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(71) Applicant (for all designated States except US): **BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM** [US/US]; 201 West 7th Street, Austin, Texas 78701 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **OLSON, Eric** [US/US]; c/o BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM, 201 West 7th Street, Austin, Texas 78701 (US). **ROOIJ, Eva van** [US/US]; c/o BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM, 201 West 7th Street, Austin, Texas 78701 (US). **FROST, Robert** [US/US]; c/o BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM, 201 West 7th Street, Austin, Texas 78701 (US).

(74) Agents: **MCLEOD, Bonnie Weiss** et al.; Cooley LLP, 777 6th Street, NW, Suite 1100, Washington, DC 20001 (US).

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(54) Title: TARGETING OF THE MIR-30 FAMILY AND LET-7 FAMILY AS A TREATMENT FOR HEART DISEASE

(57) Abstract: The present invention provides a method for designing an inhibitor that is capable of targeting multiple members of a miRNA family. In particular, the present invention provides inhibitors for reducing the expression or activity of two or members of the miR-30 family or the let-7 family. Methods of using such inhibitors for the treatment of cardiovascular disease are also disclosed.



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TARGETING OF THE MIR-30 FAMILY AND LET-7 FAMILY AS A TREATMENT FOR HEART DISEASE

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims the benefit of U.S. Provisional Application No. 61/169,538, filed April 15, 2009, which is herein incorporated by reference in its entirety.

STATEMENT OF GOVERNMENT SUPPORT

[002] This invention was made with grant support under grant no. HL53351-06 from the National Institutes of Health. The government may have certain rights in the invention.

DESCRIPTION OF THE TEXT FILE SUBMITTED ELECTRONICALLY

[003] The contents of the text file submitted electronically herewith are incorporated herein by reference in their entirety: A computer readable format copy of the Sequence Listing (filename: MIRG 015_01WO SeqList_ST25.txt, date recorded: April 8, 2010, file size 12 kilobytes).

FIELD OF THE INVENTION

[004] The present invention is related to the fields of cardiology and molecular biology. In particular, the invention provides a method of designing an inhibitor to reduce or eliminate the expression of multiple members of a microRNA family. Such inhibitors are useful in the treatment or prevention of cardiovascular diseases.

BACKGROUND OF THE INVENTION

[005] Heart disease and its manifestations, including coronary artery disease, myocardial infarction, congestive heart failure and cardiac hypertrophy, clearly present a major health risk in the United States today. The cost to diagnose, treat and support patients suffering from these diseases is well into the billions of dollars. Two particularly severe manifestations of heart disease are myocardial infarction and cardiac hypertrophy.

[006] Myocardial infarction, commonly known as a heart attack, is caused by a sudden and sustained lack of blood flow to the heart tissue, which is usually the result of a narrowing or occlusion of a coronary artery. Without adequate blood supply, the tissue becomes ischemic,

leading to the death of cardiomyocytes (*e.g.* heart muscle cells) and vascular structures. The necrotic tissue resulting from the death of the cardiomyocytes is generally replaced by scar tissue, which is not contractile, fails to contribute to cardiac function, and often plays a detrimental role in heart function by expanding during cardiac contraction, or by increasing the size and effective radius of the ventricle, for example, becoming hypertrophic.

[007] Cardiac hypertrophy is an adaptive response of the heart to virtually all forms of cardiac disease, including those arising from hypertension, mechanical load, myocardial infarction, cardiac arrhythmias, endocrine disorders, and genetic mutations in cardiac contractile protein genes. While the hypertrophic response is initially a compensatory mechanism that augments cardiac output, sustained hypertrophy can lead to dilated cardiomyopathy (DCM), heart failure, and sudden death. In the United States, approximately half a million individuals are diagnosed with heart failure each year, with a mortality rate approaching 50%.

[008] Numerous signaling pathways, especially those involving aberrant calcium signaling, drive cardiac hypertrophy and pathological remodeling (Heineke & Molkentin, 2006). Hypertrophic growth in response to stress involves different signaling pathways and gene expression patterns than physiological hypertrophy, which occurs in response to exercise. Stress-mediated myocardial hypertrophy is a complex phenomenon associated with numerous adverse consequences with distinct molecular and histological characteristics causing the heart to fibrose, dilate and decompensate which, through cardiomyocyte degeneration and death, often culminates in heart failure. As such, there has been intense interest in deciphering the underlying molecular mechanisms and in discovering novel therapeutic targets for suppressing adverse cardiac growth and ultimately failure. Understanding these mechanisms is essential to the design of new therapies to treat cardiac hypertrophy and heart failure.

[009] MicroRNAs (miRNAs) have recently been implicated in a number of biological processes including regulation of developmental timing, apoptosis, fat metabolism, and hematopoietic cell differentiation among others. MiRNAs are small, non-protein coding RNAs of about 18 to about 25 nucleotides in length that are derived from individual miRNA genes, from introns of protein coding genes, or from poly-cistronic transcripts that often encode multiple, closely related miRNAs. See review by Carrington *et al.* (*Science*, Vol. 301(5631):336-338, 2003). MiRNAs act as repressors of target mRNAs by promoting their degradation, when their sequences are perfectly complementary, or by inhibiting translation, when their sequences contain mismatches.

[0010] MiRNAs are transcribed by RNA polymerase II (pol II) or RNA polymerase III (pol III; see Qi *et al.* (2006) *Cellular & Molecular Immunology*, Vol. 3:411-419) and arise from initial transcripts, termed primary miRNA transcripts (pri-miRNAs), that are generally several thousand bases long. Pri-miRNAs are processed in the nucleus by the RNase Drosha into about 70- to about 100-nucleotide hairpin-shaped precursors (pre-miRNAs). Following transport to the cytoplasm, the hairpin pre-miRNA 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.

[0011] Numerous recent studies point to the involvement of miRNAs in the regulation of cellular responses to stress. For example, miRNAs have been identified that are up-regulated in response to stresses, such as hypoxia, nutrient deprivation, and DNA damage (Leung and Sharp (2007) *Cell*, Vol. 130(4):581-585). MiRNAs were first suggested to play a role in heart disease in a study of distinctive patterns of miRNA expression in the hearts of normal mice and mice that suffered from heart disease (van Rooij *et al.* (2006) *Proc. Natl. Acad. Sci.*, Vol. 103(48):18255-18260). Specific miRNAs were up- or down-regulated in mice that were subjected to thoracic aortic banding (TAB), a potent stimulus for pathological hypertrophy, or in response to constitutive activation of calcineurin, a stress-inducible mediator of the hypertrophic response. Importantly, several of these microRNAs were also dysregulated in failing human hearts, suggesting they established a diagnostic molecular signature for cardiac pathogenesis. Based on a hand full of genetic studies in mice and humans, it is becoming increasingly clear that miRNAs are indeed actively involved in cardiac remodeling, growth, conductance, and contractility (reviewed in van Rooij and Olson (2007) *Journal of Clinical Investigation*, Vol. 117(9):2369-2376).

[0012] Thus, there is a need in the art for novel therapeutic regulators of miRNAs for the treatment of various cardiovascular disorders, such as myocardial infarction and heart failure. Inhibitors that can target multiple related members of a specific miRNA family or multiple miRNAs implicated in the disease state are desirable.

SUMMARY OF THE INVENTION

[0013] The present invention is based, in part, on the discovery that a single antisense oligonucleotide can be designed to target multiple members of a miRNA family. In particular, the present invention provides a method of reducing expression of two or more members of a miRNA family, such as the miR-30 or let-7 family, in a cell by administering a single inhibitor capable of targeting two or more members of the miRNA family. Such miRNA family inhibitors are useful in the treatment of cardiac diseases.

[0014] In one embodiment, the present invention provides a method of reducing expression of two or more members of a miRNA family in a cell comprising contacting the cell with an inhibitor, wherein the inhibitor targets two or more members of the miRNA family, and wherein the expression or activity of the two or more miRNA family members is reduced in the cell following contact with the inhibitor. The inhibitor can be an antisense oligonucleotide or an antagomir. In some embodiments, the inhibitor is an antisense oligonucleotide having at least one chemical modification (*e.g.*, sugar or backbone modification). In one embodiment, the inhibitor is a nucleic acid comprising one or more binding sites for the miRNA family members. In another embodiment, the inhibitor is a nucleic acid comprising one or more linked antisense oligonucleotides for each of the two or more miRNA family members. The miRNA family can include, for example, miR-30, let-7, miR-29, and miR-15.

[0015] The present invention also includes a method of designing a nucleic acid for inhibiting the expression or activity of some or all members of a miRNA family. In one embodiment, the method comprises aligning nucleotide sequences of some or all the members of a miRNA family; determining a consensus sequence from said aligned nucleotide sequences; and synthesizing a nucleic acid having a sequence that is complementary to said consensus sequence, wherein the synthesized nucleic acid is capable of inhibiting the expression or activity of some or all members of the miRNA family. In some embodiments, a nucleic acid having complementarity to a consensus sequence can be designed to allow for bulges and loops at regions or individual nucleotides lacking complementarity. The miRNA family can include, for example, miR-30, let-7, miR-29, and miR-15. In certain embodiments, the miRNA family is the miR-30 family. In other embodiments, the miRNA family is the let-7 family.

[0016] In another embodiment, the present invention encompasses a method of treating or preventing pathologic cardiac hypertrophy, cardiac remodeling, heart failure, or myocardial

infarction in a subject in need thereof comprising administering to the subject an inhibitor of at least one miR-30 family member, wherein the expression or activity of the at least one miR-30 family member is reduced in heart cells of the subject following administration of the inhibitor. The at least one miR-30 family member can be miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e. In some embodiments, the expression or activity of two or more miR-30 family members is reduced following administration of the inhibitor. In a preferred embodiment, the subject is human.

[0017] In still another embodiment, the present invention provides a method of treating or preventing pathologic cardiac hypertrophy, cardiac remodeling, heart failure, or myocardial infarction in a subject in need thereof comprising administering to the subject an inhibitor of at least one let-7 family member, wherein the expression or activity of the at least one let-7 family member is reduced in heart cells of the subject following administration of the inhibitor. The at least one member of the let-7 family can be let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, and let-7i. In certain embodiments, the expression or activity of two or more let-7 family members is reduced following administration of the inhibitor. In a preferred embodiment, the subject is human.

[0018] The present invention also includes a method of modulating calcium signaling in a cell comprising delivering to the cell a modulator of one or more miR-30 family members. The cell may be *in vitro* or *in vivo*. The modulator can be an agonist of miR-30 function or an inhibitor of miR-30 function. In one embodiment, calcium signaling is reduced in the cell following delivery of a miR-30 agonist. In another embodiment, calcium signaling is increased in the cell following delivery of a miR-30 inhibitor. In some embodiments, the expression or activity of one or more genes regulated by a miR-30 family member is modulated in the cell after delivery of a miR-30 modulator. Genes regulated by miR-30 can include adrenergic receptor, endothelin receptor type A, regulator of G-protein signaling 2 (RGS2), calcium/calmodulin-dependent protein kinase II delta (CaMKIId), G protein-coupled receptor kinase 5, and Calpain 7.

[0019] The present invention also provides a method of improving endothelial function or promoting endothelial regeneration after vascular injury in a subject. In one embodiment, the method comprises administering to the subject an inhibitor of one or more miR-30 family members, wherein the expression or activity of the one or more miR-30 family members is reduced in endothelial cells of the subject following administration of the inhibitor. In certain

embodiments, the activity or expression of dimethylarginine dimethylaminohydrolase 1 is increased in the endothelial cells of the subject following administration of the inhibitor. In other embodiments, the activity or expression of nitric oxide synthase is increased in the endothelial cells of the subject following administration of the inhibitor. In a preferred embodiment, the subject is human.

[0020] The present invention includes pharmaceutical compositions comprising miRNA family inhibitors as described herein. In one embodiment, the pharmaceutical composition comprises a pharmaceutically acceptable carrier and an inhibitor of two or more members of a miRNA family. In certain embodiments, the miRNA family is the miR-30 family. In other embodiments, the miRNA family is the let-7 family. The composition can be formulated for injection, for example, for parenteral administration or catheter administration.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] Figure 1. Downregulation of miR-30 in cardiac disease. **A.** Determination of the expression of the miR-30 family members in total cardiac RNA from human heart failure patients compared to healthy individuals by microRNA micro-array. **B.** Expression of the individual miR-30 family members after myocardial infarction (MI). Total RNA from the area adjacent to the infarcted region (border zone) was collected three and fourteen days after induction of a MI by ligation of the coronary artery in C57BL/6 mice (n=6). Expression of miRNAs was determined in pooled RNA by microRNA micro-array in comparison to sham-operated mice.

[0022] Figure 2. Gene structure of miR-30 family members. **A.** Murine sequences of the miR-30 family members (SEQ ID NOs: 50-54). While miR-30a, miR-30d and miR-30e differ each only in one nucleotide, miR-30b and miR-30c have a different 3'-end. **B.** Four of the five miR-30 genes are located as two paired clusters on chromosomes 4 and 15. MiR-30a and a second copy of the miR-30c gene (miR-30c-2) are expressed independently from intergenic regions on chromosome 1. The miR-30c-1 and miR-30e cluster is located within intron 5 of the transcription factor Nfyc. E, exon.

[0023] Figure 3. Expression pattern of miR-30. **A.** Detection of individual family members of miR-30 in diverse tissues of wild-type C57BL/6 mice by Northern blotting (5µg total RNA per lane pooled from 3 animals). Due to cross-reactivity, the probes directed specifically against

miR-30b and miR-30c detect two bands of which the lower one represents miR-30b and the upper one miR-30c, which is one nucleotide longer than the other miR-30 family members. **B.** Detection of miR-30 in primary neonatal rat fibroblasts (FB) and cardiomyocytes (NRCM) by Northern blotting. Ethidium bromide-stained acrylamide gels were used as loading controls.

[0024] Figure 4. Specificity of miR-30 probes. COS cells were transfected with plasmids expressing miR-30a, the miR-30b/d-cluster, miR-30c, or miR-30e. Northern blots of lysates of cells expressing the different miR-30 plasmids were probed with oligonucleotides directed to specific miR-30 family members.

[0025] Figure 5. MiRNAs in training-induced growth of the adult heart. **A.** Representative record of the voluntary run activity of mice during a 10-day period. **B.** Ratio of ventricular weight to body weight in mice after 10 days of voluntary running (Run) compared to inactive mice (Ctr). **C.** MiRNA expression in hearts of trained mice relative to inactive mice. **D.** Expression of the miR-30 family members in the mouse heart after 10 days of voluntary running.

[0026] Figure 6. Overexpression of miR-30 increases protein synthesis in cardiomyocytes. **A.** Adenoviruses (Adv) expressing either lacZ, miR-30a or the miR-30b/d-cluster were generated and used to infect isolated neonatal rat cardiomyocytes (NRCM). Expression of miR-30a, miR-30b and miR-30d, respectively, in NRCMs was determined by Northern blotting following adenovirus infection. **B.** Protein synthesis of isolated NRCM was ascertained by determination of [³H]-isoleucine incorporation. Cells were transfected with an adenovirus expressing miR-30a (left panel) or the miR-30b/d-cluster (right panel) and stimulated with isoproterenol and phenylephrine (Iso/PE, 2.5/25 μmol/L) or insulin-like growth factor-1 (IGF-1) or left untreated. MOI, multiplicity of infection; Cpm, counts per minute.

