioside GM2, anti-HER-2, anti-p185 (Pietras *et al.*, 1998; Hanibuchi *et al.*, 1998; U.S. Patent 5,824,311). It is contemplated that one or more anti-cancer therapies may be employed with the gene silencing therapies described herein.

In active immunotherapy, an antigenic peptide, polypeptide or protein, or an autologous or allogenic tumor cell composition or "vaccine" is administered, generally with a distinct bacterial adjuvant (Ravindranath and Morton, 1991; Morton *et al.*, 1992; Mitchell *et al.*, 1990; Mitchell *et al.*, 1993).

In adoptive immunotherapy, the patient's circulating lymphocytes, or tumor infiltrated lymphocytes, are isolated *in vitro*, activated by lymphokines such as IL-2 or transduced with genes for tumor necrosis, and readministered (Rosenberg *et al.*, 1988; 1989).

4. Surgery

Approximately 60% of persons with cancer will undergo surgery of some type, which includes preventative, diagnostic or staging, curative, and palliative surgery. Curative surgery is a cancer treatment that may be used in conjunction with other therapies, such as the treatment of the present invention, chemotherapy, radiotherapy, hormonal therapy, gene therapy, immunotherapy and/or alternative therapies.

Curative surgery includes resection in which all or part of cancerous tissue is physically removed, excised, and/or destroyed. Tumor resection refers to physical removal of at least part of a tumor. In addition to tumor resection, treatment by surgery includes laser surgery, cryosurgery, electrosurgery, and microscopically controlled surgery (Mohs' surgery). It is further contemplated that the present invention may be used in conjunction with removal of superficial cancers, precancers, or incidental amounts of normal tissue.

Upon excision of part or all of cancerous cells, tissue, or tumor, a cavity may be formed in the body. Treatment may be accomplished by perfusion, direct injection or local application of the area with an additional anti-cancer therapy. Such treatment may be

repeated, for example, every 1, 2, 3, 4, 5, 6, or 7 days, or every 1, 2, 3, 4, and 5 weeks or every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months. These treatments may be of varying dosages as well.

5. Gene Therapy

In yet another embodiment, the secondary treatment is a gene therapy in which a therapeutic polynucleotide is administered before, after, or at the same time as an miRNA is administered. Delivery of an miRNA in conjunction with a vector encoding one of the following gene products may have a combined anti-hyperproliferative effect on target tissues. A variety of proteins are encompassed within the invention, some of which are described below. The proteins that induce cellular proliferation further fall into various categories dependent on function. The tumor suppressor oncogenes function to inhibit excessive cellular proliferation. Apoptosis, or programmed cell death, is an essential process for normal embryonic development, maintaining homeostasis in adult tissues, and suppressing carcinogenesis (Kerr *et al.*, 1972). The Bcl-2 family of proteins and ICE-like proteases have been demonstrated to be important regulators and effectors of apoptosis in other systems. Any of these agents are suitable combination therapies.

6. Other Agents

It is contemplated that other agents may be used with the present invention. These additional agents include immunomodulatory agents, agents that affect the upregulation of cell surface receptors and GAP junctions, cytostatic and differentiation agents, inhibitors of cell adhesion, agents that increase the sensitivity of the hyperproliferative cells to apoptotic inducers, or other biological agents. Immunomodulatory agents include tumor necrosis factor; interferon α, β, and γ; IL-2 and other cytokines; F42K and other cytokine analogs; or MIP-1, MIP-1β, MCP-1, RANTES, and other chemokines. It is further contemplated that the upregulation of cell surface receptors or their ligands such as Fas/Fas ligand, DR4 or DR5/TRAIL (Apo-2 ligand) would potentiate the apoptotic inducing abilities of the present invention by establishment of an autocrine or paracrine effect on hyperproliferative cells. Increases intercellular signaling by elevating the number of GAP junctions would increase the anti-hyperproliferative effects on the neighboring hyperproliferative cell population. In other embodiments, cytostatic or differentiation agents can be used in combination with the present invention to improve the anti-hyerproliferative efficacy of the treatments. Inhibitors

of cell adhesion are contemplated to improve the efficacy of the present invention. Examples of cell adhesion inhibitors are focal adhesion kinase (FAKs) inhibitors and Lovastatin. It is further contemplated that other agents that increase the sensitivity of a hyperproliferative cell to apoptosis, such as the antibody c225, could be used in combination with the present invention to improve the treatment efficacy.

There have been many advances in the therapy of cancer following the introduction of cytotoxic chemotherapeutic drugs. However, one of the consequences of chemotherapy is the development/acquisition of drug-resistant phenotypes and the development of multiple drug resistance. The development of drug resistance remains a major obstacle in the treatment of such tumors and therefore, there is an obvious need for alternative approaches such as gene therapy.

Another form of therapy for use in conjunction with chemotherapy, radiation therapy or biological therapy includes hyperthermia, which is a procedure in which a patient's tissue is exposed to high temperatures (up to 106°F). External or internal heating devices may be involved in the application of local, regional, or whole-body hyperthermia. Local hyperthermia involves the application of heat to a small area, such as a tumor. Heat may be generated externally with high-frequency waves targeting a tumor from a device outside the body. Internal heat may involve a sterile probe, including thin, heated wires or hollow tubes filled with warm water, implanted microwave antennae, or radiofrequency electrodes.

A patient's organ or a limb is heated for regional therapy, which is accomplished using devices that produce high energy, such as magnets. Alternatively, some of the patient's blood may be removed and heated before being perfused into an area that will be internally heated. Whole-body heating may also be implemented in cases where cancer has spread throughout the body. Warm-water blankets, hot wax, inductive coils, and thermal chambers may be used for this purpose.

E. Dosage

An miRNA, mimic or inhibitory oligonucleotide can be administered at a unit dose less than about 75 mg per kg of bodyweight, or less than about 70, 60, 50, 40, 30, 20, 10, 5, 2, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, or 0.0005 mg per kg of bodyweight, and less than 200 nmol of antagomir (*e.g.*, about 4.4 x 10^{16} copies) per kg of bodyweight, or less than 1500, 750, 300, 150, 75, 15, 7.5, 1.5, 0.75, 0.15, 0.075, 0.015, 0.0075, 0.0015, 0.00075, 0.00015 nmol of antagomir per kg of bodyweight. The unit dose, for example, can be administered by

injection (*e.g.*, intravenous or intramuscular, intrathecally, intratumorally or directly into an organ), inhalation, or a topical application.

Delivery of an miRNA, mimic or inhibitory oligonucleotide directly to an organ can be at a dosage on the order of about 0.00001 mg to about 3 mg per organ, or particularly about 0.0001-0.001 mg per organ, about 0.03-3.0 mg per organ, about 0.1-3.0 mg per organ or about 0.3-3.0 mg per organ.

Significant modulation of target gene expression may be achieved using nanomolar/submicromolar or picomolar/subnamomolar concentrations of the oligonucleotide, and it is typical to use the lowest concentration possible to achieve the desired resultant increased synthesis, *e.g.*, oligonucleotide concentrations in the 1-100 nM range are contemplated; more particularly, the concentration is in the 1-50 nM, 1-25 nM, 1-10 nM, or picomolar range. In particular embodiments, the contacting step is implemented by contacting the cell with a composition consisting essentially of the oligonucleotide.

In one embodiment, the unit dose is administered once a day, *e.g.*, or less frequently less than or at about every 2, 4, 8 or 30 days. In another embodiment, the unit dose is not administered with a frequency (*e.g.*, not a regular frequency). For example, the unit dose may be administered a single time. Because oligonucleotide agent can persist for several days after administering, in many instances, it is possible to administer the composition with a frequency of less than once per day, or, for some instances, only once for the entire therapeutic regimen.

An miRNA, mimic or inhibitory oligonucleotide featured in the invention can be administered in a single dose or in multiple doses. Where the administration of the miRNA, mimic or inhibitory oligonucleotide is by infusion, the infusion can be a single sustained dose or can be delivered by multiple infusions. Injection of the miRNA, mimic or inhibitory oligonucleotide can be directly into the tissue at or near the site of interest. Multiple injections of can be made into the tissue at or near the site.

In a particular dosage regimen, the miRNA, mimic or inhibitory oligonucleotide is injected at or near a disease site once a day for seven days, for example, into a tumor, a tumor bed, or tumor vasculature. Where a dosage regimen comprises multiple administrations, it is understood that the effective amount of the miRNA, mimic or inhibitory oligonucleotide administered to the subject can include the total amount of miRNA, mimic or inhibitory oligonucleotide administered over the entire dosage regimen. One skilled in the art will appreciate that the exact individual dosages may be adjusted somewhat depending on a variety of factors, including the specific antagomir being administered, the time of

administration, the route of administration, the nature of the formulation, the rate of excretion, the particular disorder being treated, the severity of the disorder, the pharmacodynamics of the oligonucleotide agent, and the age, sex, weight, and general health of the patient. Wide variations in the necessary dosage level are to be expected in view of the differing efficiencies of the various routes of administration. Variations in these dosage levels can be adjusted using standard empirical routines of optimization, which are well-known in the art. The precise therapeutically effective dosage levels and patterns can be determined by the attending physician in consideration of the above-identified factors.

In one embodiment, a subject is administered an initial dose, and one or more maintenance doses of an miRNA, mimic or inhibitory oligonucleotide. The maintenance dose or doses are generally lower than the initial dose, *e.g.*, one-half less of the initial dose. The maintenance doses are generally administered no more than once every 5, 10, or 30 days. Further, the treatment regimen may last for a period of time which will vary depending upon the nature of the particular disease, its severity and the overall condition of the patient. Following treatment, the patient can be monitored for changes in his condition and for alleviation of the symptoms of the disease state. The dosage of the compound may either be increased in the event the patient does not respond significantly to current dosage levels, or the dose may be decreased if an alleviation of the symptoms of the disease state is observed, if the disease state has been ablated, or if undesired side-effects are observed.

The effective dose can be administered two or more doses, as desired or considered appropriate under the specific circumstances. If desired to facilitate repeated or frequent infusions, implantation of a delivery device, *e.g.*, a pump, semi-permanent stent (*e.g.*, intravenous, intraperitoneal, intracisternal or intracapsular), or reservoir may be advisable.

Certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. It will also be appreciated that the effective dosage of the antagomir used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result and become apparent from the results of diagnostic assays. For example, the subject can be monitored after administering an antagomir composition. Based on information from the monitoring, an additional amount of the antagomir composition can be administered.

Dosing is dependent on severity and responsiveness of the disease condition to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of disease state is achieved. Optimal dosing schedules can be

calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual compounds, and can generally be estimated based on EC_{50}'s found to be effective in *in vitro* and *in vivo* animal models.

VI. EXAMPLES

The following examples are included to demonstrate particular embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute particular modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1

The inventors hypothesized that regulation of miR expression by EWS/Ets fusions represents an important mechanism controlling oncogenesis in Ewing Sarcoma. In order to identify candidate microRNAs (miRs) involved in EWS/Ets-mediated oncogenesis, they employed an EWS/Fli1 stable silencing system in Ewing Sarcoma cells, as such systems are well established and currently represent the optimal cellular context for the study of Ewing Sarcoma biology (Kinsey *et al.*, 2006; Smith *et al.*, 2006). Similar to the approach of others (Smith *et al.*, 2006), the inventors employed shRNAs targeted to the 3' end of Fli1 to target the EWS-Fli1 fusion (the unrearranged Fli1 gene is not expressed in Ewing Sarcoma), using a lentiviral delivery system. As shown in FIG. 1A, the inventors achieved potent knock-down of EWS/Fli1 using 2 such shRNAs (EFsh1 and EFsh1), compared to the off-target control (shRNA to EGFP). Importantly, this knock-down was sufficient to downregulate the expression of two established EWS-Fli1 target genes, Nkx2.2 and NR0B1 (Kinsey *et al.*, 2006; Smith *et al.*, 2006) (FIG. 1B). To further ensure the specificity of EWS/Fli1 silencing, the inventors additionally adapted the retroviral stable knock-down system of Smith *et al.*, which employs a different 3'Fli-targeting shRNA (EF2) and a different control shRNA (luciferase) (Smith *et al.*, 2006). This system effected robust EWS/Fli1 silencing of similar

potency to a lentiviral system (FIG. 1C). Thus, the inventors achieved stable and specific knock-down of the EWS-Fli1 oncoprotein in Ewing Sarcoma cells.

In order to identify EWS/Fli1-regulated miRs, the inventors performed miR microarray screening for miRs differentially expressed between A673 Ewing Sarcoma cells with stably silenced EWS/Fli1 and control. From miRs differentially expressed by array (Tables 2-3), they initially chose to focus on EWS/Fli1-repressed miRs (*i.e.*, those upregulated upon EWS/Fli1 silencing), as these represented candidate tumor suppressors and thus novel anti-tumor agents in Ewing Sarcoma. Of the 30 miRs upregulated upon EWS/Fli1 knockdown with a false discovery rate (FDR) less than or equal to 0.01, the inventors further focused on those showing the greatest change (at least 1.5-fold by array). Since individual miRs have many mRNA targets (Bartel, 2009; Ghildiyal & Zamore, 2009), they next queried whether any of these miRs shared common target pathways, using the DIANA-mirPath algorithm and Target Scan 5 (diana.cslab.ece.ntua.gr). Strikingly, 7 of the 13 miRs most highly changed by the above criteria showed predicted pro-oncogenic targets in the IGF-1 signaling pathway, including IGF-1 itself and its receptor, IGF-1R. Given the importance of this pathway in Ewing Sarcoma oncogenesis, these miRs, namely miRs 22, 100, 125b, 221, 222, 27a and 29a, were selected for further analysis as potential mediators of EWS/Fli1 oncogenesis through the IGF pathway (FIG. 1D).

First, to verify the array results, the inventors quantified relative expression levels of the miRs using qRT-PCR in all stable knock-down cell lines (shEF1, shEF2 and EF2) and controls (EGFP and luc). All EWS/Fli1-targeted shRNAs showed increased levels of these miRs relative to matched off-target controls (FIG. 1D), confirming that the changes observed were specific to EWS/Fli1 silencing. They next asked whether the miRs are expressed at lower levels in Ewing Sarcoma cell lines compared to human mesenchymal progenitor cells (hMPCs), the putative cell of origin of Ewing Sarcoma (Riggi & Stamenkovic, 2007), as would be expected of tumor suppressors. As shown in FIG. 1E, all of the miRs showed lower expression in a panel of 5 Ewing Sarcoma cell lines, compared to 2 different hMPC cell lines, supporting a candidate tumor suppressive role. Interestingly, the differences between Ewing Sarcoma cell lines and hMPCs were even greater than those due to EWS/Fli1 silencing, presumably in part reflecting the absence, rather than the reduction in levels, of EWS/Fli1.

The control of miR levels in the cell has a transcriptional component as well as post-transcriptional processing components, all of which are subject to regulation (Davis & Hata, 2009; Kim *et al.*, 2009b; Winter *et al.*, 2009). Since EWS/Fli1 is a transcriptional regulator, the inventors hypothesized that the observed changes in the levels of the mature miRs upon

EWS/Fli1 silencing could be due to regulation at the level of the miR primary transcript (pri-miR). As shown in FIG. 2A, this hypothesis was supported by measurement of pri-miR levels, all of which were increased in A673 cells with stably silenced EWS/Fli1 compared to controls. To test this hypothesis further, the inventors ectopically expressed EWS/Fli1 in 293FT cells, which do not harbor EWS/Fli1, but tolerate its expression (FIG. 2B). As shown in FIGS. 2C-D, EWS/Fli1 expression resulted in downregulation of both the primary transcript and the mature form, respectively, of miRs 100, 125b, 22, 221 and 27a. These data support transcriptional repression as the mechanism responsible for the downregulation of these miRs by EWS/Fli1. The lack of regulation of miR-29a by EWS/Fli1 in 293 cells may reflect the absence of a required cofactor, or downstream mediator. Target repression by EWS/Fli1 in Ewing Sarcoma can occur by direct or indirect mechanisms (Jedlicka, 2010). To determine if a direct mechanism is involved, the inventors searched for candidate EWS/Fli1 DNA-binding sites in the miR promoter regions and were able to identify at least one site in each (FIG. 2E). The inventors further assayed for EWS/Fli1 binding to the promoter of miR-100, the most regulated miR, using chromatin immunoprecipitation (ChIP). As shown in FIG. 2F, compared to negative (Neg) and positive (NR0B1) controls, the inventors observed enrichment of EWS/Fli1 binding to the more proximal upstream (amplicons -1324 and -510) and immediate downstream (amplicon +510) regions of the miR-100 promoter. Since these regions contain the candidate EWS/Fli1 sites (-660 and +120 (FIG. 2E)), this finding suggests that miR-100 downregulation may involve direct transcriptional repression by EWS/Fli1. The modest degree of enrichment, however, suggests that other, indirect, mechanisms may also play a role. Taken together, these data indicate that miR downregulation by EWS/Fli1 in Ewing Sarcoma involves transcriptional repression, and that, for at least some miRs, this may involve direct repression by EWS/Fli1 itself.