[0027] Figure 7. Knock-down of miR-30 by anti-miRs. **A.** Design of a specific anti-miR targeted to miR-30a (SEQ ID NO: 18). The first five nucleotides on the 5' and 3' ends are methylated at the 2' position of the sugar to prevent degradation. **B.** Northern blot analysis of lysates from isolated neonatal rat cardiomyocytes forty eight hours after transfection with the anti-miR-30a. **C.** Northern blot analysis of lysates from isolated neonatal rat cardiomyocytes forty eight hours after transfection with anti-miRs specifically directed against miR-30b or miR-30c.

[0028] Figure 8: Knock-down of miR-30 reduces protein synthesis in cardiomyocytes. Neonatal rat cardiomyocytes were transfected with various combinations of anti-miRs targeted to

miR-30a, miR-30b, and miR-30c and stimulated with isoproterenol and phenylephrine (Iso/PE, 2.5/25 $\mu\text{mol/L}$) or left untreated. Protein synthesis was measured by [^3H]-isoleucine incorporation.

[0029] Figure 9. MiR-30 transgenic mice develop severe heart failure. **A.** Photomicrographs of hearts from miR-30a and miR-30e transgenic mice compared to hearts from wild-type littermates. **B.** Kaplan-Meier analysis of survival of miR-30a and miR-30e transgenic mice relative to wild-type littermates.

[0030] Figure 10. MiR-30 targets molecules involved in Ca^{2+} -signaling in the heart. Schematic of calcium signaling pathways and predicted miR-30 targets (red outlines and red background). MiR-30 is predicted to target the adrenergic receptors (AR), the endothelin receptor type A (EtR-A), the regulator of G-protein signaling 2 (RGS2), calcium/calmodulin-dependent protein kinase II delta (CaMKIId), and Calpain 7. Several of these targets (red background) exhibited reduced mRNA expression in 3-week old transgenic mice overexpressing miR-30a in cardiac tissue.

[0031] Figure 11. Generation of miR-30 eraser mice. **A.** Schematic illustrating two different eraser constructs for downregulating expression of miR-30 family members in cardiac tissue. Nucleic acids containing sequences complementary to the mature miR-30 family members are placed under the control of the alpha myosin heavy chain promoter (αMHC). **B.** Western blot analysis of cardiac tissue isolated from miR-30 eraser mice expressing antisense sequences to miR-30a, miR-30d, and miR-30e (30ade eraser). Expression of total CaMKII (left) and activated CaMKII (right) is increased in the eraser mice.

[0032] Figure 12. MiR-30 targets GRK-5 directly. **A.** The 3'-UTR of the G protein-coupled receptor kinase 5 (GRK5) has a putative target site for miR-30b (SEQ ID NO: 51) that is conserved across species (SEQ ID NOs: 55-59). **B.** Luciferase expression in COS cells co-expressing a luciferase gene fused to a 3'-UTR of GRK5 and either the miR-30b/d-cluster or a let-7a/d/f cluster.

[0033] Figure 13. Expression of let-7 family members in cardiac disease. **A.** Cardiac expression of let-7a, let-7d, and let-7f in calcineurin transgenic (Cn-Tg) mice, a model of cardiac disease, in comparison to wild-type (Wt) littermates. **B.** Cardiac expression of let-7a, let-7d, and let-7f in transgenic mice expressing the let-7a,d,f-cluster under the control of the $\alpha\text{-MHC}$ promoter (let-7 Tg) in comparison to Wt littermates.

[0034] Figure 14. Let-7 transgenic mice have a high variability in cardiomyocyte size.

Transgenic mice overexpressing the let-7a,d,f-cluster under the control of the alpha-MHC promoter exhibit a high variability in cardiomyocyte size (right two panels) relative to wild-type litter mates (left panel).

DETAILED DESCRIPTION OF THE INVENTION

[0035] The present invention is based, in part, on the discovery that several miRNA families are regulated in various forms of cardiac disease, and all members of such miRNA families are regulated in the same direction. The inventors have surprisingly found that a single inhibitor can be designed to target multiple members of a miRNA family. In particular, the present invention provides a method of designing a nucleic acid for inhibiting the expression or activity of some or all members of a miRNA family. Such inhibitors are capable of reducing the expression or activity of some or all members of the miRNA family in a cell. These inhibitors can be used to treat various forms of heart disease in a subject. Accordingly, the present invention also provides a method of treating or preventing pathologic cardiac hypertrophy, cardiac remodeling, heart failure, or myocardial infarction in a subject in need thereof by administering to the subject an inhibitor of two or more members of a miRNA family.

[0036] In one embodiment, the present invention provides a method of designing a nucleic acid for inhibiting the expression or activity of some or all members of a miRNA family comprising aligning nucleotide sequences of some or all the members of a miRNA family; determining a consensus sequence from said aligned nucleotide sequences; and synthesizing a nucleic acid having a sequence that is complementary to said consensus sequence, wherein the synthesized nucleic acid is capable of inhibiting the expression or activity of some or all members of the miRNA family.

[0037] As used herein, a “consensus sequence” is a sequence containing one or more variable nucleotides, and is determined by aligning and comparing the nucleotide sequences of two or more miRNA family members. In a preferred embodiment, the mature sequences of the miRNA family members are compared. In another embodiment, the minor or star sequences of the miRNA family members are compared. Alternatively, in some embodiments, the pre-miRNA or pri-miRNA sequences of each of the miRNA family members can be aligned and compared to determine a consensus sequence. The consensus sequence can be determined from nucleotide

sequences of two or more members, three or more members, four or more members, five or more members, six or more members, seven or more members, eight or more members, or all members of a miRNA family. Any miRNA family can be used in the method as long as it would be desirable to inhibit expression of multiple members or all members of the miRNA family.

Non-limiting examples of miRNA families that are suitable for use in the methods of the invention include the miR-29 family (*e.g.* miR-29a, miR-29b, and miR-29c), the miR-15 family (miR-15, miR-16, miR-195, miR-424, and miR-497), the miR-30 family (miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e), and the let-7 family (let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, and let-7i). In one embodiment, the miRNA family is the miR-30 family. In another embodiment, the miRNA family is the let-7 family.

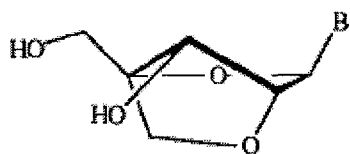
[0038] The present invention also includes a method of reducing expression of two or more members of a miRNA family in a cell comprising contacting the cell with an inhibitor, wherein the inhibitor targets two or more members of the miRNA family, and wherein the expression or activity of the two or more miRNA family members is reduced in the cell following contact with the inhibitor. In some embodiments, the cell is *in vitro*. In other embodiments, the cell is *in vivo*. In another embodiment, the cell is a cardiomyocyte.

[0039] As used herein, “target” refers to the ability of the inhibitor to bind to and/or interfere with the endogenous activity of the miRNA molecule. By way of example, an inhibitor that targets two members of a miRNA family is capable of binding to and/or interfering with the endogenous activity of those two miRNA family members. In some embodiments, the inhibitor is a nucleic acid designed according to the methods of the invention as described herein.

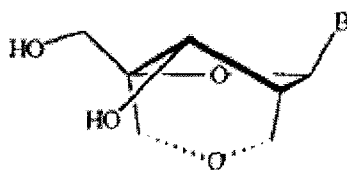
[0040] In certain embodiments, the inhibitor of two or more members of a miRNA family is an antisense oligonucleotide. The antisense oligonucleotides can include ribonucleotides, deoxyribonucleotides, or a combination thereof. It is understood that when the RNA sequences disclosed herein are used in embodiments that require deoxyribonucleotides, a thymidine residue is substituted for a uridine residue. Similarly, when the DNA sequences disclosed herein are used in embodiments that require ribonucleotides, a uridine residue is substituted for a thymidine residue.

[0041] In some embodiments, the antisense oligonucleotides have at least one chemical modification (*e.g.*, sugar or backbone modification). For instance, suitable antisense oligonucleotides may be comprised of one or more “conformationally constrained” or bicyclic

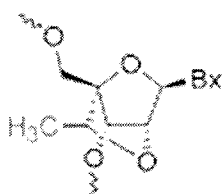
sugar nucleoside modifications (BSN) that confer enhanced thermal stability to complexes formed between the oligonucleotide containing BSN and their complementary microRNA target strand. For example, in one embodiment, the antisense oligonucleotides contain at least one “locked nucleic acid.” Locked nucleic acids (LNAs) contain the 2'-O, 4'-C-methylene ribonucleoside (structure A) wherein the ribose sugar moiety is in a “locked” conformation. In another embodiment, the antisense oligonucleotides contain at least one 2', 4'-C-bridged 2' deoxyribonucleoside (CDNA, structure B). *See, e.g.*, U.S. Patent No. 6,403,566 and Wang *et al.* (1999) *Bioorganic and Medicinal Chemistry Letters*, Vol. 9: 1147-1150, both of which are herein incorporated by reference in their entireties. In yet another embodiment, the antisense oligonucleotides contain at least one modified nucleoside having the structure shown in structure C. The antisense oligonucleotides targeting two or more members of a miRNA family can contain combinations of BSN (LNA, CDNA and the like) or other modified nucleotides, and ribonucleotides or deoxyribonucleotides.



A



B



C

[0042] Alternatively, the antisense oligonucleotides can comprise peptide nucleic acids (PNAs), which contain a peptide-based backbone rather than a sugar-phosphate backbone. Other modified sugar or phosphodiester modifications to the antisense oligonucleotide are also contemplated. For instance, 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. Patent Nos. 6,693,187 and 7,067,641, which are herein incorporated by reference in their entireties). In one embodiment, antisense oligonucleotides targeting two or more miRNA family members contain 2'-O-methyl sugar modifications on each base and are linked by phosphorothioate linkages. Antisense oligonucleotides, particularly those of shorter lengths (*e.g.*, less than 15 nucleotides) can comprise one or more affinity enhancing modifications, such as, but not limited to, LNAs, bicyclic nucleosides, phosphonoformates, 2' O-alkyl modifications and the like. 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 No. 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. For instance, to facilitate *in vivo* delivery and stability, the antisense oligonucleotide may be linked to a steroid, such as cholesterol moiety, a vitamin, a fatty acid, a carbohydrate or glycoside, a peptide, or other small molecule ligand at its 3' end.

[0043] Antisense oligonucleotides useful for inhibiting the activity of two or more miRNA family members are about 5 to about 25 nucleotides in length, about 10 to about 30 nucleotides in length, or about 20 to about 25 nucleotides in length. In certain embodiments, antisense oligonucleotides targeting two or more miRNA family members are about 8 to about 18 nucleotides in length, and in other embodiments about 12 to about 16 nucleotides in length. Antisense oligonucleotides can comprise a sequence that is at least partially complementary to a mature miRNA sequence from one or more of the family members. "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. In some embodiments, the antisense oligonucleotide can be substantially complementary to a mature miRNA family member sequence, that is at least about 90%, 95%, 96%, 97%, 98%, or 99% complementary to a target polynucleotide sequence. In one embodiment, the antisense oligonucleotide comprises a

sequence that is at least partially complementary to a consensus sequence derived from some or all of the members of the miRNA family. In another embodiment, the antisense oligonucleotide is 100% complementary to a consensus sequence derived from some or all of the members of the miRNA family. In some embodiments, the antisense oligonucleotides may be designed to allow for bulges or loops at regions or at individual sites lacking identity between family members. Such bulges or loops may range in length from two to about five or about 10 or about 15 or more nucleotides.

[0044] In some embodiments, the antisense oligonucleotides are antagomirs. "Antagomirs" are single-stranded, chemically-modified ribonucleotides that are at least partially complementary to at least one of the miRNA family members or a consensus sequence derived from the members of the miRNA family. Antagomirs may comprise one or more modified nucleotides, such as bicyclic sugar nucleoside modifications or 2'-O-methyl-sugar modifications. In some embodiments, antagomirs comprise only modified nucleotides. Antagomirs can also comprise one or more phosphorothioate linkages resulting in a partial or full phosphorothioate backbone. To facilitate *in vivo* delivery and stability, the antagomir can be linked to a steroid (*e.g.*, cholesterol) or other moiety (*e.g.*, a vitamin, a fatty acid, a carbohydrate or glycoside, a peptide, or other small molecule ligand) at its 3' end. Antagomirs suitable for inhibiting two or more miRNA family members can 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. The antagomirs can be at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% complementary to a mature miRNA family member sequence or a consensus sequence derived from some or all miRNA family members. In some embodiments, the antagomir may be substantially complementary to a mature miRNA family member sequence or miRNA family consensus sequence, that is at least about 90%, 95%, 96%, 97%, 98%, or 99% complementary to a target polynucleotide sequence. In other embodiments, the antagomirs are 100% complementary to a consensus sequence derived from some or all of the members of the miRNA family. Antagomirs may also be designed to form bulges or loops at regions lacking identity between miR family members.

[0045] In certain embodiments, the inhibitor of two or more members of a miRNA family is a nucleic acid comprising one or more linked antisense oligonucleotides for each of the two or more miRNA family members. By way of example, an inhibitor for three particular members of

a miRNA family can contain at least one antisense oligonucleotide for each of those three particular miRNA family members. The antisense oligonucleotides for each of the family members can be adjacent to one another in the nucleic acid (*e.g.* no linker sequences) or can be separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more linker nucleotides. The linker nucleotides can, in some embodiments, contain cleavage sequences for endonucleases, such that each of the antisense oligonucleotides are liberated from the larger nucleic acid sequence following delivery of the nucleic acid sequence to the cell or expression of the nucleic acid in the cell, or alternatively may be separated by cleavage *in vitro*. The inhibitor nucleic acid can contain one or more, two or more, three or more, four or more, or five or more antisense oligonucleotides for each family member.