The inventors next examined the effects of the miRs on regulation of their predicted targets in the IGF signaling pathway. Activation of the IGF signaling pathway in Ewing Sarcoma has an important autocrine component. Indeed, expression of IGF-1 itself is positively regulated by the EWS/Fli1 oncoprotein (Cironi *et al.*, 2008; Herrero-Martin *et al.*, 2009; Riggi *et al.*, 2005). Strikingly, four of the miRs, namely miRs 27a, 29a and 221/222, were predicted to target the IGF-1 3'UTR. They thus tested whether overexpression of these miRs has an effect on IGF-1 expression. As shown in FIG. 3A, overexpression miR27a resulted in a robust (~30%) decrease in IGF-1 production per cell. Overexpression of miRs 29a and 221 did not lead to similar changes (data not shown). Thus, miR-27a negatively regulates IGF-1 expression in Ewing Sarcoma. Target prediction algorithms also identified

the IGF-1 receptor (IGF-1R) as a candidate target of miRs 100 and 22. As shown in FIG. 3B, miR-100 overexpression in A673 cells resulted in a decrease in the IGF-1R protein level, as determined by immunoblotting, compared to control.

Overexpression of miR-22 did not affect IGF-1R protein levels (data not shown). MiR-100 has previously also been shown to also target mTOR (mammalian/mechanistic Target of Rapamycin), a downstream mediator of IGF signaling with pro-oncogenic functions (Ciuffreda *et al.,* 2010), in other systems (Nagaraja *et al.*, 2010; Wang *et al.*, 2008a). The inventors thus quantified mTOR levels in the same miR-100 overexpression experiments, and were able to verify downregulation in A673 cells (FIG. 3C). Target prediction algorithms also identified RSK1 (Ribosomal Protein S6 Kinase A1), a substrate of Erk in the MAPK arm of the IGF signaling pathway with pro-oncogenic functions (Carriere *et al.*, 2008), as a candidate target for miR-125b. As shown in FIG. 3D, overexpression of miR-125b in A673 cells resulted in a downregulation of RSK1 protein levels. In order to verify that regulation of the above targets was through a direct mechanism, the inventors tested miR regulation of the predicted 3'UTR sites (FIG. 3E) using the siCHECK dual luciferase reporter system. Two previous studies have demonstrated direct regulation of the mTOR 3'UTR by miR-100 (Nagaraja *et al.*, 2010; Wang *et al.*, 2008a); the inventors thus focused their studies on the novel miR-target pairs. As shown in FIG. 3F, compared to negative control, miR-100 robustly inhibited reporter expression through its predicted site in the IGF-1R 3'UTR, but had no effect when the miR seed sequence was mutated. Similarly, miR-125b specifically inhibited reporter activity through its predicted site in the RSK1 3'UTR (FIG. 3F). Surprisingly, the inventors were unable to demonstrate direct regulation of the IGF-1 3'UTR by miR-27a (FIG. 3F), suggesting that the regulation of IGF-1 protein levels occurs via other mechanisms. Thus, a number of the EWS/Fli1-repressed miRs converge on prooncogenic targets in the IGF signaling pathway, with at least some exerting their effects directly through 3'UTR interactions.

The IGF signaling pathway plays a critical pro-oncogenic role in Ewing Sarcoma, including regulation of cell growth (Scotlandi *et al.*, 1996; Yee *et al.*, 1990). The inventors therefore overexpressed each of the EF-repressed miRs in A673 Ewing Sarcoma cells, and determined effects on cell growth. MiR overexpression was achieved by transient transfection of miR mimics, which the inventors found to be the most effective means to approximate miR expression differences between Ewing Sarcoma cell lines and MPCs (FIG. 2B; up to 30 to 76-fold difference). As shown in FIG. 4A, overexpression of each miR resulted in

inhibition of cell growth, with miRs 22, 125b and 221 showing the strongest effect. MiR overexpression levels in these experiments are shown in FIG. 4B.

In order to further probe the function of miR-100, the most strongly EWS/Fli1-regulated miR in the group (FIG. 2A), the inventors generated A673 cells stably overexpressing miR-100 using a lentiviral delivery system of a miR-100 precursor. Using this system, they were able to achieve ~6-7-fold overexpression (FIG. 4C), which was less than the difference between Ewing Sarcoma cell lines and MPCs, as well as A673 cells with strong EWS/Fli1 knock-down and controls. Strikingly, even at such modest overexpression levels, miR-100 robustly inhibited anchorage-independent cell growth, as assayed by colony formation in soft agar, by ~35% (FIGS. 4D and 4E). Taken together, these findings support tumor suppressive functions for miRs 22, 100, 125b, 221, 27a and 29a in Ewing Sarcoma, in part through negative regulation of pro-oncogenic components of the IGF-1 signaling pathway.

The inventors further examined published data on EWS/Fli1-regulated genes for possible additional relevant targets of these miRs. Kinsey *et al.* (2006) identified a group of 34 genes upregulated by EWS/Fli1 in three different Ewing Sarcoma cell lines. When these were subjected to a miR target prediction algorithm (TargetScan), 9 of the genes emerged as candidate targets of miRs 22, 100, 125b, 221/222, 27a and 29a (Table 1). Moreover, 6 of the genes were predicted to be targeted by a single member of this group, miR-125b. One of these target genes, GSTM4, has been demonstrated to have a pro-oncogenic function downstream of EWS/Fli1 in Ewing Sarcoma (Luo *et al.*, 2009). Thus, miR repression may represent one mechanism of induction of these target genes by EWS/Fli1. Moreover, repression of these EWS/Fli1 targets may represent an additional mechanism by which these miRs exert tumor suppressive effects.

Table 1 – EWS/Fli1-Upregulated Genes from Kinsey *et al.* (Mol. Cancer Res. 2006) Predicted to be Targeted by EWS-/Fli1-Downregulated miRs from Screen

	100	125b	22	221/222	27a	29a
MKI67		X		X		
NMI						X
GSTM4		X				
CCDC21	X	X				
FDX1		X				
PRUNE				X		
DICER1		X		X		
SEPHS1			X			
SSX2		X		X		

Table 2 - miRs Upregulated Upon EWS/Fli1 Silencing

miR	FDR	fold-change	miR sequence	SEQ ID NO
hsa-miR-146a	0.00033	3.4	ugagaacugaauuccauggguu	SEQ ID NO:1
hsa-miR-21	0.00903	2.8		
hsa-miR-22	0.00045	2.4	aagcugccaguugaagaacugu	SEQ ID NO:2
hsa-miR-100	0.00171	2.1	aacccguagauccgaacuugug	SEQ ID NO:3
hsa-miR-125b	0.00239	2.1	ucccugagacccuaacuuguga	SEQ ID NO:4
hsa-miR-146b-5p	0.00068	2		
hsa-miR-222	0.00275	1.9	agcuacaucuggcuacugggu	SEQ ID NO:5
hsa-miR-584	0.01125	1.8		
hsa-miR-221	0.00406	1.8	agcuacauugucugcuggguuuc	SEQ ID NO:6
hsa-miR-199a/b-3p	0.01343	1.6		
hsa-miR-29a	0.00613	1.6	uagcaccaucugaaaucgguua	SEQ ID NO:7
hsa-miR-27a/b	0.01343	1.5	uucacaguggcuaaguuccgc (27a)	SEQ ID NO:8
hsa-miR-193b	0.00239	1.4	cgggguuuugagggcgagauga	SEQ ID NO:9
hsa-miR-99a	0.00193	1.4		
hsa-miR-549	0.00275	1.3		
hsa-miR-95	0.00275	1.3		
hsa-miR-99b	0.0036	1.3		
hsa-miR-127-3p	0.00459	1.3		
hsa-miR-941	0.00825	1.3		
hsa-miR-203	0.01125	1.3		
hsa-miR-574-3p	0.00925	1.2		
hsa-miR-186*	0.00372	1.2		
hsa-miR-493	0.00707	1.2		
hsa-miR-922	0.0118	1.2		
hsa-miR-30a	0.00825	1.2		
hsa-miR-603	0.0121	1.2		
hsa-miR-125b-1*	0.01415	1.1		
hsa-miR-214*	0.00905	1.1		
hsa-miR-649	0.00406	1.1		
has-miR-223	0.00276	1.1	cguguauuugacaagcugaguu	SEQ ID NO:10