[0046] In one embodiment, the inhibitor targets two or more family members of the miR-30 family. The miR-30 family consists of five family members: miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e. In humans, miR-30b and miR-30d are expressed as a cluster from an intergenic region of chromosome 8. MiR-30e and one copy of miR-30c (miR-30c-1) are expressed as a cluster from an intron of the gene encoding the NYFC transcription factor on chromosome 1. MiR-30a and a second copy of miR-30c (miR-30c-2) are expressed independently from introns of protein coding genes on chromosome 6. The seed region (*e.g.* bases spanning the 5' two to eight nucleotides of the mature miRNA sequence) for all miR-30 family members is highly conserved (UGUAAACAUC; SEQ ID NO: 19). Each of the pre-miRNA sequences for each miR-30 family member is processed into a mature sequence and a star sequence. The star sequence is processed from the other arm of the stem loop structure. The pre-miRNA (*e.g.* stem-loop sequences), mature, and star sequences for each of the miR-30 family members is given below:

Human pre-miR-30a (SEQ ID NO: 1)

5' -GCGACUGUAA ACAUCCUCGA CUGGAAGCUG UGAAGCCACA GAUGGGCUUU
CAGUCGGAUG UUUGCAGCUG C -3'

Human miR-30a mature (SEQ ID NO: 2)

5' -UGUAAACAUC CUCGACUGGA AG-3'

Human miR-30a* (SEQ ID NO: 3)

5' -CUUUCAGUCG GAUGUUUGCA GC-3'

Human pre-miR-30b (SEQ ID NO: 4)

5' -ACCAAGUUUC AGUUCAUGUA AACAUCCUAC ACUCAGCUGU AAUACAUGGA
UUGGCUGGGA GGUGGAUGUU UACUUCAGCU GACUUGGA-3'

Human miR-30b mature (SEQ ID NO: 5)

5' -UGUAAACAUC CUACACUCAG CU-3'

Human miR-30b* (SEQ ID NO: 6)

5' -CUGGGAGGUG GAUGUUUACU UC-3'

Human pre-miR-30c-1 (SEQ ID NO: 7)

5' -ACCAUGCUGU AGUGUGUGUA AACAUCCUAC ACUCUCAGCU GUGAGCUCAA
GGUGGCUGGG AGAGGGUUGU UUACUCCUUC UGCCAUGGA-3'

Human pre-miR-30c-2 (SEQ ID NO: 8)

5' -AGAUACUGUA AACAUCCUAC ACUCUCAGCU GUGGAAAGUA AGAAAGCUGG
GAGAAGGCUG UUUACUCUUU CU-3'

Human miR-30c mature (SEQ ID NO: 9)

5' -UGUAAACAUC CUACACUCUC AGC-3'

Human miR-30c-1* (SEQ ID NO: 10)

5' -CUGGGAGAGG GUUGUUUACU CC-3'

Human miR-30c-2* (SEQ ID NO: 11)

5' -CUGGGAGAAG GCUGUUUACU CU-3'

Human pre-miR-30d (SEQ ID NO: 12)

5' -GUUGUUGUAA ACAUCCCCGA CUGGAAGCUG UAAGACACAG CUAAGCUUUC
AGUCAGAUGU UUGCUGCUAC-3'

Human miR-30d mature (SEQ ID NO: 13)

5' -UGUAAACAUC CCCGACUGGA AG-3'

Human miR-30d* (SEQ ID NO: 14)

5' -CUUUCAGUCA GAUGUUUGCU GC-3'

Human pre-miR-30e (SEQ ID NO: 15)

5' -GGGCAGUCUU UGCUACUGUA AACAUCCUUG ACUGGAAGCU GUAAGGUGUU
CAGAGGAGCU UUCAGUCGGA UGUUUACAGC GGCAGGCUGC CA-3'

Human miR-30e mature (SEQ ID NO: 16)

5' -UGUAAACAUC CUUGACUGGA AG-3'

Human miR-30e* (SEQ ID NO: 17)

5' -CUUUCAGUCG GAUGUUUACA GC-3'

[0047] The expression or activity of two or more, three or more, or four or more miR-30 family members can be reduced in the cell following contact with the inhibitor. In one embodiment, the inhibitor reduces the activity or expression of miR-30a, miR-30b, and miR-30c in the cell. In another embodiment, the inhibitor reduces the activity or expression of miR-30a, miR-30d, and miR-30e in the cell. Exemplary inhibitors that reduce the expression of miR-30a, miR-30d, and miR-30e include, but are not limited to, nucleic acids comprising the sequence of 5'-CUUCCAGUCGAGGAUGUUUACA-3' (SEQ ID NO: 18); 5'-CUUCCAGUCUAGGAUGUUUACA-3' (SEQ ID NO: 60); 5'-CUUCCAGUCGGGAUGUUUACA-3' (SEQ ID NO: 63); or 5'-CUUCCAGUCAAGGAUGUUUACA-3' (SEQ ID NO: 64).

[0048] In another embodiment, the expression or activity of all five miR-30 family members (*e.g.* miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e) is reduced in the cell following contact with the inhibitor. In some embodiments, the inhibitor for reducing the expression or activity of all five miR-30 family members in a cell is a nucleic acid comprising the sequence of 5'-CUUCGAGUCGAGGAUGUUUACA-3' (SEQ ID NO: 61). In other embodiments, the inhibitor for reducing the expression or activity of all five miR-30 family members in a cell is a nucleic acid comprising one or more miR-30 binding sites. The term "miR-30 binding site" as used herein refers to a nucleotide sequence that is capable of binding a mature sequence of miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e. Preferably, a miR-30 binding site comprises a sequence that is at least partially complementary (*e.g.* 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%) to the miR-30 seed sequence (SEQ ID NO: 19). In one embodiment, the miR-30 binding site has a sequence that is complementary to SEQ ID NO: 19. The nucleic acid comprising one or more miR-30 binding sites may be from about 20 to about 500 nucleotides in length, about 25 to about 400 nucleotides in length, about 30 to about 300 nucleotides in length, about 40 to about 200 nucleotides in length, or about 50 to about 100 nucleotides in length. For example, the nucleic acid may be 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 225, 250, 275, 300, 350, 400, 500 nucleotides in length. The nucleic acid may contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, or 20 miR-30 binding sites. The multiple miR-30 binding sites may be adjacent or may be separated by spacers of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more nucleotides.

[0049] In certain embodiments, the inhibitor is a nucleic acid comprising one or more linked antisense oligonucleotides for each of the two or more miR-30 family members. In one embodiment, the nucleic acid comprises one or more linked antisense oligonucleotides for each of miR-30a, miR-30d, and miR-30e. The nucleic acid can have a sequence comprising SEQ ID NO: 18, SEQ ID NO: 63, and SEQ ID NO: 64 separated by one or more linker nucleotides. In another embodiment, the nucleic acid comprises two or more linked antisense oligonucleotides for each of miR-30a, miR-30d, and miR-30e (see Example 4 and Figure 11A). In another embodiment, the nucleic acid comprises one or more linked antisense oligonucleotides for each of miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e. The nucleic acid can have a sequence comprising SEQ ID NO: 18, SEQ ID NO: 63, SEQ ID NO: 64, 5'-AGCUGAGUGUAGGAUGUUUACA-3' (SEQ ID NO: 65), and 5'-

GCUGAGAGUGUAGGAUGUUUACA-3' (SEQ ID NO: 66) separated by one or more linker nucleotides. In still another embodiment, the nucleic acid comprises two or more linked antisense oligonucleotides for each of miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e (see Example 4 and Figure 11A).

[0050] In another embodiment, the inhibitor targets two or more family members of the let-7 family. The let-7 family consists of eight family members: let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, and let-7i. In humans, a first copy of let-7a (let-7a-1) is expressed as a cluster with let-7d and a first copy of let-7f (let-7f-1) from an intergenic region of chromosome 9. A second copy of let-7a (let-7a-2) is expressed as a cluster with miR-100 from an intergenic region on chromosome 11. A third copy of let-7a (let-7a-3) is expressed as a cluster with let-7b from a coding region of the RP4 gene on chromosome 22. Let-7c is expressed as a cluster with miR-99a from within an open reading frame on chromosome 21, while let-7e is expressed as a cluster with miR-99b and miR-125a from an intergenic region on chromosome 19. A second copy of let-7f (let-7f-2) is expressed in a cluster with miR-98 from within an intron of the HUWE-1 gene on the X-chromosome. Let-7g is expressed from an intron of the WDR82 gene on chromosome 3, and let-7i is expressed from an intergenic region of chromosome 12. The seed region for all let-7 family members is highly conserved (GAGGUAGUAG (SEQ ID NO: 49)). Each of the pre-miRNA sequences for each let-7 family member is processed into a mature sequence and a star sequence. The star sequence is processed from the other arm of the stem loop structure. The pre-miRNA (*e.g.* stem-loop sequences), mature, and star sequences for each of the let-7 family members is given below:

Human pre-let-7a-1 (SEQ ID NO: 20)

5' -UGGGAUGAGG UAGUAGGUUG UAUAGUUUUA GGGUCACACC CACCACUGGG
AGAUAAACUAU ACAAUUCUACU GUCUUUCCUA-3'

Human let-7a mature (SEQ ID NO: 21)

5' -UGAGGUAGUA GGUUGUAUAG UU-3'

Human let-7a* (SEQ ID NO: 22)

5' -CUAUACAAUC UACUGUCUUU C-3'

Human pre-let-7a-2 (SEQ ID NO: 29)

5' -AGGUUGAGGU AGUAGGUUGU AUAGUUUAGA AUUACAUCAA GGGAGUAAC
UGUACAGCCU CCUAGCUUUC CU-3'

Human let-7a-2* (SEQ ID NO: 30)

5' -CUGUACAGCC UCCUAGCUUU CC-3'

Human pre-let-7a-3 (SEQ ID NO: 31)

5' -GGGUGAGGUA GUAGGUUGUA UAGUUUGGG CUCUGCCCUG CUAUGGGAUA
ACUAUACAAU CUACUGUCUU UCCU-3'

Human pre-let-7b (SEQ ID NO: 32)

5' -CGGGGUGAGG UAGUAGGUUG UGUGGUUUA GGCAGUGAU GUUGCCCCUC
GGAAGUAAC UAUACAACCU ACUGCCUUC CUG-3'

Human let-7b mature (SEQ ID NO: 33)

5' -UGAGGUAGUA GGUUGUGUGG UU-3'

Human let-7b* (SEQ ID NO: 34)

5' -CUAUACAACC UACUGCCUUC CC-3'

Human pre-let-7c (SEQ ID NO: 35)

5' -GCAUCCGGGU UGAGGUAGUA GGUUGUAUGG UUUAGAGUUA CACCCUGGGA
GUUAACUGUA CAACCUUCUA GCUUCCUUG GAGC-3'

Human let-7c mature (SEQ ID NO: 36)

5' -UGAGGUAGUA GGUUGUAUGG UU-3'

Human let-7c* (SEQ ID NO: 37)

5' -UAGAGUUACA CCCUGGGAGU UA-3'

Human pre-let-7d (SEQ ID NO: 23)

5' -CCUAGGAAGA GGUAGUAGGU UGCAUAGUUU UAGGGCAGGG AUUUUGCCCA
CAAGGAGGUA ACUAUACGAC CUGCUGCCUU UCUUAGG-3'

Human let-7d mature (SEQ ID NO: 24)

5' -AGAGGUAGUA GGUUGCAUAG UU-3'

Human let-7d* (SEQ ID NO: 25)

5' -CUAUACGACC UGCUGCCUUU CU-3'

Human pre-let-7e (SEQ ID NO: 38)

5' -CCCGGGCUGA GGUAGGAGGU UGUUAUAGUUG AGGAGGACAC CCAAGGAGAU
CACUAUACGG CCUCCUAGCU UUCCCCAGG-3'

Human let-7e mature (SEQ ID NO: 39)

5' -UGAGGUAGGA GGUUGUAUAG UU-3'

Human let-7e* (SEQ ID NO: 40)

5' -CUAUACGGCC UCCUAGCUUU CC-3'

Human pre-let-7f-1 (SEQ ID NO: 26)

5' -UCAGAGUGAG GUAGUAGAUU GUAUAGUUGU GGGGUAGUGA UUUUACCCUG
UUCAGGAGAU AACUAUACAA UCUAUUGCCU UCCCUGA-3'

Human let-7f mature (SEQ ID NO: 27)

5' -UGAGGUAGUA GAUUGUAUAG UU-3'

Human let-7f-1* (SEQ ID NO: 28)

5' -CUAUACAAUC UAUUGCCUUC CC-3'

Human pre-let-7f-2 (SEQ ID NO: 41)

5' -UGUGGGAUGA GGUAGUAGAU UGUUAUAGUUU UAGGGUCAUA CCCCAUCUUG
GAGAUAACTA UACAGUCUAC UGUCUUUCCC ACG-3'

Human let-7f-2* (SEQ ID NO: 42)

5' -CUAUACAGUC UACUGUCUUU CC-3'

Human pre-let-7g (SEQ ID NO: 43)

5' -AGGCUGAGGU AGUAGUUUGU ACAGUUUGAG GGUCUAUGAU ACCACCCGGU
ACAGGAGAUU ACUGUACAGG CCACUGCCUU GCCA-3'

Human let-7g mature (SEQ ID NO: 44)

5' -UGAGGUAGUA GUUUGUACAG UU-3'

Human let-7g* (SEQ ID NO: 45)

5' -CUGUACAGGC CACUGCCUUG C-3'

Human pre-let-7i (SEQ ID NO: 46)

5' -CUGGCUGAGG UAGUAGUUUG UGCUGUUGGU CGGGUUGUGA CAUUGCCCCG
UGUGGAGAUU ACUGCGCAAG CUACUGCCUU GCUA-3'

Human let-7i mature (SEQ ID NO: 47)

5' -UGAGGUAGUA GUUUGUGCUG UU-3'

Human let-7i* (SEQ ID NO: 48)

5' -CUGCGCAAGC UACUGCCUUG CU-3'

[0051] The expression or activity of two or more, three or more, four or more, five or more, six or more, or seven or more let-7 family members can be reduced in the cell following contact with the inhibitor. In one embodiment, the inhibitor reduces the activity or expression of let-7a, let-7d, and let-7f in the cell. In another embodiment, the inhibitor reduces the activity or expression of

let-7a, let-7b, let-7c, let-7d, and let-7f in the cell. In still another embodiment, the expression or activity of all eight let-7 family members (*e.g.* let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, and let-7i) is reduced in the cell following contact with the inhibitor. In some embodiments, the inhibitor for reducing the expression or activity of all eight let-7 family members in a cell is a nucleic acid comprising the sequence of 5'-AACUAUACAACCUACUACCUCA-3' (SEQ ID NO: 62). In other embodiments, the inhibitor for reducing the expression or activity of all eight let-7 family members in a cell is a nucleic acid comprising one or more let-7 binding sites. The term "let-7 binding site" as used herein refers to a nucleotide sequence that is capable of binding a mature sequence of let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, and let-7i. Preferably, a let-7 binding site comprises a sequence that is at least partially complementary (*e.g.* 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%) to the let-7 seed sequence (SEQ ID NO: 49). In one embodiment, the let-7 binding site has a sequence that is complementary to SEQ ID NO: 49. The nucleic acid comprising one or more let-7 binding sites may be from about 20 to about 500 nucleotides in length, about 25 to about 400 nucleotides in length, about 30 to about 300 nucleotides in length, about 40 to about 200 nucleotides in length, or about 50 to about 100 nucleotides in length. For example, the nucleic acid may be 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 225, 250, 275, 300, 350, 400, 500 nucleotides in length. The nucleic acid may contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, or 20 let-7 binding sites. The multiple let-7 binding sites may be adjacent or may be separated by spacers of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more nucleotides.

[0052] In certain embodiments, the inhibitor is a nucleic acid comprising one or more linked antisense oligonucleotides for each of the two or more let-7 family members. In one embodiment, the nucleic acid comprises one or more linked antisense oligonucleotides for each of let-7a, let-7d, and let-7f. In another embodiment, the nucleic acid comprises one or more linked antisense oligonucleotides for each of let-7a, let-7b, let-7c, let-7d, and let-7f. In still another embodiment, the nucleic acid comprises one or more linked antisense oligonucleotides for each of let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, and let-7i. The antisense oligonucleotides for each let-7 family member can be separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more linker nucleotides.

[0053] The miRNA family inhibitors of the invention (*e.g.* miR-30, let-7, miR-29, and miR-15 family inhibitors) can be delivered to a cell by an expression vector encoding any of inhibitor

nucleic acids as described herein. A "vector" is a composition of matter which can be used to deliver a nucleic acid of interest to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term "vector" includes an autonomously replicating plasmid or a virus. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, and the like. An expression construct can be replicated in a living cell, or it can be made synthetically. For purposes of this application, the terms "expression construct," "expression vector," and "vector," are used interchangeably to demonstrate the application of the invention in a general, illustrative sense, and are not intended to limit the invention.

[0054] In one embodiment, an expression vector for expressing a miRNA family inhibitor comprises a promoter operably linked to a polynucleotide encoding an antisense oligonucleotide, wherein the sequence of the expressed antisense oligonucleotide is partially or perfectly complementary to a mature sequence of one member of the miRNA family or a consensus sequence derived from some or all members of the miRNA family. In another embodiment, an expression vector for expressing a miRNA family inhibitor comprises a promoter operably linked to a polynucleotide encoding a nucleic acid comprising one or more miRNA family binding sites. The phrase "operably linked" or "under transcriptional control" as used herein means that the promoter is in the correct location and orientation in relation to a polynucleotide to control the initiation of transcription by RNA polymerase and expression of the polynucleotide. In another embodiment, the expression vector may encode a single nucleic acid that comprises one or more linked antisense oligonucleotides for each member of the miRNA family, wherein the single nucleic acid is operably linked to a promoter.

[0055] As used herein, a "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. Suitable promoters include, but are not limited to RNA pol I, pol II, pol III, and viral promoters (*e.g.* human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, and the Rous sarcoma virus long terminal repeat). In one embodiment, the promoter is a tissue specific promoter. Of particular interest are muscle specific promoters, and more particularly, cardiac specific promoters. These include the myosin light chain-2 promoter (Franz *et al.* (1994) *Cardioscience*, Vol. 5(4):235-43; Kelly *et al.* (1995) *J. Cell Biol.*, Vol.

129(2):383-396), the alpha actin promoter (Moss *et al.* (1996) *Biol. Chem.*, Vol. 271(49):31688-31694), the troponin 1 promoter (Bhavsar *et al.* (1996) *Genomics*, Vol. 35(1):11-23); the Na⁺/Ca²⁺ exchanger promoter (Barnes *et al.* (1997) *J. Biol. Chem.*, Vol. 272(17):11510-11517), the dystrophin promoter (Kimura *et al.* (1997) *Dev. Growth Differ.*, Vol. 39(3):257-265), the alpha7 integrin promoter (Ziober and Kramer (1996) *J. Bio. Chem.*, Vol. 271(37):22915-22), the brain natriuretic peptide promoter (LaPointe *et al.* (1996) *Hypertension*, Vol. 27(3 Pt 2):715-22) and the alpha B-crystallin/small heat shock protein promoter (Gopal-Srivastava (1995) *J. Mol. Cell. Biol.*, Vol. 15(12):7081-7090), alpha myosin heavy chain promoter (Yamauchi-Takahara *et al.* (1989) *Proc. Natl. Acad. Sci. USA*, Vol. 86(10):3504-3508) and the ANF promoter (LaPointe *et al.* (1988) *J. Biol. Chem.*, Vol. 263(19):9075-9078). In one embodiment, the promoter is the alpha myosin heavy chain promoter.

[0056] In certain embodiments, the promoter operably linked to a polynucleotide encoding a miRNA family inhibitor may be an inducible promoter. Inducible promoters are known in the art and include, but are not limited to, tetracycline promoter, metallothionein IIA promoter, heat shock promoter, steroid/thyroid hormone/retinoic acid response elements, the adenovirus late promoter, and the inducible mouse mammary tumor virus LTR.

[0057] Methods of delivering expression constructs and nucleic acids to cells are known in the art and can include, for example, calcium phosphate co-precipitation, electroporation, microinjection, DEAE-dextran, lipofection, transfection employing polyamine transfection reagents, cell sonication, gene bombardment using high velocity microprojectiles, and receptor-mediated transfection.

[0058] The miRNA family inhibitors as described herein can be employed to treat various cardiac diseases in which the miRNA family is dysregulated. For instance, the inventors surprisingly discovered that all five members of the miR-30 family are downregulated in cardiac tissue from failing hearts, and overexpression of miR-30 family members leads to heart failure and premature death (see Examples 1 and 3). The let-7 family is upregulated in cardiac disease states and appears to control cardiomyocyte growth and proliferation (see Example 5). Other miRNA families also appear to play a role in cardiac hypertrophy and cardiac remodeling. The miR-15 family members (miR-15, miR-16, miR-195, miR-424, and miR-497) are upregulated in response to stress, and inhibition of this family increases cell survival following infarction. MiR-29 family members (miR-29a, miR-29b, and miR-29c) are predicted to target IGF-1, AMP-

activated Kinase and PPAR delta, all of which have been shown to be involved in the regulation of physiological cardiomyocyte hypertrophy. Therefore, downregulation of the miR-29 family in myocytes during physiological training promotes hypertrophy of cardiac and skeletal myocytes. Thus, the present invention also provides a method of treating or preventing pathologic cardiac hypertrophy, heart failure, cardiac remodeling, or myocardial infarction in a subject in need thereof comprising administering to the subject an inhibitor of two or more members of a miRNA family, wherein the expression or activity of the two or more miRNA family members is reduced in heart cells of the subject following administration of the inhibitor. The miRNA family can include, but is not limited to, miR-30, let-7, miR-15, and miR-29.

[0059] As used herein, the term “subject” or “patient” refers to any vertebrate including, without limitation, humans and other primates (*e.g.*, chimpanzees and other apes and monkey species), farm animals (*e.g.*, cattle, sheep, pigs, goats and horses), domestic mammals (*e.g.*, dogs and cats), laboratory animals (*e.g.*, rodents such as mice, rats, and guinea pigs), and birds (*e.g.*, domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like). In some embodiments, the subject is a mammal. In other embodiments, the subject is a human.

[0060] In one embodiment, the present invention includes a method of treating or preventing pathologic cardiac hypertrophy, cardiac remodeling, heart failure, or myocardial infarction in a subject in need thereof comprising administering to the subject an inhibitor of at least one miR-30 family member, wherein the expression or activity of the at least one miR-30 family member is reduced in heart cells of the subject following administration of the inhibitor. The at least one miR-30 family can be selected from the group consisting of miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e. Preferably, the expression or activity of two or more miR-30 family members is reduced following administration of the inhibitor. In some embodiments, the expression or activity of miR-30a, miR-30d, and miR-30e is reduced following administration of the inhibitor. In other embodiments, the expression or activity of miR-30a, miR-30b, and miR-30c is reduced following administration of the inhibitor. In certain embodiments, the expression or activity of miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e is reduced following administration of the inhibitor.

[0061] The inhibitor can be any of the inhibitory molecules described herein. For instance, the inhibitor can be an antisense oligonucleotide, a modified antisense oligonucleotide, an

antagomir, or a nucleic acid containing one or more miR-30 binding sites. In some embodiments, the inhibitor targets a mature sequence of one or more miR-30 family members (e.g. SEQ ID NOs: 2, 5, 9, 13, and 16) or a star sequence of one or more miR-30 family members (e.g. SEQ ID NOs: 3, 6, 10, 11, 14, and 17). In certain embodiments, inhibitors of miR-30 family members are antisense oligonucleotides or antagomirs comprising a sequence that is partially or perfectly complementary to a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, and SEQ ID NO: 17.

[0062] In some embodiments, inhibitors of one or more miR-30 family members are chemically-modified antisense oligonucleotides. In one embodiment, an inhibitor of a miR-30 family member is a chemically-modified antisense oligonucleotide comprising a sequence substantially complementary to a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, and SEQ ID NO: 17. As used herein “substantially complementary” refers to a sequence that is at least about 95%, 96%, 97%, 98%, 99%, or 100% complementary to a target polynucleotide sequence.

[0063] Antisense oligonucleotides may comprise a sequence that is substantially complementary to a precursor miRNA sequence (pre-miRNA) for one or more miR-30 family members (e.g. pre-miR-30a, pre-miR-30b, pre-miR-30c, pre-miR-30d, or pre-miR-30e). In some embodiments, the antisense oligonucleotide comprises a sequence that is substantially complementary to a sequence located outside the stem-loop region of the pre-miRNA sequence. In one embodiment, an inhibitor of a miR-30 family member is an antisense oligonucleotide having a sequence that is substantially complementary to a pre-miRNA sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 12, and SEQ ID NO: 15.

[0064] In another embodiment, the present invention includes a method of treating or preventing pathologic cardiac hypertrophy, cardiac remodeling, heart failure, or myocardial infarction in a subject in need thereof comprising administering to the subject an inhibitor of at least one let-7 family member, wherein the expression or activity of the at least one let-7 family member is reduced in heart cells of the subject following administration of the inhibitor. The at least one let-7 family can be selected from the group consisting of let-7a, let-7b, let-7c, let-7d, let-7e, let-

7f, let-7g, and let-7i. Preferably, the expression or activity of two or more let-7 family members is reduced following administration of the inhibitor. In some embodiments, the expression or activity of let-7a, let-7d, and let-7f is reduced following administration of the inhibitor. In other embodiments, the expression or activity of let-7a, let-7b, let-7c, let-7d, and let-7f is reduced following administration of the inhibitor. In certain embodiments, the expression or activity of let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, and let-7i is reduced following administration of the inhibitor.

[0065] In some embodiments, the inhibitor targets a mature sequence of one or more let-7 family members (*e.g.* SEQ ID NOs: 21, 24, 27, 33, 36, 39, 44, and 47) or a star sequence of one or more miR-30 family members (*e.g.* SEQ ID NOs: 22, 25, 28, 30, 34, 37, 40, 42, 45, and 48). In certain embodiments, inhibitors of let-7 family members are antisense oligonucleotides or antagomirs comprising a sequence that is partially or perfectly complementary to a sequence selected from the group consisting of SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 47, and SEQ ID NO: 48.

[0066] In some embodiments, inhibitors of one or more let-7 family members are chemically-modified antisense oligonucleotides. In one embodiment, an inhibitor of a let-7 family member is a chemically-modified antisense oligonucleotide comprising a sequence substantially complementary to a sequence selected from the group consisting of SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 47, and SEQ ID NO: 48.

[0067] Antisense oligonucleotides may comprise a sequence that is substantially complementary to a precursor miRNA sequence (pre-miRNA) for one or more let-7 family members (*e.g.* pre-let-7a, pre-let-7b, pre-let-7c, pre-let-7d, pre-let-7e, pre-let-7f, pre-let-7g, and pre-let-7i). In some embodiments, the antisense oligonucleotide comprises a sequence that is substantially complementary to a sequence located outside the stem-loop region of the pre-miRNA sequence. In one embodiment, an inhibitor of a let-7 family member is an antisense oligonucleotide having a sequence that is substantially complementary to a pre-miRNA sequence selected from the

group consisting of SEQ ID NO: 20, SEQ ID NO: 23, SEQ ID NO: 26, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 35, SEQ ID NO: 38, SEQ ID NO: 41, SEQ ID NO: 43, and SEQ ID NO: 46.

[0068] Preferably, administration of a miRNA family inhibitor (*e.g.* inhibitor of a miR-30 or let-7 family member) results in the improvement of one or more symptoms of pathologic cardiac hypertrophy, heart failure, cardiac remodeling, or myocardial infarction in the subject, or delays the transition from cardiac hypertrophy to heart failure. The one or more improved symptoms can be, for example, increased exercise capacity, increased cardiac ejection volume, decreased left ventricular end diastolic pressure, decreased pulmonary capillary wedge pressure, increased cardiac output, increased cardiac index, lowered pulmonary artery pressures, decreased left ventricular end systolic and diastolic dimensions, decreased left and right ventricular wall stress, decreased wall tension, increased quality of life, and decreased disease related morbidity or mortality. In addition, use of inhibitors of the miR-30 or let-7 family members can prevent cardiac hypertrophy and its associated symptoms from arising. In one embodiment, administration of an inhibitor of one or more miR-30 or let-7 family members to a subject suffering from myocardial infarction can reduce infarct size by decreasing the loss of heart cells. In another embodiment, cardiac function is stabilized in a subject suffering from myocardial infarction following administration of an inhibitor of one or more miR-30 or let-7 family members.

[0069] The present invention also encompasses a method of modulating calcium signaling in a cell comprising delivering to the cell a modulator of one or more miR-30 family members. The one or more miR-30 family members is selected from the group consisting of miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e. In preferred embodiments, the cell is a cardiomyocyte. The cardiomyocyte can be *in vitro* or *in vivo*. As used herein, a “modulator” is a molecule that regulates the expression or activity of one or more miR-30 family members. Modulators can be agonists of miR-30 family member function or they can be inhibitors of miR-30 family member function. Modulators can include proteins, peptides, polypeptides, polynucleotides, oligonucleotides, or small molecules.

[0070] In one embodiment, the modulator is an agonist of one or more miR-30 family members, and calcium signaling is reduced in the cell following delivery of the agonist. The expression or activity of one or more genes regulated by a miR-30 family member can be reduced in the cell

following delivery of the miR-30 agonist. For instance, the expression or activity of one or more genes selected from the group consisting of adrenergic receptor, endothelin receptor type A, regulator of G-protein signaling 2 (RGS2), calcium/calmodulin-dependent protein kinase II delta (CaMKIId), G protein-coupled receptor kinase 5, and Calpain 7 is reduced in the cell following delivery of the agonist.

[0071] In another embodiment, the modulator is an inhibitor of one or more miR-30 family members, and calcium signaling is increased in the cell following delivery of the inhibitor. The expression or activity of one or more genes regulated by a miR-30 family member can be increased in the cell following delivery of the miR-30 inhibitor. For instance, the expression or activity of one or more genes selected from the group consisting of adrenergic receptor, endothelin receptor type A, regulator of G-protein signaling 2 (RGS2), calcium/calmodulin-dependent protein kinase II delta (CaMKIId), G protein-coupled receptor kinase 5, and Calpain 7 is increased in the cell following delivery of the inhibitor.

[0072] The present invention also provides a method of improving endothelial function or promoting endothelial regeneration after vascular injury in a subject in need thereof. In one embodiment, the method comprises administering to the subject an inhibitor of one or more miR-30 family members, wherein the expression or activity of the one or more miR-30 family members is reduced in endothelial cells of the subject following administration of the inhibitor. The one or more miR-30 family members can be miR-30a, miR-30b, miR-30c, miR-30d, or miR-30e. Endothelial function is the normal function of endothelial cells lining vessels, and can include, but is not limited to, mediation of coagulation, platelet adhesion, immune function, control of volume and electrolyte content of the intravascular and extravascular spaces, production of nitric oxide, and response to vasoactive substances.

[0073] In some embodiments, the activity or expression of dimethylarginine dimethylaminohydrolase 1 is increased in the endothelial cells of the subject following administration of the inhibitor. In other embodiments, the activity or expression of nitric oxide synthase is increased in the endothelial cells of the subject following administration of the inhibitor. In certain embodiments, vasodilation of vessels is increased in the subject following administration of the inhibitor.

[0074] The present invention also includes pharmaceutical compositions comprising a pharmaceutically acceptable carrier and an effective dose of an inhibitor of two or more

members of a miRNA family. The miRNA family can be miR-30, let-7, miR-15, or miR-29. In some embodiments, the miRNA family is the miR-30 family. In other embodiments, the miRNA family is the let-7 family. An “effective dose” is an amount sufficient to effect a beneficial or desired clinical result. An effective dose of an miRNA inhibitor of the invention may be about 1 mg/kg to about 100 mg/kg, about 2.5 mg/kg to about 50 mg/kg, or about 5 mg/kg to about 25 mg/kg. The precise determination of what would be considered an effective dose may be based on factors individual to each patient, including their size, age, type of disorder to be treated (*e.g.* myocardial infarction, heart failure, pathologic hypertrophy), and nature of inhibitor (*e.g.* expression construct, antisense oligonucleotide, modified antisense oligonucleotide, etc). Therefore, dosages can be readily ascertained by those of ordinary skill in the art from this disclosure and the knowledge in the art.

[0075] Where clinical applications are contemplated, pharmaceutical compositions will be prepared 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.