Table 3 - miRs Down-Regulated Upon EWS/Fli1 Silencing

miR	FDR	fold-change	miR sequence	SEQ ID NO
hsa-miR-93	0.00275	-2	caaagugcuguucgugcagguag	SEQ ID NO:11
hsa-miR-484	0.0004	-2		
hsa-miR-106a/17	0.0044 2	-1.9	aaaagugcuuacagugcagguag (106a) caaagugcuuacagugcagguag (17)	SEQ ID NO: 12 SEQ ID NO:13
hsa-miR-92a	0.00033	-1.9	uauugcacuugucccggccugu	SEQ ID NO:14
hsa-miR-25	0.00239	-1.8	cauugcacuugucucggucuga	SEQ ID NO:15
hsa-miR-20a	0.00956	-1.8	uaaagugcuuauagugcagguag	SEQ ID NO:16
hsa-miR-15b	0.00275	-1.7		
hsa-miR-92b	0.00059	-1.7	uauugcacucgucccggccucc	SEQ ID NO:17
hsa-miR-103/107	0.00275	-1.5		
hsa-miR-324-5p	0.00406	-1.5		
hsa-miR-423-3p	0.00239	-1.5		
hsa-miR-130a	0.01125	-1.4		
hsa-miR-324-3p	0.00427	-1.4		
hsa-miR-320	0.00059	-1.3		
hsa-let-7d	0.00613	-1.3		
hsa-miR-106b*	0.00059	-1.3		
hsa-miR-130b	0.00239	-1.3		
hsa-miR-760	0.01327	-1.3		
hsa-miR-378	0.00275	-1.3		
hsa-miR-532-3p	0.00275	-1.3		
hsa-miR-665	0.00459	-1.2		
hsa-miR-886-5p	0.00595	-1.2		
hsa-miR-574-5p	0.00527	-1.2		
hsa-miR-940	0.00956	-1.2		
hsa-miR-296-3p	0.00825	-1.2		
hsa-miR-186	0.01103	-1.2		
hsa-miR-181d	0.01322	-1.2		
hsa-miR-575	0.01475	-1.1		
hsa-miR-604	0.00406	-1.1		
hsa-miR-345	0.01103	-1.1		
hsa-miR-875-3p	0.00066	-1.1		

FIGS. 7A-C show *in vivo* studies using miR-146a in mice bearing Ewing Sarcoma. FIG. 7A shows relative overexpression of miR-146a in cell lines and tumors arising therefrom. Only modest reduction in expression is seen in the tumors. FIG. 7B shows that more animals remain tumor free for longer periods of time when the tumors express miR-146a. FIG. 7C shows a dramatic reduction in tumor weight when the tumors express miR-146a. FIG. 8 shows the effect of overexpressing miR-193b and miR-223 in a Ewing Sarcoma cell line. Compared to non-targeting miR control, miRs 193b and 223 each result in a marked reduction in colony formation in a clonogenic assay, a stringent assay of cell growth approximating *in vivo* tumor formation.

* *

All of the methods and apparatus disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of particular embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and apparatus and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

VII. REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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PCT Appln. WO 9743450
PCT Appln. WO 9839352
PCT Appln. WO 9914226
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CLAIMS

1. A method of diagnosing Ewing sarcoma in a subject comprising:

   (a) obtaining a sample from said subject; and

   (b) assessing said sample for one or more miRNAs selected from the group consisting of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, miR-193b, miR-223, miR-146a, miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a and miR-92b;

   wherein a decreased level of one or more of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, miR-193b, miR-223, or miR-146a, or an increased level of one or more of miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a or miR-92b, as compared to a sample from normal subject, indicates that said subject has a Ewing sarcoma.

2. The method of claim 1, wherein said sample is a biopsy or resected tumor tissue.

3. The method of claim 1, wherein assessing comprises microarray hybridization.

4. The method of claim 1, wherein 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or all 14 of said miRNAs are assessed.

5. The method of claim 1, wherein at least one of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, miR-193b, miR-223, or miR-146a and at least one of miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a or miR-92b are assessed.

6. The method of claim 1 or 5, wherein all of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, miR-193b, miR-223, and miR-146a are assessed.

7. The method of claim 1 or 5, wherein all of miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a and miR-92b are assessed.

8. The method of claim 7, wherein all miRNAs but miR-17 are assessed.

9. The method of claim 7, wherein all miRNAs but miR-17, miR-92a and miR-92b are assessed.

10. The method of claim 1, wherein one or more of miR-25, miR-93, miR-106a, miR-92a, miR-20a or miR-92b are assessed.

11. The method of claim 1, wherein one or more of miR-25, miR-93, miR-106a and miR-20a are assessed.

12. The method of claim 1, wherein either or both of miR-20a and miR-106a are not assessed.

13. The method of claim 1, wherein miR-25 and miR-93 are assessed.

14. The method of claim 1, wherein miR-100, miR-125b, and 29a are not assessed.

15. The method of claim 1, wherein miR-22, miR-221, miR-27a, and miR-146a are assessed.

16. A method of treating a subject with Ewing sarcoma comprising providing to said subject one or more miRNAs selected from the group consisting of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-193b, miR-223, miR-29a, and/or miR-146a and/or an miRNA mimic thereof.

17. The method of claim 16, wherein providing comprises administration into a vein, artery, tumor or tumor vasculature.

18. The method of claim 17, wherein said one or more miRNAs is formulated in a lipid vehicle.

19. The method of claim 16, wherein providing comprises administering to said subject an expression vector that expresses on or more of said miRNAs.

20. The method of claim 19, wherein said expression vector is a viral expression vector.

21. The method of claim 16, wherein one, two, three or four of said miRNAs or mimics thereof is provided.

22. The method of claim 16, wherein each of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-193b, miR-223, miR-29a and miR-146a or mimics thereof are provided.

23. The method of claim 16, wherein one or more of said miRNAs or mimics thereof is provided more than once.

24. The method of claim 22, wherein each of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, miR-193b, miR-223 and miR-146a, or mimics thereof, are provided more than once.

25. The method of claim 16, wherein one, two or all three of miR-100, miR-125b, and 29a are not provided to said subject.

26. A method of treating a subject with Ewing sarcoma comprising providing to said subject one or more antagomirs for an miRNA selected from the group consisting of miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a and/or miR-92b.

27. The method of claim 26, wherein providing comprises administration into a vein, artery, tumor or tumor vasculature.

28. The method of claim 27, wherein said one or more antagomirs is formulated in a lipid vehicle.

29. The method of claim 26, wherein providing comprises administering to said subject an expression vector that expresses on or more of said antagomirs.

30. The method of claim 29, wherein said expression vector is a viral expression vector.

31. The method of claim 26, wherein one, two, three or four of said antagomirs is provided.

32. The method of claim 26, wherein antagomirs for each of miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a and/or miR-92b are provided.

33. The method of claim 26, wherein one or more of said antagomirs is provided more than once.

34. The method of claim 32, wherein antagomirs for each of miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a and/or miR-92b are provided more than once.

35. The method of claim 26, wherein antagomirs for one, two, three or all four of miR-106a, miR-17, miR-92a and miR-92b are not provided to said subject.

36. The method of claim 35, wherein antagomirs for one, two, three, four or all five of miR-106a, miR-17, miR-92a, miR-20a and miR-92b are not provided to said subject.

37. A kit comprising probes for a plurality of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, miR-146a, miR-193b, miR-223, miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a and miR-92b.

38. The kit of claim 37, wherein said kit provides probes for 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or all 14 of said miRNAs.

39. The kit of claim 37, wherein said probes are labeled.

40. The kit of claim 37, further comprising one or more buffers or diluents.

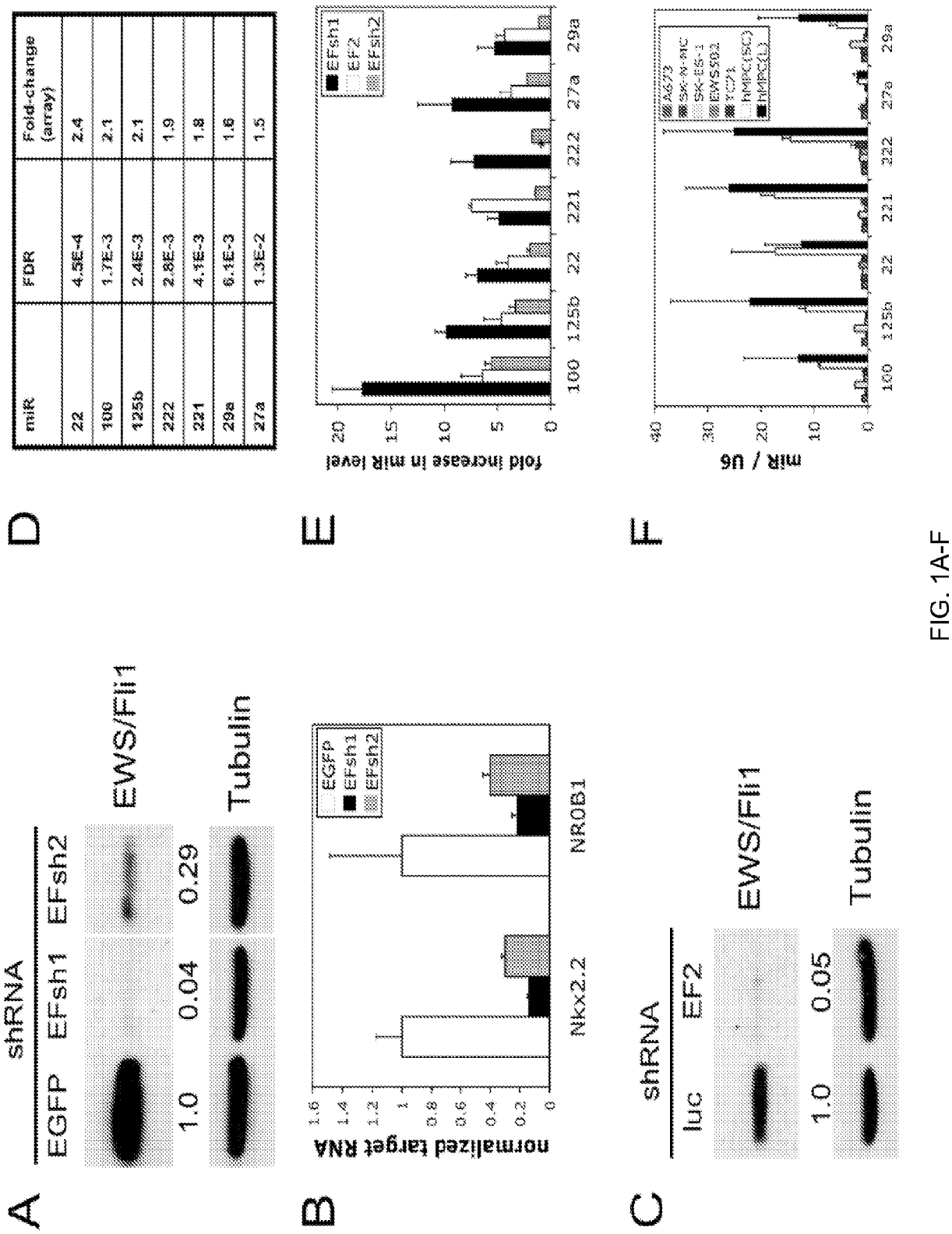
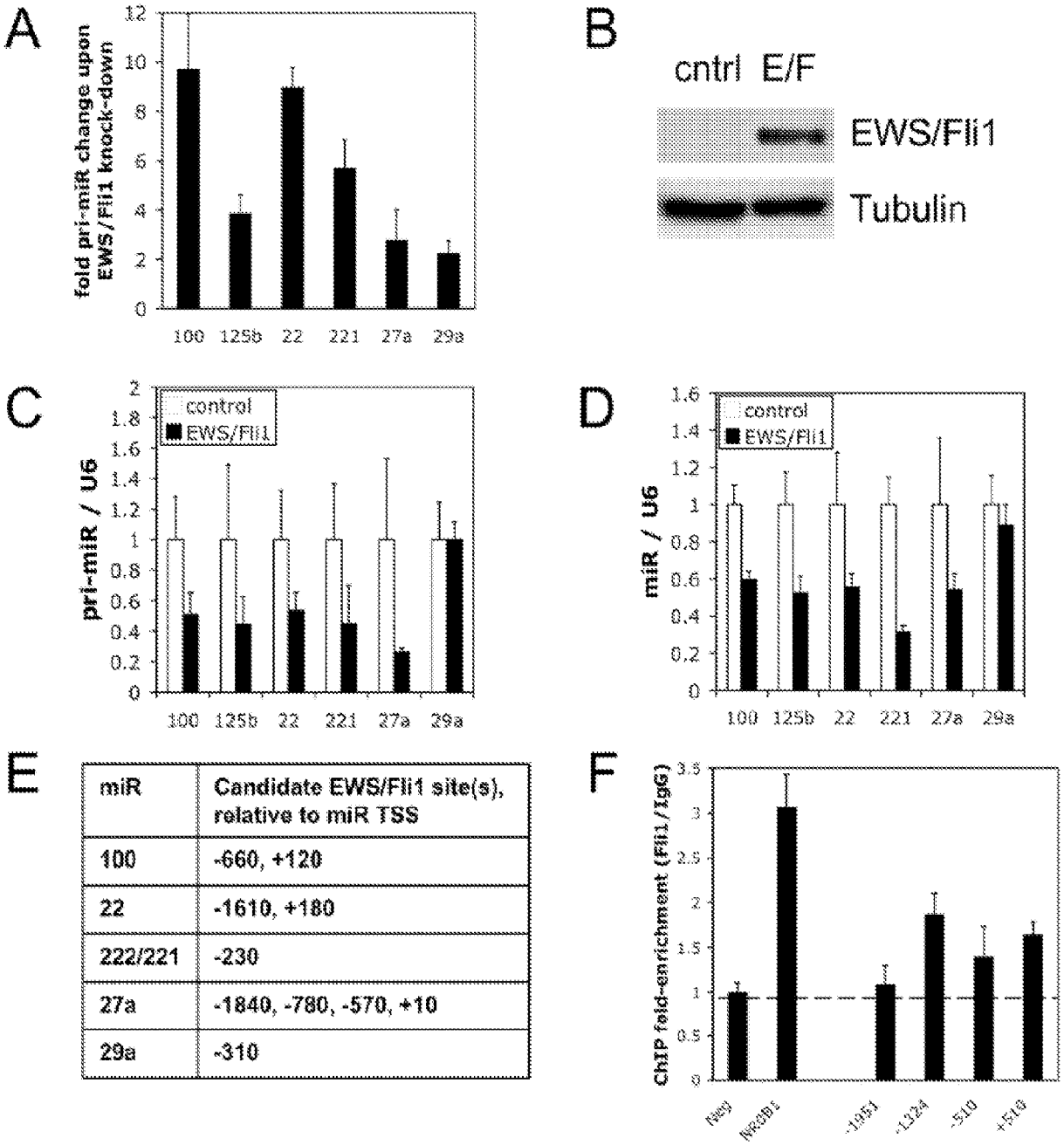

A
shRNA
EGFP
EFsh1
EFsh2
EWS/Fli1
1.0
0.04
0.29
Tubulin
B
EGFP
EFsh1
EFsh2
normalized target RNA
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Nkx2.2
NR0B1
C
shRNA
luc
EF2
EWS/Fli1
1.0
0.05
Tubulin
D
miR
FDR
Fold-change (array)
22
4.5E-4
2.4
100
1.7E-3
2.1
125b
2.4E-3
2.1
222
2.8E-3
1.9
221
4.1E-3
1.8
29a
6.1E-3
1.6
27a
1.3E-2
1.5
E
EFsh1
EF2
EFsh2
fold increase in miR level
20
15
10
5
0
100
125b
22
221
222
27a
29a
F
A673
SK-N-MC
SK-ES-1
EWS502
TC71
RMPC(ESC)
RMPC(LL)
miR / U6
40
30
20
10
0
100
125b
22
221
222
27a
29a

FIG. 1A-F



E

miR	Candidate EWS/Fli1 site(s), relative to miR TSS
100	-660, +120
22	-1610, +180
222/221	-230
27a	-1840, -780, -570, +10
29a	-310