[0076] In one embodiment, the pharmaceutical composition comprises an effective dose of an inhibitor of two or more members of the miR-30 family. In some embodiments, the inhibitor reduces the activity or expression of miR-30a, miR-30b, and miR-30c. In other embodiments, the inhibitor reduces the activity or expression of miR-30a, miR-30d, and miR-30e. The inhibitor can be a nucleic acid comprising a sequence selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 60, SEQ ID NO: 63, and SEQ ID NO: 64. In certain embodiments, the inhibitor reduces the activity or expression of miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e. In such embodiments, the inhibitor can be a nucleic acid comprising the sequence of SEQ ID NO: 61. In other such embodiments, the inhibitor can be a nucleic acid comprising one or more miR-30 binding sites. The one or more miR-30 binding sites can have a sequence that is at least partially complementary to a miR-30 seed sequence (SEQ ID NO: 19).

[0077] In another embodiment, the pharmaceutical composition comprises an effective dose of an inhibitor of two or more members of the let-7 family. In some embodiments, the inhibitor reduces the activity or expression of let-7a, let-7d, and let-7f. In other embodiments, the inhibitor reduces the activity or expression of let-7a, let-7b, let-7c, let-7d, and let-7f. In certain embodiments, the inhibitor reduces the activity or expression of let-7a, let-7b, let-7c, let-7d, let-

7e, let-7f, let-7g, and let-7i. In such embodiments, the inhibitor can be a nucleic acid comprising the sequence of SEQ ID NO: 62. In other such embodiments, the inhibitor can be a nucleic acid comprising one or more let-7 binding sites. The one or more let-7 binding sites can have a sequence that is at least partially complementary to a let-7 seed sequence (SEQ ID NO: 49).

[0078] In still another embodiment, the pharmaceutical composition comprises an effective dose of a miRNA family inhibitor, wherein the inhibitor is a nucleic acid comprising one or more linked antisense oligonucleotides for each of the two or more miRNA family members.

Pharmaceutical compositions comprising effective doses of any of the inhibitors described herein are also contemplated.

[0079] Colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes, may be used as delivery vehicles for the inhibitors of miRNA family function, polynucleotides encoding miRNA family inhibitors, or constructs expressing particular miRNA family inhibitors. Commercially available fat emulsions that are suitable for delivering the nucleic acids of the invention to cardiac and skeletal muscle tissues include Intralipid®, Liposyn®, Liposyn® II, Liposyn® III, Nutrilipid, and other similar lipid emulsions. A preferred colloidal system for use as a delivery vehicle *in vivo* is a liposome (*i.e.*, an artificial membrane vesicle). The preparation and use of such systems is well known in the art. Exemplary formulations are also disclosed in US 5,981,505; US 6,217,900; US 6,383,512; US 5,783,565; US 7,202,227; US 6,379,965; US 6,127,170; US 5,837,533; US 6,747,014; and WO03/093449, which are herein incorporated by reference in their entireties.

[0080] One will generally desire to employ appropriate salts and buffers to render delivery vehicles stable and allow for uptake by target cells. Aqueous compositions of the present invention comprise an effective amount of the delivery vehicle comprising the inhibitor polynucleotides (*e.g.* liposomes or other complexes or expression vectors) dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. The phrases “pharmaceutically acceptable” or “pharmacologically acceptable” refers 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 solvents, buffers, solutions, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like acceptable for use in formulating pharmaceuticals, such as

pharmaceuticals suitable for administration to humans. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredients of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions, provided they do not inactivate the vectors or polynucleotides of the compositions.

[0081] The active compositions of the present invention may include classic pharmaceutical preparations. Administration of these compositions according to the present invention may be via any common route so long as the target tissue is available via that route. This includes oral, nasal, or buccal. Alternatively, administration may be by intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection, or by direct injection into cardiac tissue. Pharmaceutical compositions comprising miRNA inhibitors, polynucleotides encoding miRNA family inhibitors or expression constructs comprising inhibitor sequences may also be administered by catheter systems or systems that isolate coronary circulation for delivering therapeutic agents to the heart. Various catheter systems for delivering therapeutic agents to the heart and coronary vasculature are known in the art. Some non-limiting examples of catheter-based delivery methods or coronary isolation methods suitable for use in the present invention are disclosed in U.S. Patent No. 6,416,510; U.S. Patent No. 6,716,196; U.S. Patent No. 6,953,466, WO 2005/082440, WO 2006/089340, U.S. Patent Publication No. 2007/0203445, U.S. Patent Publication No. 2006/0148742, and U.S. Patent Publication No. 2007/0060907, which are all herein incorporated by reference in their entireties. Such compositions would normally be administered as pharmaceutically acceptable compositions as described herein.

[0082] The active compounds may also be administered parenterally or intraperitoneally. By way of illustration, solutions of the active compounds 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 generally contain a preservative to prevent the growth of microorganisms.

[0083] The pharmaceutical forms suitable for injectable use or catheter delivery include, for example, sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. Generally, these preparations are sterile

and fluid to the extent that easy injectability exists. Preparations should be stable under the conditions of manufacture and storage and should be preserved against the contaminating action of microorganisms, such as bacteria and fungi. Appropriate solvents or dispersion media may contain, 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 and 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.

[0084] Sterile injectable solutions may be prepared by incorporating the active compounds in an appropriate amount into a solvent along with any other ingredients (for example as enumerated above) as desired, 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 desired other ingredients, *e.g.*, as enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation include vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient(s) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0085] The compositions of the present invention generally may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include, for example, acid addition salts (formed with the free amino groups of the protein) derived from inorganic acids (*e.g.*, hydrochloric or phosphoric acids, or from organic acids (*e.g.*, acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups of the protein can also be derived from inorganic bases (*e.g.*, sodium, potassium, ammonium, calcium, or ferric hydroxides) or from organic bases (*e.g.*, isopropylamine, trimethylamine, histidine, procaine and the like.

[0086] Upon formulation, solutions are preferably administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations may

easily be administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral administration in an aqueous solution, for example, the solution generally is suitably buffered and the liquid diluent first rendered isotonic for example with sufficient saline or glucose. Such aqueous solutions may be used, for example, for intravenous, intramuscular, subcutaneous and intraperitoneal administration. Preferably, sterile aqueous media are employed as is known to those of skill in the art, particularly in light of the present disclosure. By way of illustration, a single dose may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards. In some embodiments, the pharmaceutical compositions of the invention can be packaged in a kit for parenteral administration.

[0087] This invention is further illustrated by the following additional examples that should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the Figures, are incorporated herein by reference in their entirety.

EXAMPLES

Example 1. MiR-30 family members are downregulated in cardiac tissue following heart failure and myocardial infarction

[0088] To identify miRNAs that are regulated in cardiac disease, a miRNA microarray was performed on RNA isolated from cardiac tissue from human heart failure patients suffering from idiopathic dilated cardiomyopathy and healthy control individuals. Interestingly, all members of the miR-30 family were down-regulated in the samples from failing hearts (Figure 1A). To determine if down-regulation of the miR-30 family was characteristic of cardiac disease, expression of the individual miR-30 family members was examined in mice following induction of myocardial infarction by ligation of the coronary artery. Total RNA isolated from the area

adjacent to the infarcted region (border zone) was collected three and fourteen days following induction of the infarction. As shown in Figure 1B, all five miR-30 family members were down-regulated in comparison to sham controls.

[0089] The miR-30 family consists of five members: miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e. The murine mature sequences for the miR-30 family members are shown in Figure 2A. While miR-30a, miR-30d and miR-30e differ each only in one nucleotide, miR-30b and miR-30c have a different 3'-end. However, all five members share the same seed region having a sequence of UGUAAACAUC (SEQ ID NO: 19). In mice, the majority of the miR-30 genes are located as two paired clusters. MiR-30c-1 is clustered with miR-30e on chromosome 4, and miR-30b is clustered with miR-30d in an intergenic region on chromosome 15 (Figure 2B). MiR-30a and miR-30c-2 are expressed independently from intergenic regions on chromosome 1. The miR-30c-1/miR-30e cluster is located within intron 5 of the transcription factor Nfyc (Figure 2B).

[0090] To determine the expression level of the different miR-30 family members, Northern analysis was performed on different tissue types (5µg total RNA per lane pooled from 3 animals). All miR-30 family members were highly expressed in heart, skeletal muscle, brain, kidney and lung (Figure 3A). Due to cross-reactivity, the probes directed specifically against miR-30b and miR-30c detect two bands of which the lower one represents miR-30b and the upper one miR-30c, which is one nucleotide longer than the other miR-30 family members (Figure 3A). Northern analysis of fibroblasts and cardiomyocytes revealed that the expression of the miR-30 family members in the heart was predominantly cardiomyocyte specific (Figure 3B).

[0091] To begin to determine the individual functions of the individual miR-30 family members, expression constructs containing the different miR-30 family members were generated. COS cells were transfected with plasmids expressing miR-30a, the miR-30b/d-cluster, miR-30c, or miR-30e. Northern blot analysis revealed some cross-reactivity among the probes for specific family members (Figure 4). The probe directed against miR-30a weakly detected miR-30d and miR-30e, and vice versa. The probe directed against miR-30b weakly detected miR-30c, and vice versa. Mature miR-30c, which is one nucleotide longer than the other miR-30 family members, runs accordingly a little bit higher on the acrylamide gel. Interestingly, pre-miR-30a is longer than the other pre-miRNAs of the miR-30 family, which is reflected by the slower migration of the upper band in lysates from cells expressing miR-30a (Figure 4). These data provide

information on the location of tolerable basepair mismatches within the mature sequences such that oligonucleotides can still bind. Thus, it is possible to design an oligonucleotide that will bind all or a subset of the miR-30 family members.

[0092] To further explore the role of the miR-30 family in cardiac remodeling, cardiac miRNA expression was determined in a setting of physiological cardiac remodeling (*e.g.* running-induced cardiac hypertrophy), which is thought to be beneficial and to increase cardiac output (Figure 5A). Mice were allowed to run voluntarily on an exercise wheel in their home cage for ten days and their activity was recorded (Figure 5A). The ratio of ventricular weight to body weight was increased in mice that were allowed to voluntarily run as compared to sedentary controls (Figure 5B), reflecting physiological cardiac hypertrophy in response to the exercise. Expression of miRNAs in cardiac tissue from animals that experienced running revealed that several miRNAs, including all members of the miR-30 family, were downregulated (Figure 5C and D). These data, in combination with the results from the experiments examining miRNA expression in response to pathological cardiac remodeling described above, suggest that downregulation of miR-30 in general may be necessary for cardiac remodeling or may counterbalance excessive myocardial remodeling. Thus, inhibition of the miR-30 family members may have a therapeutic benefit in conditions characterized by pathological cardiac hypertrophy, such as heart failure.

Example 2. MiR-30 regulates protein synthesis in cardiomyocytes in response to hypertrophic stimuli

[0093] To specifically study the role of the miR-30 family in cardiomyocytes, adenoviruses carrying a transgene encoding for miR-30a or the miR-30b/miR-30d cluster were generated. Isolated neonatal rat cardiomyocytes (NRCM) were infected with adenoviruses expressing either lacZ, miR-30a or the miR-30b/d-cluster. Expression of the specific miR-30 family member was increased in cells following infection with the adenovirus expressing the miR-30 family member (Figure 6A). NRCMs transfected with increasing titers of an adenovirus expressing miR-30a (Figure 6B, left) or the miR-30b/d-cluster (Figure 6B, right) were stimulated with isoproterenol and phenylephrine (Iso/PE, 2.5/25 $\mu\text{mol/L}$), insulin-like growth factor-1 (IGF-1), or left untreated. Protein synthesis was determined by [^3H]-isoleucine incorporation in the isolated NRCMs following stimulation. Overexpression of miR-30, in combination with hypertrophic

stimuli like isoproterenol or IGF-1, appeared to increase protein synthesis (Figure 6B), which is likely due to hypertrophy of the cardiomyocytes. These data indicate that miR-30 family members may play an active role in cardiac remodeling, and could be therapeutic targets in the treatment of pathological cardiac hypertrophy.

[0094] In a next series of experiments, an anti-miR approach was used to knockdown miR-30 in NRCMs *in vitro*. An anti-miR sequence targeted to miR-30a was designed. The first five nucleotides on both the 5' and 3' ends were methylated at the 2' position of the sugars to prevent degradation (Figure 7A). Transfection of isolated NRCMs with the anti-miR-30a oligonucleotide abolished endogenous expression of miR-30a as well as miR-30d and miR-30e within forty eight hours (Figure 7B). The expression of miR-30b and miR-30c was also partially reduced in NRCMs after transfection with the anti-miR-30a oligonucleotide (Figure 7B). Antisense oligonucleotides targeted to either the miR-30b or miR-30c sequences effectively eliminated expression of these miR-30 family members (Figure 7C). These results show that multiple miR-30 family members can be targeted by one oligonucleotide. An anti-miR oligonucleotide that closely overlaps with the 3' ends of the miR-30b and miR-30c sequences is likely to enable one to target all five of the miR-30 family members using one oligonucleotide.

[0095] NRCMs were transfected with various combinations of anti-miRs directed to miR-30a, miR-30b, and miR-30c. Protein synthesis was measured by [³H]-isoleucine incorporation following stimulation with isoproterenol and phenylephrine. Interestingly, knockdown of miR-30 in cardiomyocytes in the presence of the hypertrophic stimulus isoproterenol / PE reduced the level of protein synthesis, suggesting that a reduction in cardiomyocyte hypertrophy occurred (Figure 8).

Example 3. Overexpression of miR-30 causes heart failure and premature death

[0096] To further examine the role of the miR-30 family in cardiac remodeling, transgenic mice overexpressing one or more of the miR-30 family members in cardiac tissue were generated. MiR-30a, the miR-30b/d cluster, miR-30c-2 and miR-30e were overexpressed specifically in the heart under the control of the α -myosin heavy chain (MHC) promoter. Northern analysis confirmed that the miR-30 family member and its precursor were upregulated in cardiac tissue isolated from independent transgenic lines (data not shown). Transgenic mice overexpressing miR-30a or miR-30e specifically in the heart exhibited significant cardiac hypertrophy as

compared to wild-type litter mates (Figure 9A). MiR-30a and miR-30e transgenic animals developed severe dilative heart failure and died prematurely (Figure 9B). These data provide further evidence that the miR-30 family contributes to cardiac hypertrophy, and downregulation of the miR-30 family could be an effective therapeutic approach to treating heart disease.

Example 4. MiR-30 family members target calcium signaling molecules

[0097] Based on sequence analysis, miR-30 is predicted to target several molecules involved in calcium signaling and handling within the cell. Such targets include the adrenergic receptors (AR), the endothelin receptor type A (EtR-A), the regulator of G-protein signaling 2 (RGS2), calcium/calmodulin-dependent protein kinase II delta (CaMKIId) and Calpain 7 (Figure 10). Messenger RNA expression of several of these predicted targets, including EtR-A, RGS2, Calpain 7, and CaMKIId, was reduced in 3-week old transgenic mice overexpressing miR-30a under the control of the α MHC promoter (*e.g.* cardiac-specific), indicating that these molecules are *in vivo* targets of miR-30a. Furthermore, calsequestrin, the sarcoplasmic reticulum Ca^{2+} -storage protein, was strongly downregulated in these mice suggesting alterations in calcium cycling.

[0098] MiR-30 eraser mice were generated to further explore *in vivo* targets of the miR-30 family. Specifically, a nucleic acid containing sequences complementary to the mature miR-30a, miR-30d, and miR-30e sequences (ade eraser) or a nucleic acid containing sequences complementary to the mature miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e sequences (abcde eraser) was placed under the control of the α MHC promoter (Figure 11A). Transgenic mice overexpressing these nucleic acids exhibited a downregulation of the miR-30 family members in cardiac tissue. Western blot analysis of cardiac tissue isolated from ade eraser mice revealed an upregulation of both total and activated CaMKII as compared to wild-type littermates (Figure 11B) providing further evidence that CaMKII is an *in vivo* target for miR-30.

[0099] G protein-coupled receptor kinase 5 (GRK5), which is associated with the β 1-adrenergic receptor, contains a putative target site for miR-30 (Figure 12A). To determine if GRK5 was a true target for miR-30, luciferase assays were conducted in COS cells transfected with a construct expressing the miR-30b/miR-30d cluster. A luciferase gene fused to the 3'UTR from GRK5 was co-expressed in COS cells with either a miR-30b/miR-30d expression construct or a let-7a/let-7d/let-7f expression construct. Expression of the miR-30b/d-cluster repressed the

expression of luciferase, while expression of the let-7 cluster, which has no predicted target site in the 3'UTR of GRK5, had no effect (Figure 12B).

[00100] Another predicted target of miR-30 is dimethylarginine dimethylaminohydrolase 1 (DDAH1). DDAH degrades methylarginines, including asymmetric dimethylarginine (ADMA) and NG-monomethyl-L-arginine (MMA), which inhibit nitric oxide synthase. Inhibition of DDAH leads to accumulation of methylarginines, which in turn blocks nitric oxide synthesis causing vasoconstriction. Reduced DDAH function has been implicated in the impairment of vascular relaxation observed in patients afflicted with cardiovascular disease. Expression of DDAH1 was reduced in miR-30a transgenic mice indicating that DDAH1 is an *in vivo* target of miR-30. In addition, a downregulation of miR-30 was observed following vascular injury (data not shown). Such a downregulation of miR-30 would result in increased DDAH1 levels, followed by decreased ADMA and increased NOS activity, which would facilitate vascular relaxation. Therefore, downregulation of miR-30 after vascular injury would enhance endothelial function and restore endothelial regeneration.

Example 5. Let-7 is upregulated in a cardiac hypertrophy model

[00101] Another miRNA family that has been implicated in cardiac disease is the let-7 family. All members of the let-7 family were found to be upregulated in cardiac tissue isolated from human heart failure patients (data not shown). Similarly, members of the let-7 family are also upregulated in calcineurin transgenic mice, a model of stress-induced cardiac hypertrophy (Figure 13A). There are eight members of the let-7 family: let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, and let-7i. In mice, let-7a-1, let-7d, and let-7f-1 are clustered in an intergenic region on chromosome 13, and let-7c-2 and let-7b are clustered in an intergenic region on chromosome 15. Let-7e is clustered with miR-99b and miR-125a in an intergenic region on chromosome 17. Let-7g is expressed from an intron of a protein coding gene on chromosome 9 and let-7i is expressed from an intergenic region on chromosome 10. The expression pattern of let-7 shows that the let-7a-1/let-7d/let-7f-1 cluster and the let-7b/let-7c-2 cluster are highly expressed in the heart (data not shown).

[00102] To further examine the role of let-7 in cardiac disease, transgenic mice expressing the let-7a/let-7d/let-7f cluster under the alpha-MHC promoter (let-7 transgenics) were generated. The level of cardiac expression of the let-7 members in the let-7 transgenic mice was comparable

to the let-7 expression levels in calcineurin transgenic mice, a model of stress-induced cardiac hypertrophy (Figure 13B). Shortly after birth, the let-7 transgenic mice exhibit considerable variability in cardiomyocyte size, suggesting that let-7 regulates cardiomyocyte growth, differentiation, and/or proliferation (Figure 14). The cardiac tissue shown in Figure 14 is from a transgenic line in which the transgene likely integrated in the X-chromosome. Since in females, one of the two X-chromosomes is inactivated, only every other cardiomyocyte would express the transgene construct. The small cells shown in the figure are likely to express the transgene construct, while the large cells are “wild-type” cells that show a compensatory growth. The fact that males would express the transgene in every cardiomyocyte could explain why males live only a couple days after postnatal activation of the α -MHC promoter. These results suggest that targeting the let-7 family could be a novel approach to control cardiac hypertrophy in various disease conditions.

[00103] It is understood that the disclosed invention is not limited to the particular methodology, protocols and materials described as these can vary. It is also understood that the terminology used herein is for the purposes of describing particular embodiments only and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

[00104] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

Claims:

1. A method of reducing expression of two or more members of a miRNA family in a cell comprising contacting the cell with an inhibitor, wherein the inhibitor targets two or more members of the miRNA family, and wherein the expression or activity of the two or more miRNA family members is reduced in the cell following contact with the inhibitor.
2. The method of claim 1, wherein the miRNA family is the miR-30 family or the let-7 family.
3. The method of claim 2, wherein said two or more miR-30 family members are selected from the group consisting of miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e.
4. The method of claim 2, wherein said two or more let-7 family members are selected from the group consisting of let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, and let-7i.
5. The method of claim 1, wherein the inhibitor is an antisense oligonucleotide.
6. The method of claim 5, wherein the antisense oligonucleotide has at least one chemical modification.
7. The method of claim 6, wherein said at least one chemical modification is a bicyclic sugar nucleoside modification.
8. The method of claim 5, wherein the antisense oligonucleotide is about 5 to about 25 nucleotides in length.
9. The method of claim 5, wherein the antisense oligonucleotide has a sequence that is at least partially complementary to a consensus sequence derived from said two or more members of the miRNA family.

10. The method of claim 3, wherein the inhibitor reduces the activity or expression of miR-30a, miR-30d, and miR-30e.
11. The method of claim 10, wherein the inhibitor is a nucleic acid comprising a sequence selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 60, SEQ ID NO: 63, and SEQ ID NO: 64.
12. The method of claim 3, wherein the inhibitor reduces the activity or expression of miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e.
13. The method of claim 12, wherein the inhibitor is a nucleic acid comprising the sequence of SEQ ID NO: 61.
14. The method of claim 12, wherein the inhibitor is a nucleic acid comprising one or more miR-30 binding sites.
15. The method of claim 14, wherein the miR-30 binding site has a sequence that is complementary to SEQ ID NO: 19.
16. The method of claim 4, wherein the inhibitor reduces the activity or expression of let-7a, let-7d, and let-7f.
17. The method of claim 4, wherein the inhibitor reduces the activity or expression of let-7a, let-7b, let-7c, let-7d, and let-7f.
18. The method of claim 4, wherein the inhibitor reduces the activity or expression of let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, and let-7i.
19. The method of claim 18, wherein the inhibitor is a nucleic acid comprising the sequence of SEQ ID NO: 62.

20. The method of claim 18, wherein the inhibitor is a nucleic acid comprising one or more let-7 binding sites.
21. The method of claim 20, wherein the let-7 binding site has a sequence that is complementary to SEQ ID NO: 49.
22. The method of claim 1, wherein the inhibitor is a nucleic acid comprising one or more linked antisense oligonucleotides for each of the two or more miRNA family members.
23. The method of claim 1, wherein the cell is *in vitro* or *in vivo*.
24. The method of claim 1, wherein the cell is a cardiomyocyte.
25. A method of designing a nucleic acid for inhibiting the expression or activity of some or all members of a miRNA family comprising:
aligning nucleotide sequences of some or all the members of a miRNA family;
determining a consensus sequence from said aligned nucleotide sequences; and
synthesizing a nucleic acid having a sequence that is complementary to said consensus sequence, wherein the synthesized nucleic acid is capable of inhibiting the expression or activity of some or all members of the miRNA family.
26. The method of claim 25, wherein the miRNA family is the miR-30 family or the let-7 family.
27. A method of treating or preventing pathologic cardiac hypertrophy, cardiac remodeling, heart failure, or myocardial infarction in a subject in need thereof comprising administering to the subject an inhibitor of at least one miR-30 family member, wherein the expression or activity of the at least one miR-30 family member is reduced in heart cells of the subject following administration of the inhibitor.

28. The method of claim 27, wherein the at least one miR-30 family member is selected from the group consisting of miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e.
29. The method of claim 27, wherein the expression or activity of two or more miR-30 family members is reduced.
30. A method of treating or preventing pathologic cardiac hypertrophy, cardiac remodeling, heart failure, or myocardial infarction in a subject in need thereof comprising administering to the subject an inhibitor of at least one let-7 family member, wherein the expression or activity of the at least one let-7 family member is reduced in heart cells of the subject following administration of the inhibitor.
31. The method of claim 30, wherein said at least one let-7 family member is selected from the group consisting of let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, and let-7i.
32. The method of claim 30, wherein the expression or activity of two or more let-7 family members is reduced.
33. A method of modulating calcium signaling in a cell comprising delivering to the cell a modulator of one or more miR-30 family members.
34. The method of claim 33, wherein said one or more miR-30 family members is selected from the group consisting of miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e.
35. The method of claim 33, wherein the modulator is an agonist of one or more miR-30 family members and wherein calcium signaling is reduced in the cell following delivery of the agonist.
36. The method of claim 35, wherein the expression or activity of one or more genes selected from the group consisting of adrenergic receptor, endothelin receptor type A, regulator of G-protein signaling 2 (RGS2), calcium/calmodulin-dependent protein kinase II delta (CaMKIId), G

protein-coupled receptor kinase 5, and Calpain 7 is reduced in the cell following delivery of the agonist.

37. The method of claim 33, wherein the modulator is an inhibitor of one or more miR-30 family members and wherein calcium signaling is increased in the cell following delivery of the inhibitor.

38. The method of claim 37, wherein the expression or activity of one or more genes selected from the group consisting of adrenergic receptor, endothelin receptor type A, regulator of G-protein signaling 2 (RGS2), calcium/calmodulin-dependent protein kinase II delta (CaMKIId), G protein-coupled receptor kinase 5, and Calpain 7 is increased in the cell following delivery of the inhibitor.

39. The method of claim 33, wherein the cell is a cardiomyocyte.

40. The method of claim 39, wherein the cardiomyocyte is *in vivo* or *in vitro*.

41. A method of improving endothelial function or promoting endothelial regeneration after vascular injury in a subject comprising administering to the subject an inhibitor of one or more miR-30 family members, wherein the expression or activity of the one or more miR-30 family members is reduced in endothelial cells of the subject following administration of the inhibitor.

42. The method of claim 41, wherein said one or more miR-30 family members is selected from the group consisting of miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e.

43. The method of claim 41, wherein the activity or expression of dimethylarginine dimethylaminohydrolase 1 is increased in the endothelial cells of the subject following administration of the inhibitor.

44. The method of claim 41, wherein the activity or expression of nitric oxide synthase is increased in the endothelial cells of the subject following administration of the inhibitor.

45. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an inhibitor of two or more members of a miRNA family.
46. The composition of claim 45, wherein the miRNA family is the miR-30 family or the let-7 family.
47. The composition of claim 46, wherein said two or more miR-30 family members are selected from the group consisting of miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e.
48. The composition of claim 46, wherein said two or more let-7 family members are selected from the group consisting of let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, and let-7i.
49. The composition of claim 47, wherein the inhibitor reduces the activity or expression of miR-30a, miR-30d, and miR-30e.
50. The composition of claim 49, wherein the inhibitor is a nucleic acid comprising a sequence selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 60, SEQ ID NO: 63, and SEQ ID NO: 64.
51. The composition of claim 47, wherein the inhibitor reduces the activity or expression of miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e.
52. The composition of claim 51, wherein the inhibitor is a nucleic acid comprising the sequence of SEQ ID NO: 61.
53. The composition of claim 51, wherein the inhibitor is a nucleic acid comprising one or more miR-30 binding sites.
54. The composition of claim 53, wherein the miR-30 binding site has a sequence that is complementary to SEQ ID NO: 19.

55. The composition of claim 48, wherein the inhibitor reduces the activity or expression of let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, and let-7i.

56. The composition of claim 55, wherein the inhibitor is a nucleic acid comprising the sequence of SEQ ID NO: 62.

57. The composition of claim 55, wherein the inhibitor is a nucleic acid comprising one or more let-7 binding sites.

58. The composition of claim 57, wherein the let-7 binding site has a sequence that is complementary to SEQ ID NO: 49.

59. The composition of claim 45, wherein the inhibitor is a nucleic acid comprising one or more linked antisense oligonucleotides for each of the two or more miRNA family members.

60. The composition of claim 45, wherein the composition is formulated for injection.

61. The composition of claim 45 in combination with a kit for parenteral administration.

62. The composition of claim 61, wherein parenteral administration is intravenous, subcutaneous, or catheter administration.

63. The composition of claim 45, wherein the inhibitor is an antisense oligonucleotide.

64. The method of claim 63, wherein the antisense oligonucleotide has at least one chemical modification.

65. The method of claim 64, wherein said at least one chemical modification is a bicyclic sugar nucleoside modification.

66. The method of claim 63, wherein the antisense oligonucleotide is about 5 to about 25 nucleotides in length.

67. The method of claim 63, wherein the antisense oligonucleotide has a sequence that is at least partially complementary to a consensus sequence derived from said two or more members of the miRNA family.