FIG. 2A-F

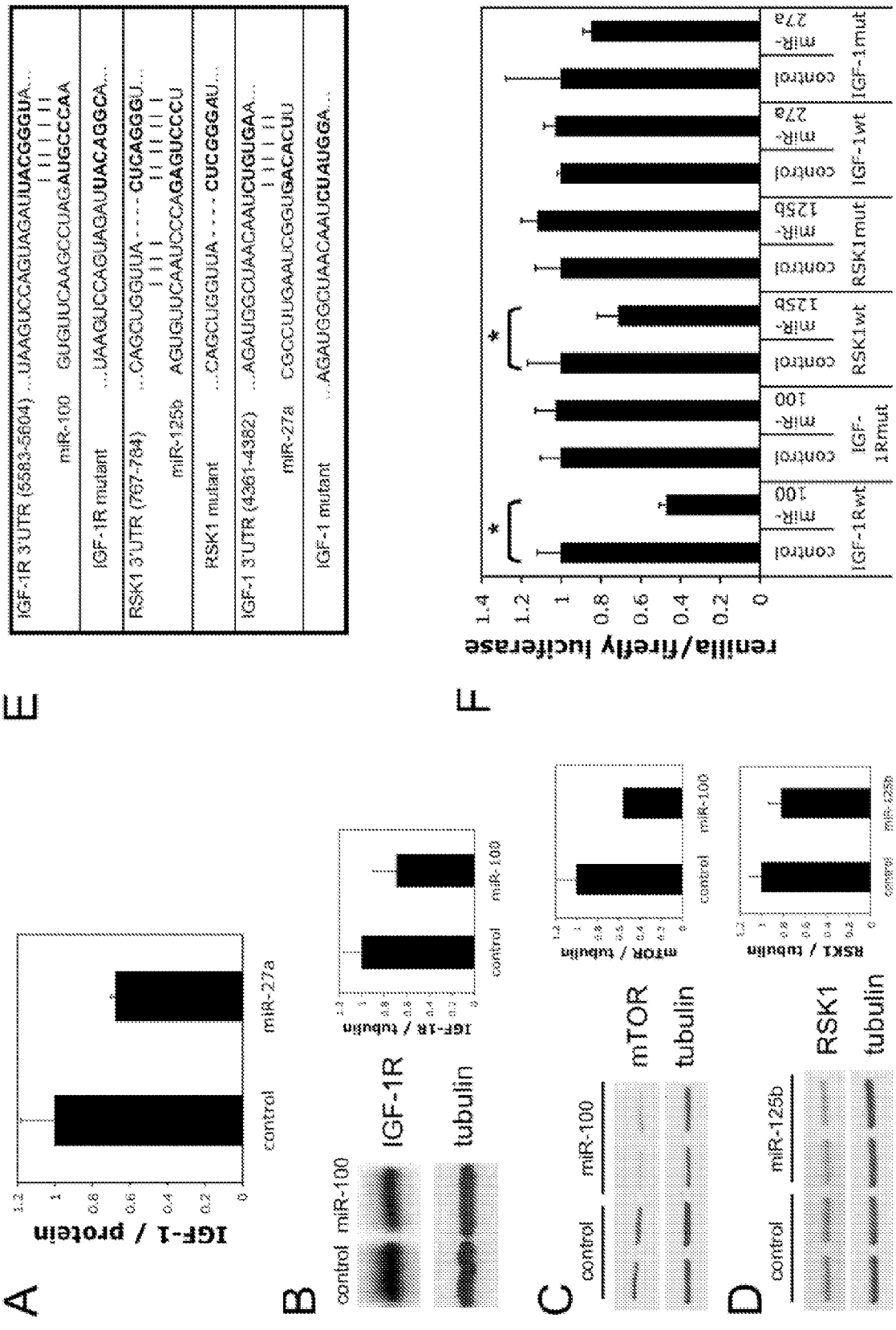

A
IGF-1 / protein
control
miR-27a
B
control miR-100
IGF-1R
tubulin
IGF-1R / tubulin
control
miR-100
C
control
miR-100
mTOR
tubulin
mTOR / tubulin
control
miR-100
D
control
miR-125b
RSK1
tubulin
RSK1 / tubulin
control
miR-125b
E
IGF-1R 3'UTR (5583-5604) ...UAAGUCCAGUAGAUUACGGGUA...
miR-100 GUGUUCAAGCCUAGAUGCCCAA
IGF-1R mutant ...UAAGUCCAGUAGAUUACAGGCA...
RSK1 3'UTR (767-784) ...CAGCUGGUUA - - - - CUCAGGGU...
miR-125b AGUGUUCAAUCCCAGAGUCCCU
RSK1 mutant ...CAGCUGGUUA - - - - CUCGGGAU...
IGF-1 3'UTR (4361-4382) ...AGAUGGCUAACAAUCUGUGAA...
miR-27a CGCCUUGAAUCCGUGACACUU
IGF-1 mutant ...AGAUGGCUAACAAUCUAUGGA...
F
renilla/firefly luciferase
control
miR-100
IGF-1Rwt
control
miR-100
IGF-1Rmut
control
miR-125b
RSK1wt
control
miR-125b
RSK1mut
control
miR-27a
IGF-1wt
control
miR-27a
IGF-1mut

FIG. 3A-F

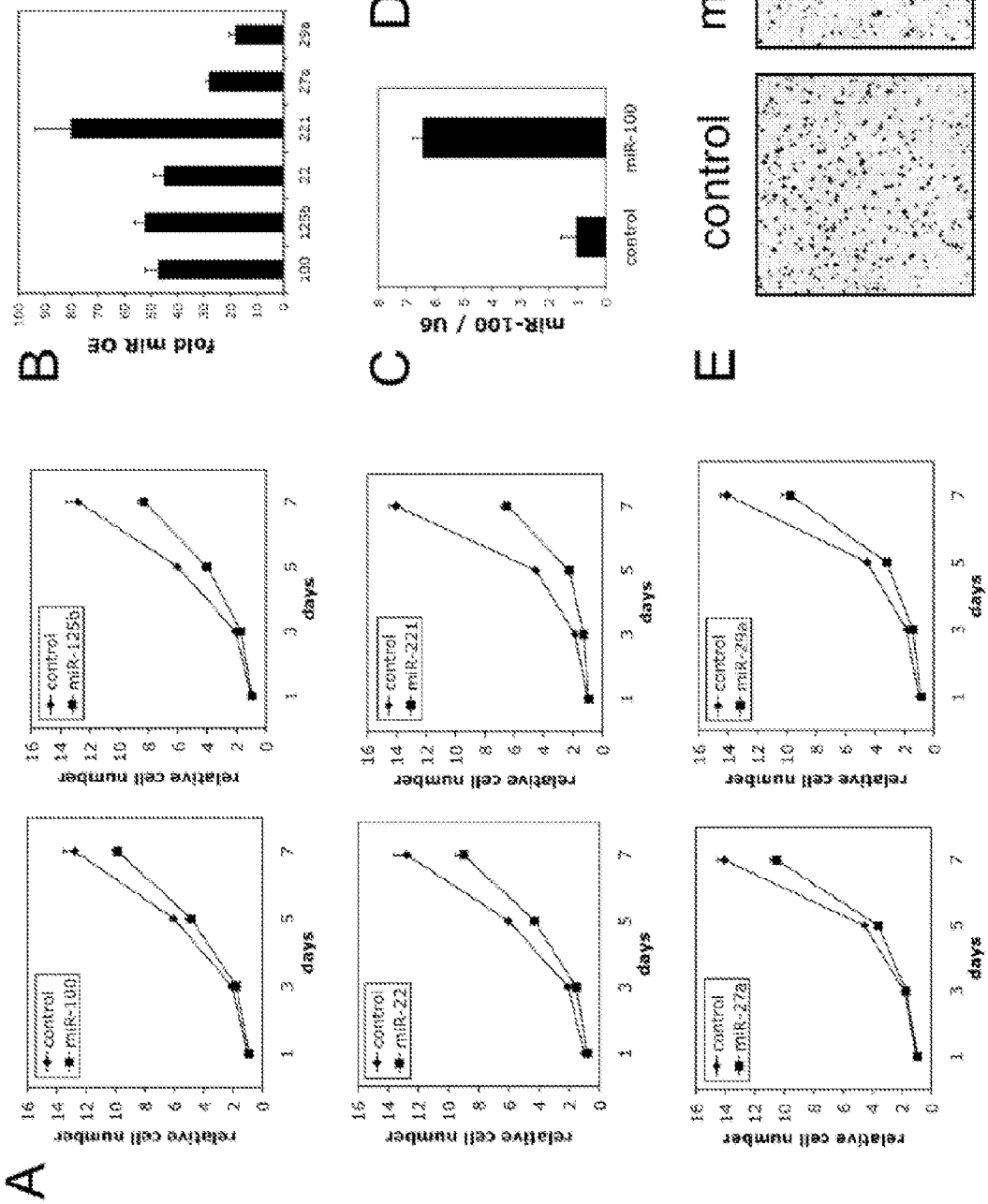

A
B
C
D
E
relative cell number
days
control
miR-100
miR-125b
miR-22
miR-221
miR-27a
miR-292a
fold mir OE
miR-100 / U6
number of colonies
control
miR-100
miR-125b
miR-221
miR-27a
miR-292a
control
miR-100

FIG. 4A-E

miRs upregulated upon EWS/Fli1 silencing

miR	FDR	fold-change
hsa-miR-146a	0.00033	3.4
hsa-miR-21	0.00903	2.8
hsa-miR-22	0.00045	2.4
hsa-miR-100	0.00171	2.1
hsa-miR-125b	0.00239	2.1
hsa-miR-146b-5p	0.00068	2
hsa-miR-222	0.00275	1.9
hsa-miR-584	0.01125	1.8
hsa-miR-221	0.00406	1.8
hsa-miR-199a-3p;hsa-miR-199b-3p	0.01343	1.6
hsa-miR-29a	0.00613	1.6
hsa-miR-27a;hsa-miR-27b	0.01343	1.5
hsa-miR-193b	0.00239	1.4
hsa-miR-99a	0.00193	1.4
hsa-miR-549	0.00275	1.3
hsa-miR-95	0.00275	1.3
hsa-miR-99b	0.0036	1.3
hsa-miR-127-3p	0.00459	1.3
hsa-miR-941	0.00825	1.3
hsa-miR-203	0.01125	1.3
hsa-miR-574-3p	0.00925	1.2
hsa-miR-186*	0.00372	1.2
hsa-miR-493	0.00707	1.2
hsa-miR-922	0.0118	1.2
hsa-miR-30a	0.00825	1.2
hsa-miR-603	0.0121	1.2
hsa-miR-125b-1*	0.01415	1.1
hsa-miR-214*	0.00905	1.1
hsa-miR-649	0.00406	1.1