FIGURE 1

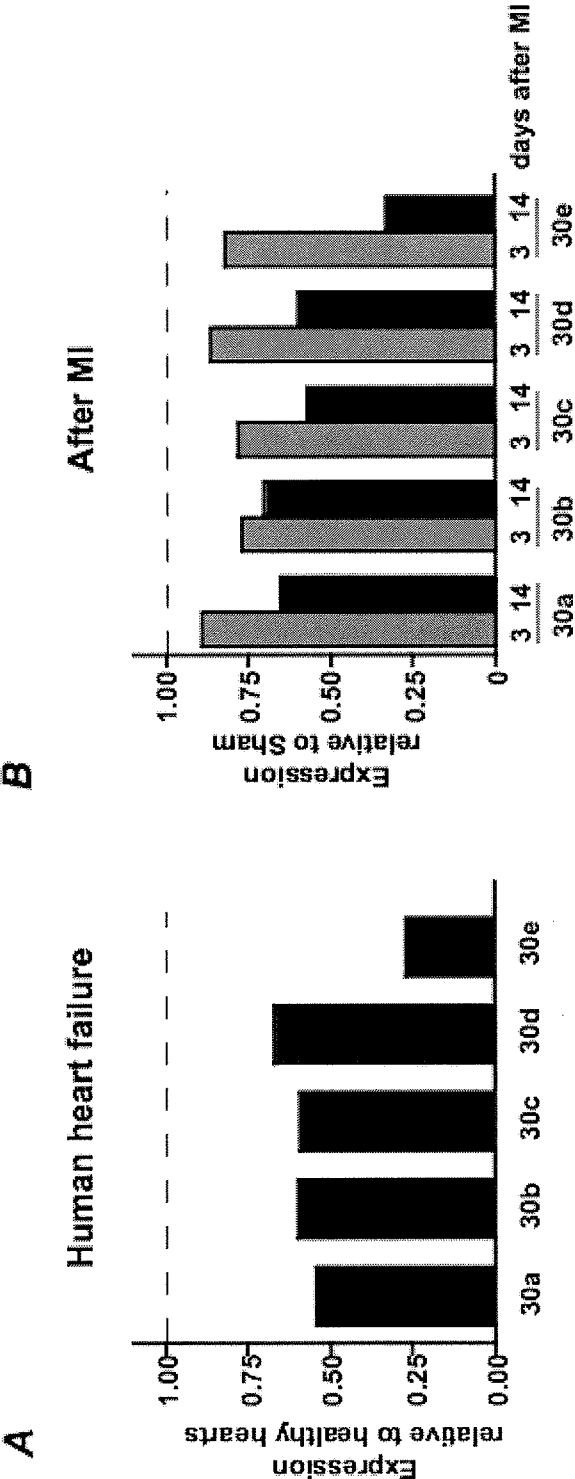


FIGURE 2

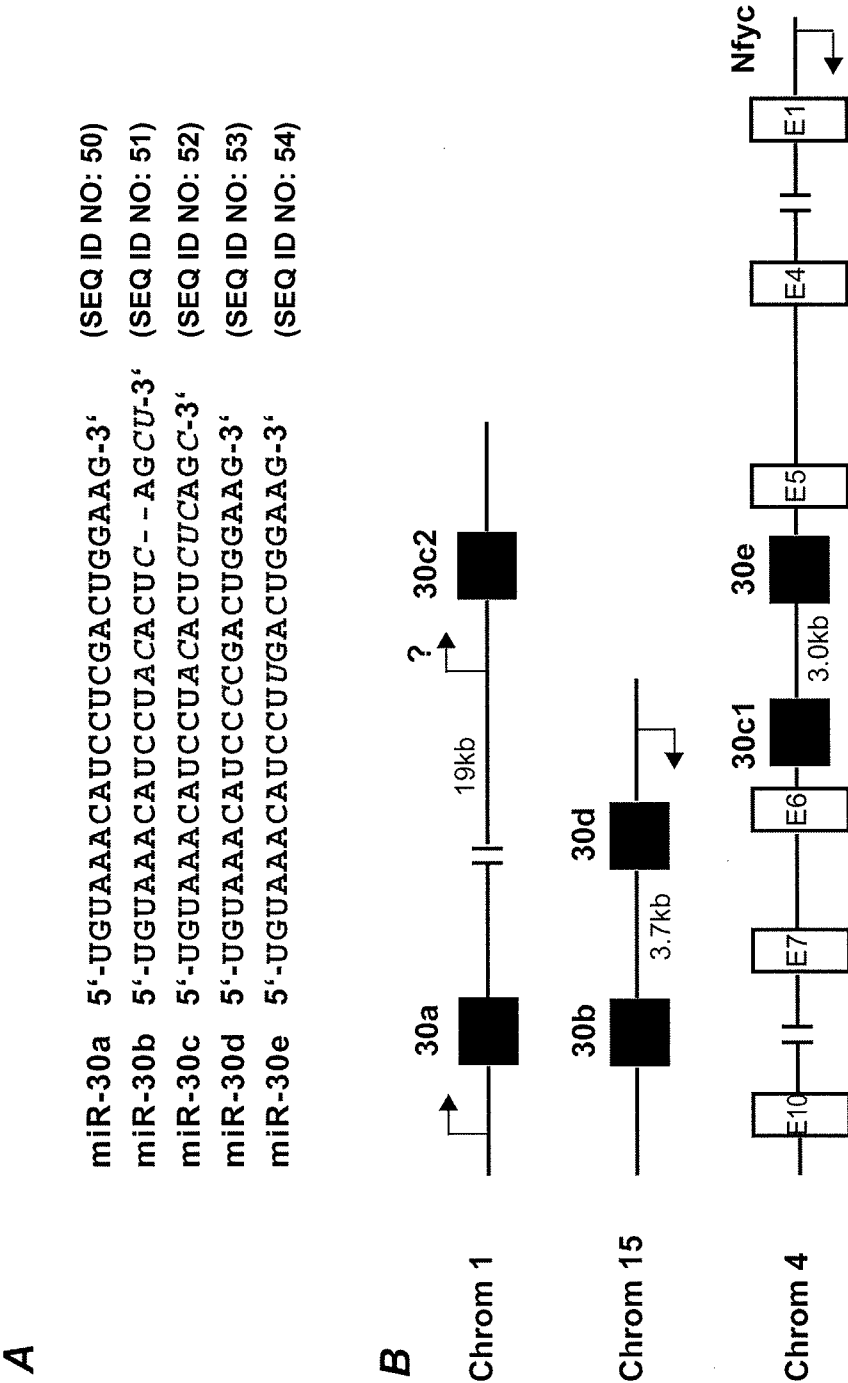


FIGURE 3

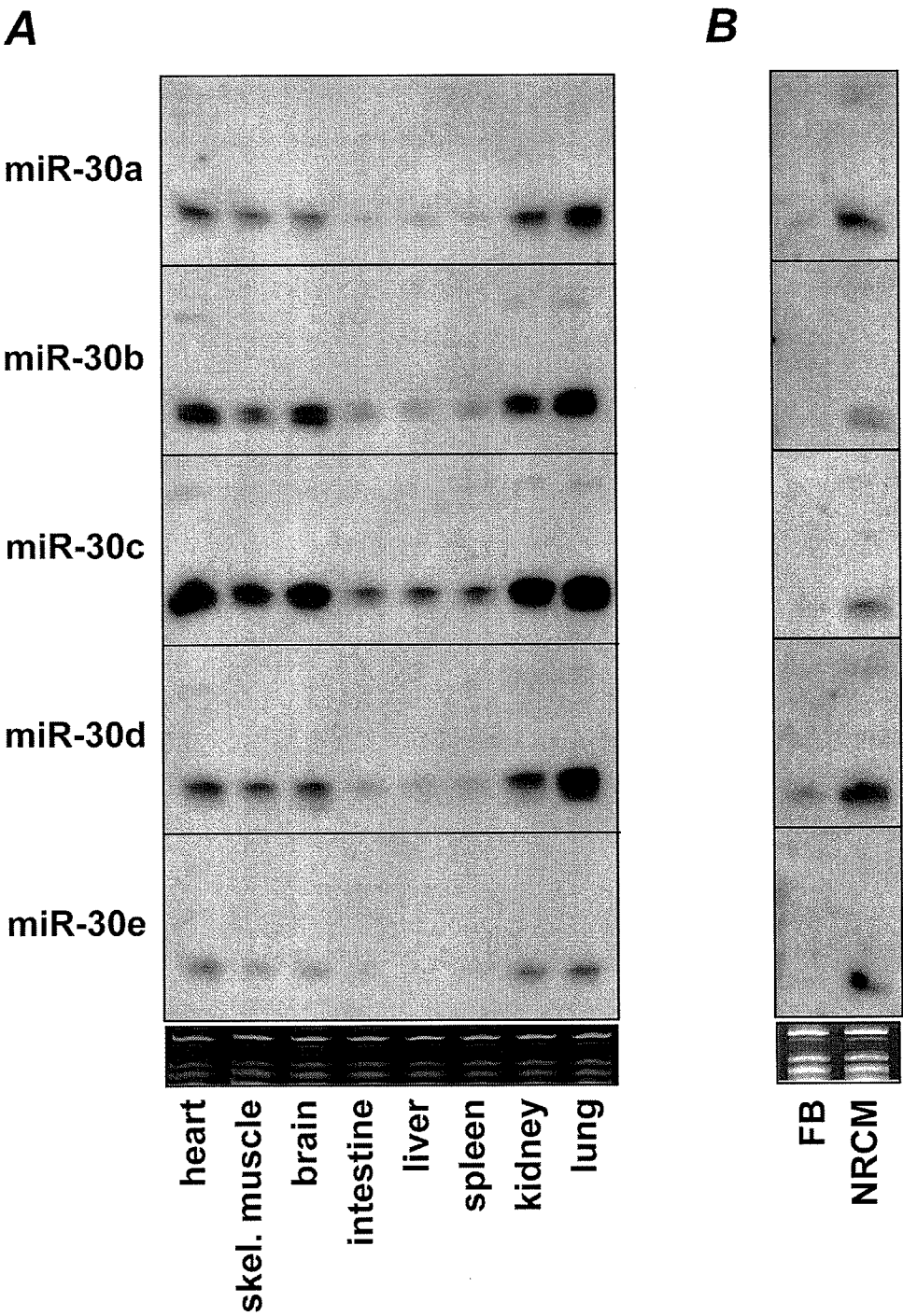


FIGURE 4

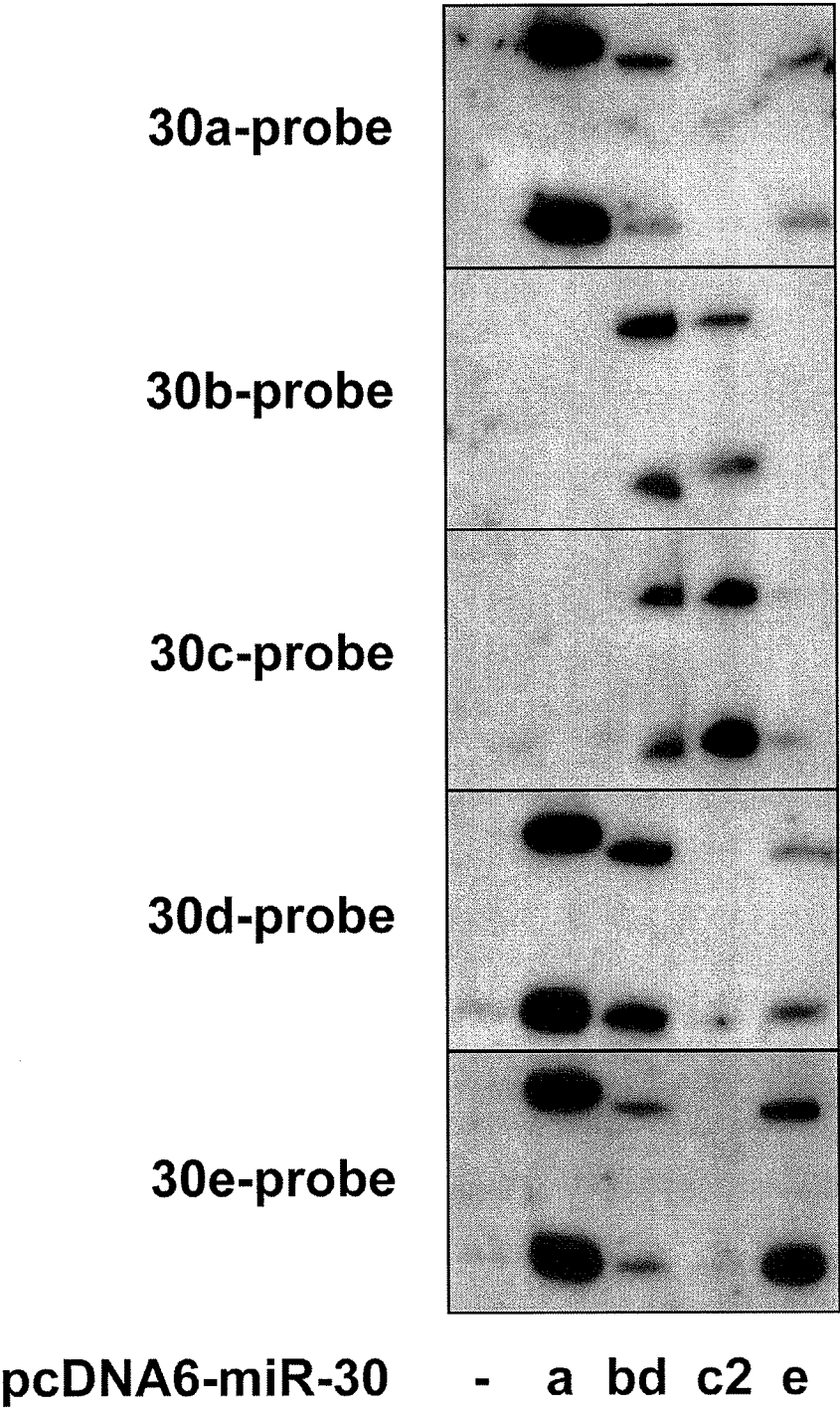


FIGURE 5A

A

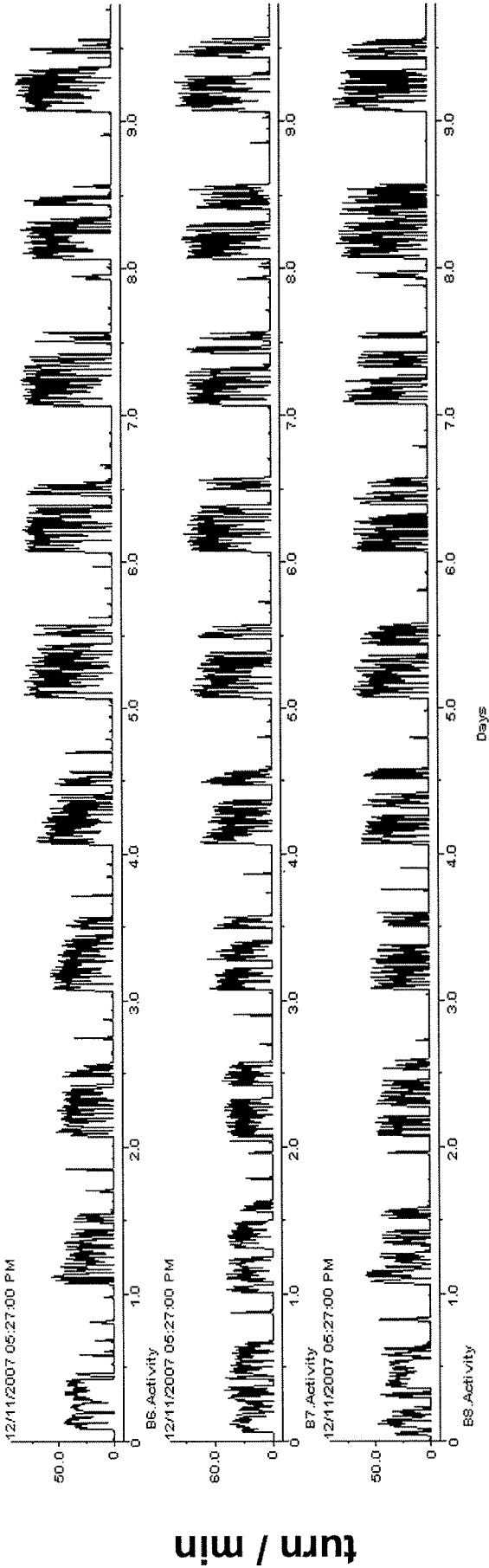


FIGURE 5B-D

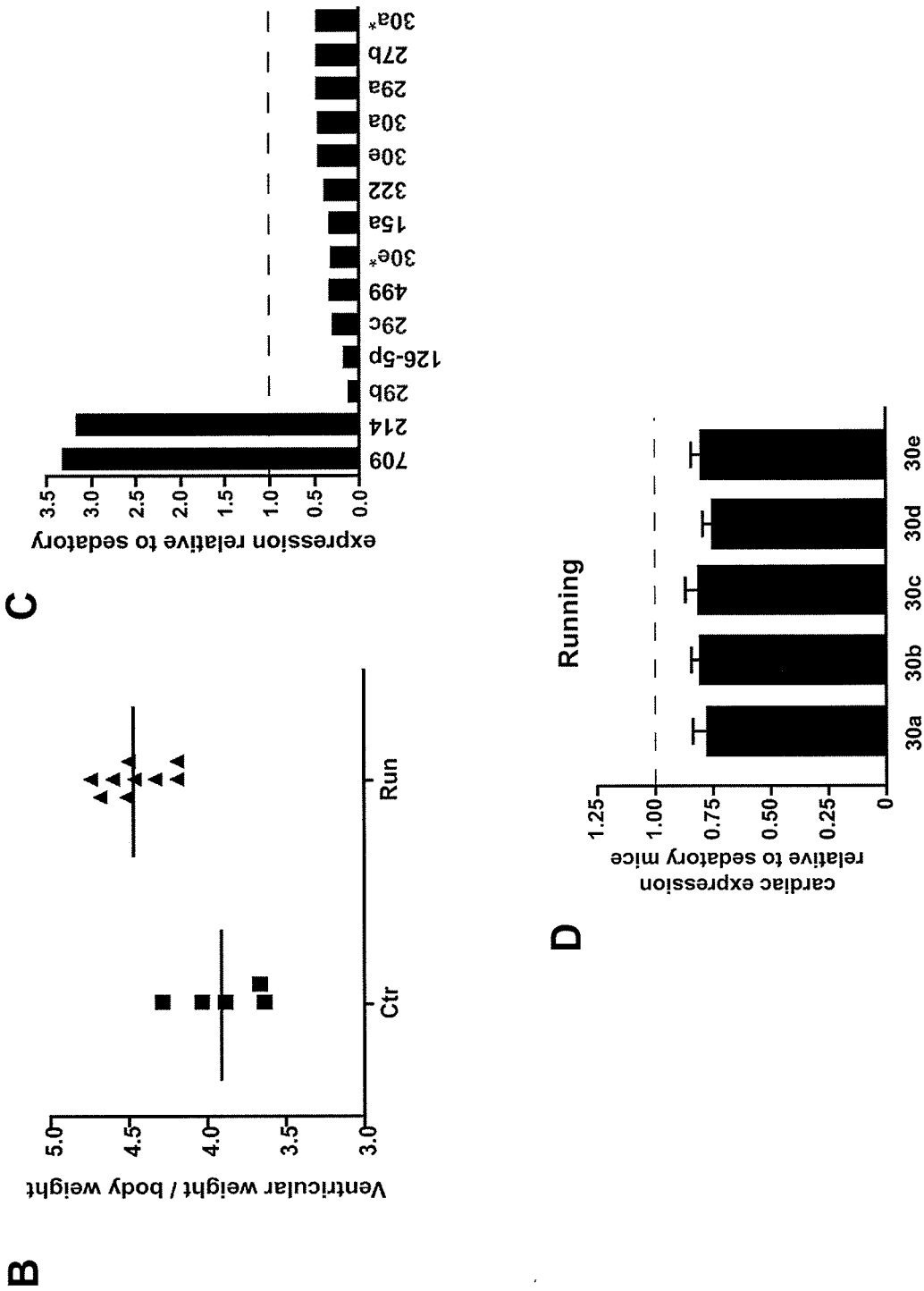