FIG. 5

miRs downregulated upon EWS/Fli1 silencing

miR	FDR	fold-change
hsa-miR-93	0.00275	-2
hsa-miR-484	0.0004	-2
hsa-miR-106a;hsa-miR-17	0.00442	-1.9
hsa-miR-92a	0.00033	-1.9
hsa-miR-25	0.00239	-1.8
hsa-miR-20a	0.00956	-1.8
hsa-miR-15b	0.00275	-1.7
hsa-miR-92b	0.00059	-1.7
hsa-miR-103;hsa-miR-107	0.00275	-1.5
hsa-miR-324-5p	0.00406	-1.5
hsa-miR-423-3p	0.00239	-1.5
hsa-miR-130a	0.01125	-1.4
hsa-miR-324-3p	0.00427	-1.4
hsa-miR-320	0.00059	-1.3
hsa-let-7d	0.00613	-1.3
hsa-miR-106b*	0.00059	-1.3
hsa-miR-130b	0.00239	-1.3
hsa-miR-760	0.01327	-1.3
hsa-miR-378	0.00275	-1.3
hsa-miR-532-3p	0.00275	-1.3
hsa-miR-665	0.00459	-1.2
hsa-miR-886-5p	0.00595	-1.2
hsa-miR-574-5p	0.00527	-1.2
hsa-miR-940	0.00956	-1.2
hsa-miR-296-3p	0.00825	-1.2
hsa-miR-186	0.01103	-1.2
hsa-miR-181d	0.01322	-1.2
hsa-miR-575	0.01475	-1.1
hsa-miR-604	0.00406	-1.1
hsa-miR-345	0.01103	-1.1
hsa-miR-875-3p	0.00066	-1.1

FIG. 5(cont.)

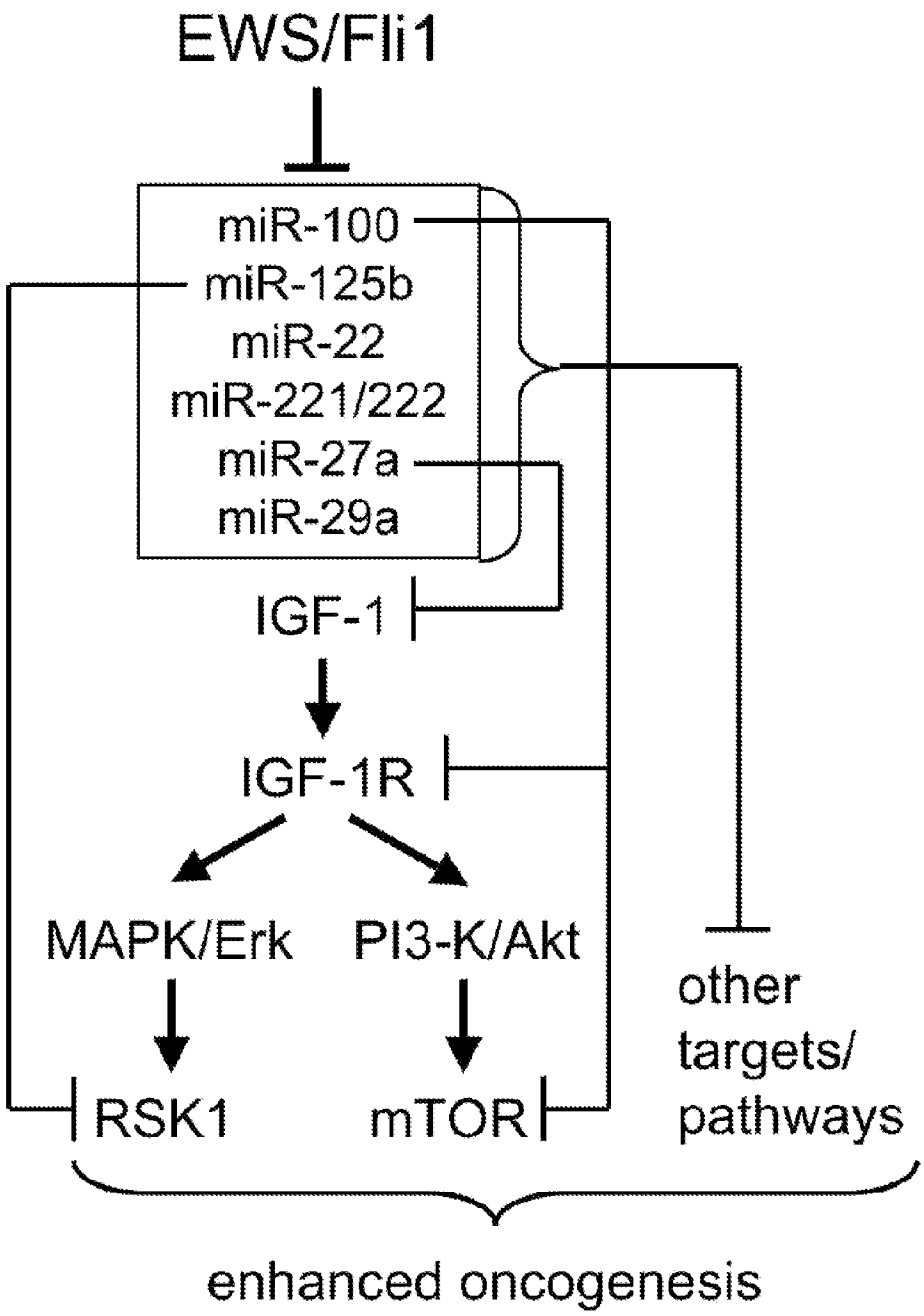

EWS/Fli1
miR-100
miR-125b
miR-22
miR-221/222
miR-27a
miR-29a
IGF-1
IGF-1R
MAPK/Erk
PI3-K/Akt
other
targets/
pathways
RSK1
mTOR
enhanced oncogenesis

FIG. 6

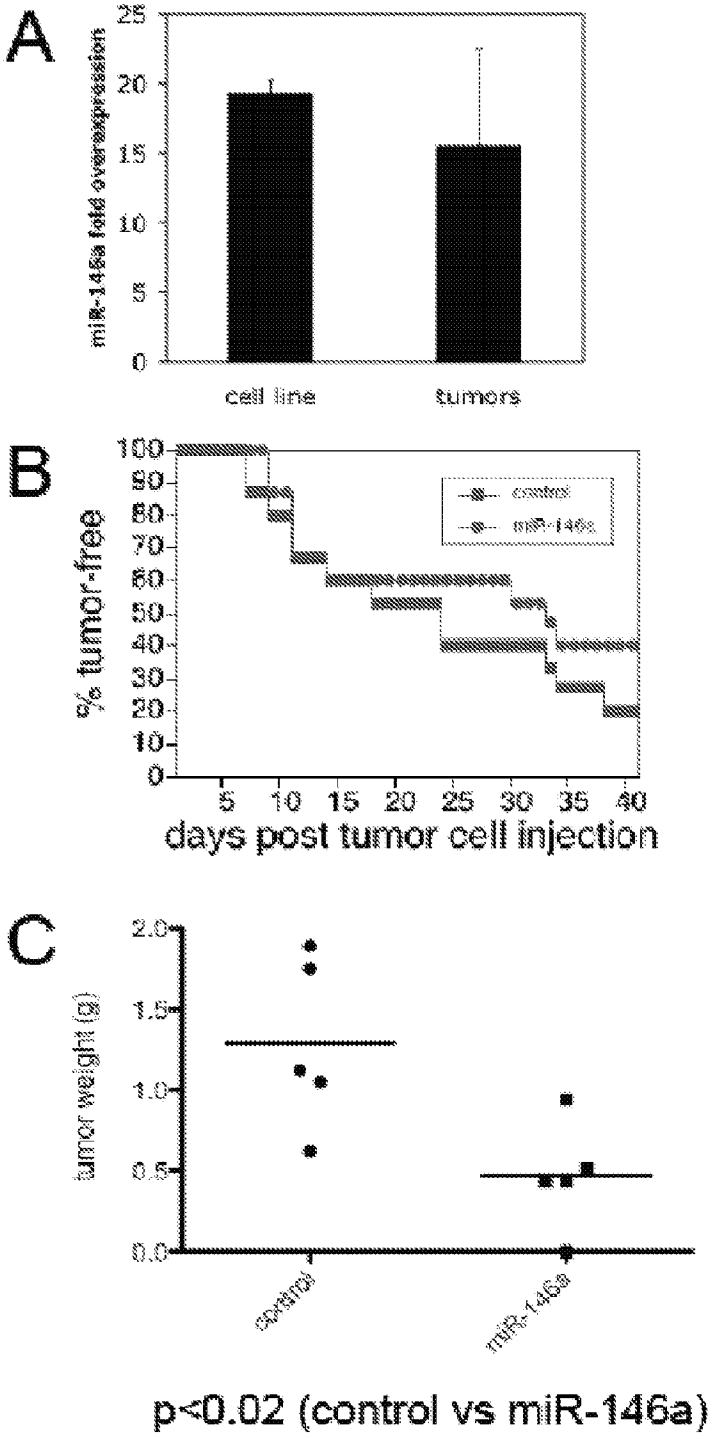

A
miR-146a fold overexpression
25
20
15
10
5
0
cell line
tumors
B
% tumor-free
100
90
80
70
60
50
40
30
20
10
0
5 10 15 20 25 30 35 40
days post tumor cell injection
control
miR-146a
C
tumor weight (g)
2.0
1.5
1.0
0.5
0.0
control
miR-146a
p<0.02 (control vs miR-146a)

FIG. 7A-C

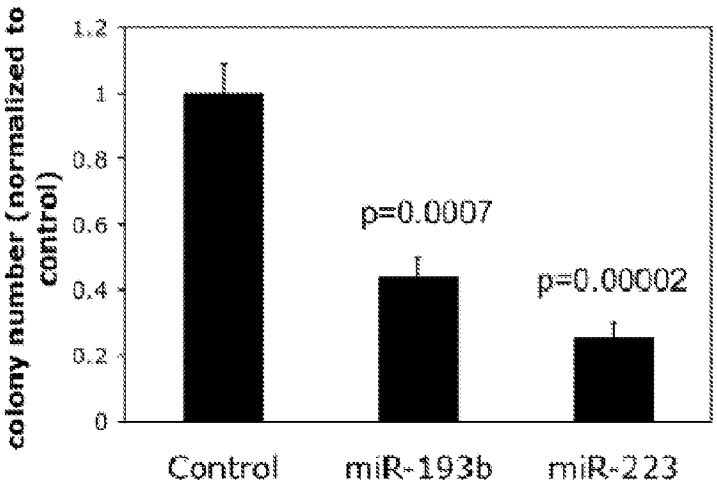

colony number (normalized to control)
1.2
1
0.8
0.6
0.4
0.2
0
p=0.0007
p=0.00002
Control
miR-193b
miR-223

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/33176

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/48 (2012.01)
USPC - 435/6.1, 6.14; 436/64
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
435/6.1, 6.14; 436/64

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
435/6.11; 436/63, 94 (text search - see search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Dialog Classic (USPT, PGPB, EPAB, JPAB and Dialog Classic (INSPEC, NTIS, MEDLINE, CHEMENG) Ewing sarcoma, miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, miR-193b, miR-223, miR-146a, miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a, miR-92b, microarray hybridization, increase, decrease, biopsy, resected tumor tissue

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2010/0298407 A1 (Mendell et al.) 25 November 2010 (25.11.2010) paras [0198]; [0191]; [0017]; [0195]; [0196]; [0232]	1-3, 5
A	US 2010/0203544 A1 (CROCE et al.) 12 August 2010 (12.08.2010)	1-3, 5
A	BAN et al. "Hsa-mir-145 is the top EWS-FLI1-repressed microRNA involved in a positive feedback loop in Ewing's sarcoma". Oncogene 5 May 2011 (Epub 10 January 2011); Vol. 30, No. 18; pg 2173-80. doi: 10.1038/onc.2010.581	1-3, 5

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
28 August 2012 (28.08.2012)	21 SEP 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/33176

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

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

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

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

3. ☒ Claims Nos.: 4 and 6-15
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+: claims 1-15, directed to a method of diagnosing Ewing Sarcoma in a subject, comprising:
(a) obtaining a sample from the subject; and

- Please see extra sheet for continuation -

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

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

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

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-15

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

Continuation of Box III: Lack of Unity of Invention

(b) assessing said sample for one or more miRNAs selected from the group consisting of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, miR-193b, miR-223, miR-146a, miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a and miR-92b; wherein a decreased level of one or more of miR-100, miR-l25b, miR-22, miR-221, miR-27a, miR-29a, miR-193b, miR-223, or miR-146a, or an increased level of one or more of miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a or miR-92b, as compared to a sample from normal subject, indicates that said subject has a Ewing sarcoma; wherein the first invention is limited to miR-100 (claims 1-3, and 5). (Applicants may opt for additional markers to be searched by specifying the marker(s) and paying an additional invention search fee for each elected marker. Note that the specific claims to be searched will depend upon the Applicants elections).

Groups II+: claims 16-25, directed to a method of treating a subject with Ewing sarcoma comprising providing to said subject one or more miRNAs selected from the group consisting of miR-100, miR125b, miR-22, miR-221, miR-27a, miR-193b, miR-223, miR-29a, and/or miR-146a and/or an miRNA mimic thereof; wherein the first invention is limited to miR-100 (claims 16-20, and 23) (Applicants may opt for additional markers to be searched by specifying the marker(s) and paying an additional invention search fee for each elected marker. Note that the specific claims to be searched will depend upon the Applicants elections).

Groups III+, claims 26-36, directed to a method of treating a subject with Ewing sarcoma comprising providing to said subject one or more antagomirs for an miRNA selected from the group consisting of miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a and/or miR -92b; wherein the first invention is limited to miR-25 (claims 26-31, 33, 35 and 36) (applicants may opt for additional miRNAs to be searched by specifying the miRNA, and paying an additional invention search fee for each elected miRNA).

Groups IV+: claims 37-40, directed to a kit comprising probes for a plurality of miR-100, miR-125b, miR-22, miR-221, miR-27a, miR-29a, miR-146a, miR-193b, miR-223, miR-25, miR-93, miR-106a, miR-17, miR-92a, miR-20a and miR-92b; wherein the first invention is limited to miR-100 and miR-125b (Applicants may opt for additional miRNAs to be searched by specifying the miRNA, and paying an additional invention search fee for each elected miRNA. Note: based on Claim 38 and pg. 4, ln 33- pg. 5, ln 3, the minimum number required for a plurality is two).

Finally, should the applicants opt to pay for a search of any of the above additional groups (e.g. II+, III+) and to also pay for the search of specific additional miRNAs; the additional elected miRNA species will be searched in all Groups for which an additional invention search payment has been made.

The inventions listed as Groups I+ to IV+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of the claims of Groups I+ to IV+ are indicated in the Group descriptions, above.

The only common technical element shared by the above groups is that they are related to miRNAs or probes for miRNAs. Groups I+ to III+ share the further common technical element of being related to miRNAs associated with Ewing Sarcoma. The claims of Groups I+ share the common technical elements of being related to a method of diagnosing Ewing Sarcoma in a subject, comprising: (a) obtaining a sample from the subject; and (b) assessing said sample for one or more miRNAs, wherein an increase or decrease in expression of the miRNA as compared to a sample from normal subject, indicates that said subject has a Ewing sarcoma.

These common technical elements do not represent an improvement over the prior art of the article entitled "Hsa-mir-145 is the top EWS -FLI1-repressed microRNA involved in a positive feedback loop in Ewing's sarcoma" by Ban et al. (hereinafter "Ban"), which teaches wherein miR-145 is decreased in Ewing's Sarcoma cells (abstract), relative to control cells (MPCs; pg. 2173, col 2, para 1) as well as wherein administration of miR-145 to Ewing's Sarcoma cells halted growth of Ewing's Sarcoma cells (abstract). Although Ban does not explicitly discloses a method of diagnosis of Ewing Sarcoma in a subject by assessing the level of an miRNA in a sample from a patient relative to that in a sample from a normal individual, it would have been obvious to a person skilled in the art to extend the association taught by Ban from cell lines to samples derived from subjects, wherein the comparison of the level of miR-145, as taught by Ban, in samples from a subject to those of normal subjects would have been an obvious means of diagnosis of Ewing Sarcoma, based on the teaching of Ban.

The claims of Groups I+, II+ and IV+ share the common technical element of being related to miR-100. The claims of Groups II+ share the common technical elements of being related to a method of treating a subject with Ewing sarcoma comprising providing to said subject one or more miRNAs, or a mimic thereof. This common technical element does not improve upon the prior art of US 2010/0298407 A1 to Mendell et al., which teaches compositions comprising miR-100 for the treatment of neoplasia (para [0006], [0007]), wherein the compositions may further comprise antagomirs (para [0159]), and the neoplasia treated may be Ewing's sarcoma (Ewing's tumor; para [0105]).

The claims of Groups III+ share the common technical element of being related to a method of treating a subject with Ewing sarcoma comprising providing to said subject one or more antagomirs for an miRNA. This common technical element does not improve upon the prior art of US 2010/0216865 A1 to Elias, which teaches treatment of a number of conditions, including Ewing family sarcomas (para [0016]) using miRNAs (para [0021]), or inhibitors thereof, including antagomirs (para [0045]).

The claims of Groups IV+ share the common technical elements of being related to a kit comprising a plurality of miRNA probes. This common technical element does not improve upon the prior art of US 2011/0076675 A1 to Jacobsen et al., which discloses probes and methods for the detection of ribonucleic acids (abstract), including miRNAs (para [0013]). Jacobsen further discloses a kit comprising a plurality of probes for miRNAs (para [0254]).

Therefore, the inventions of Groups I+ to IV+ lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.