FIGURE 6A-B

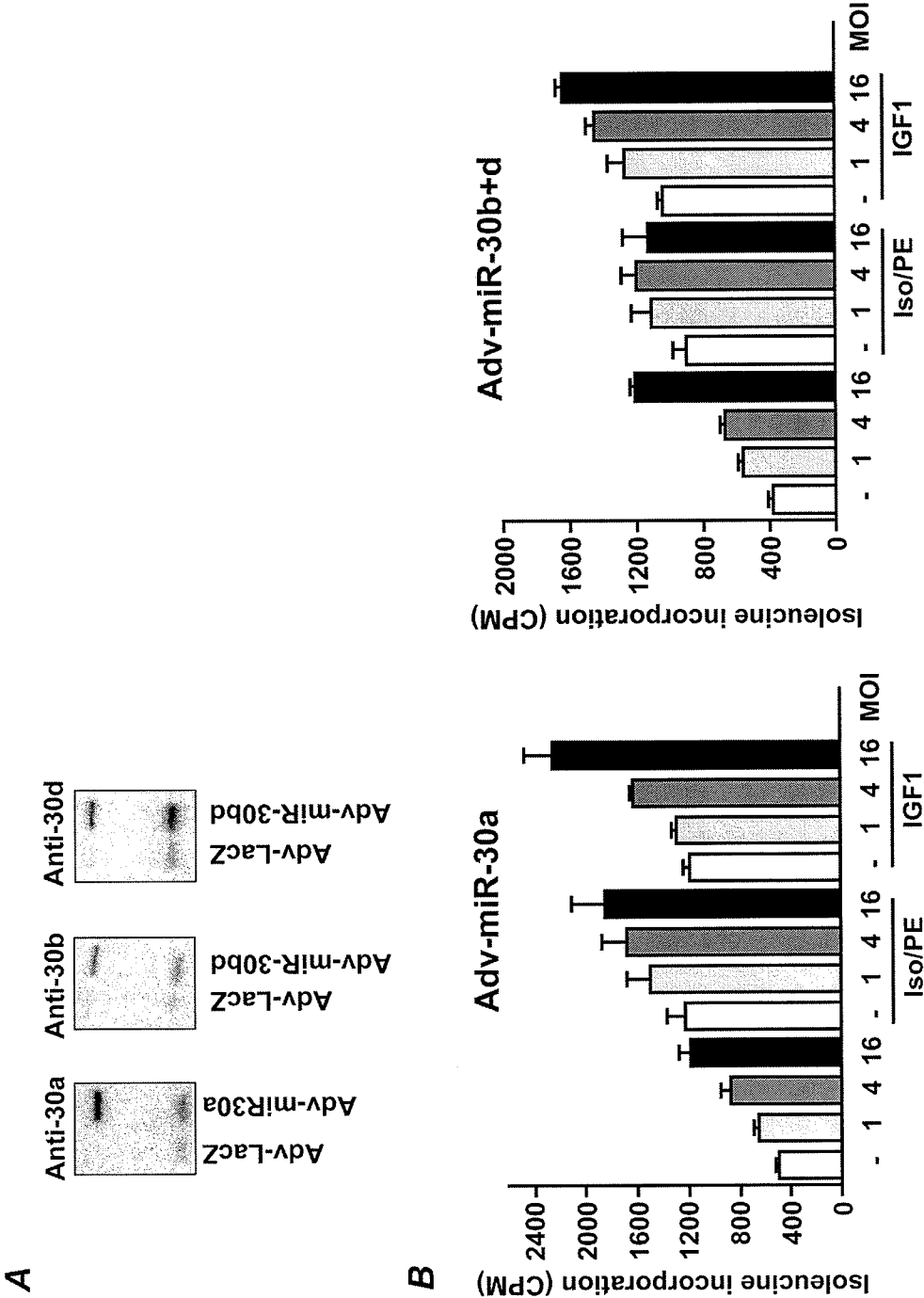


FIGURE 7A-C

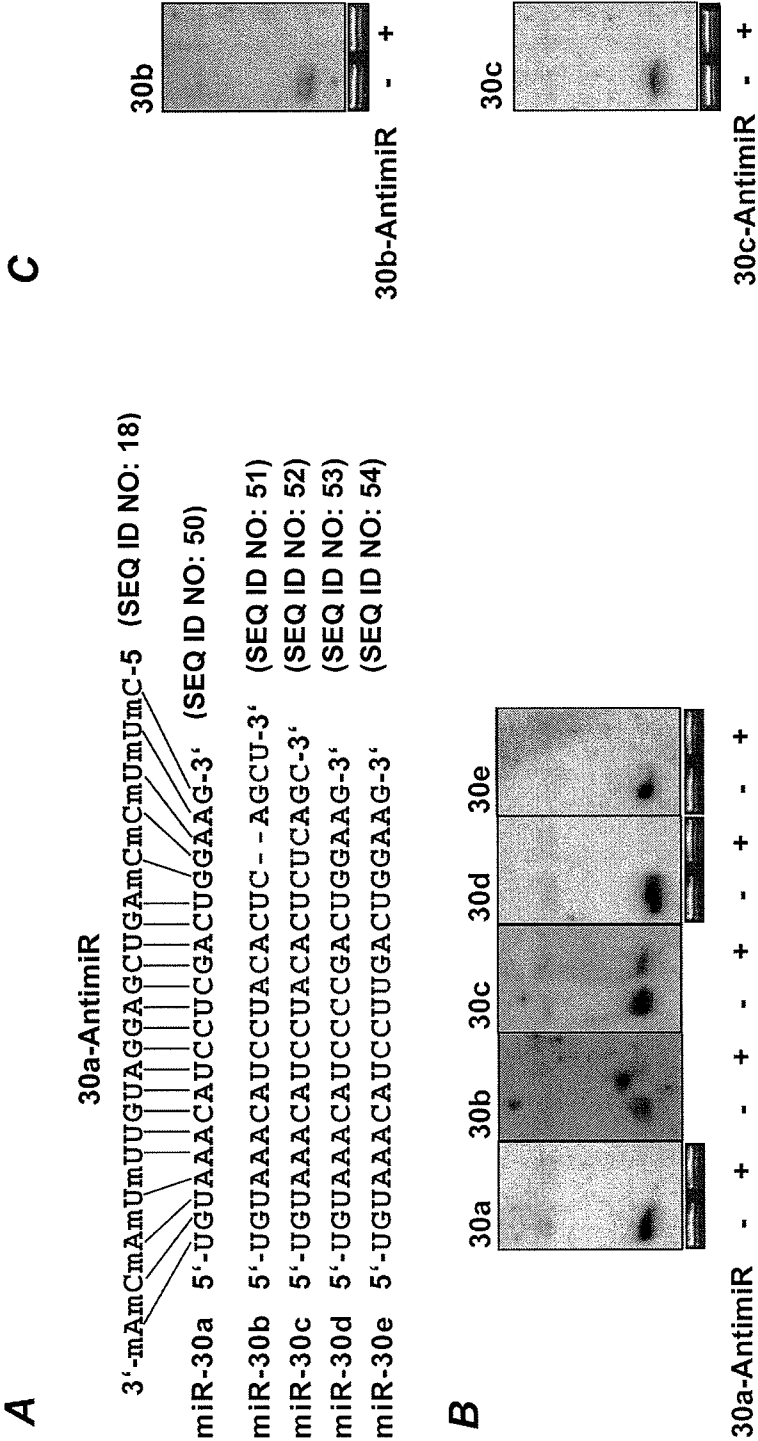


FIGURE 8

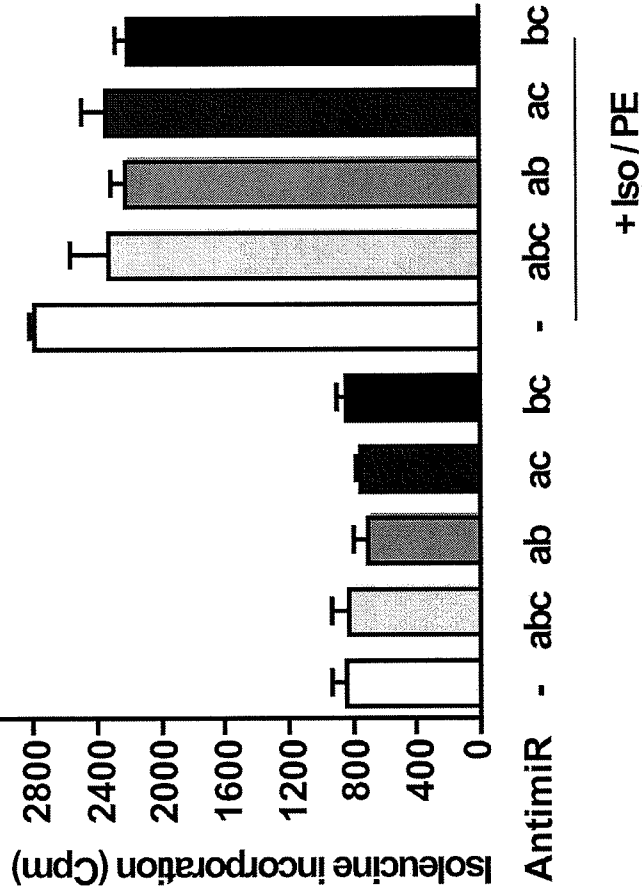


FIGURE 9A-B

A miR-30 transgenic mice develop severe heart failure

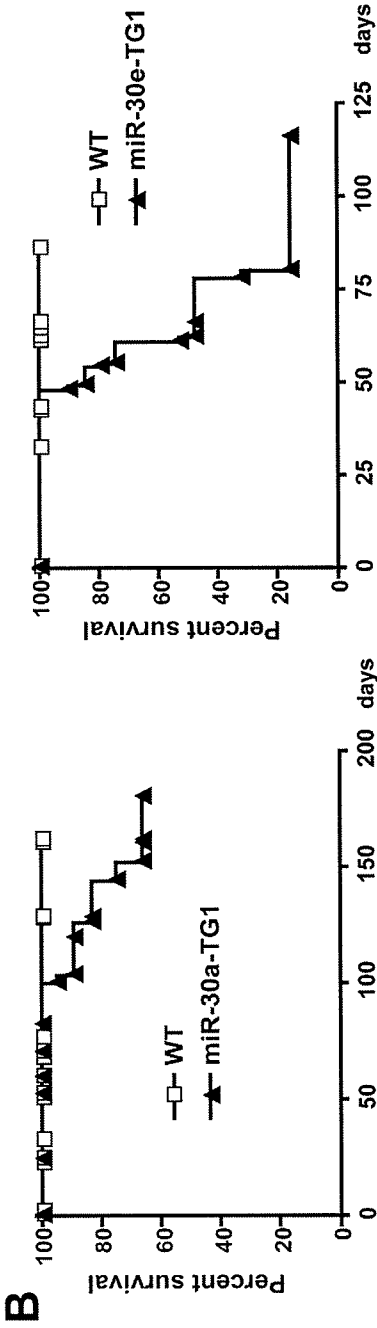
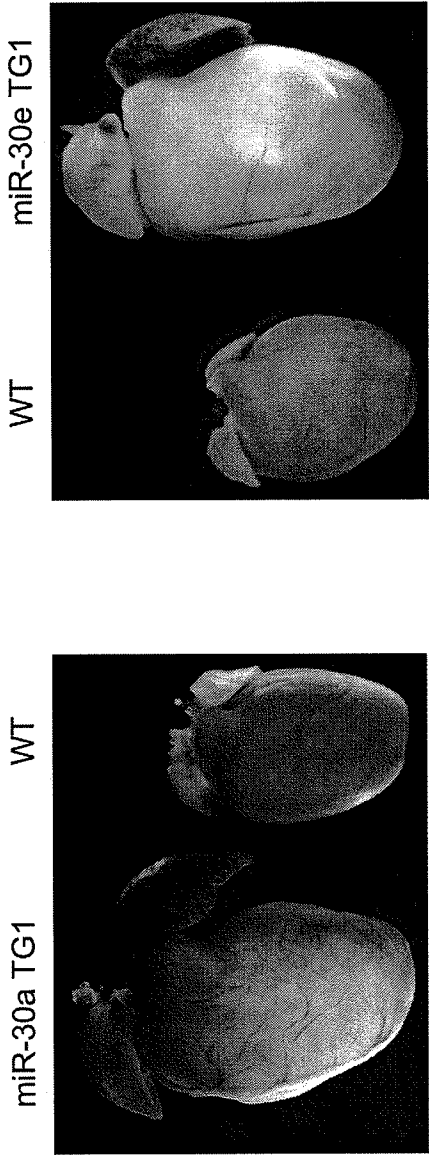


FIGURE 10

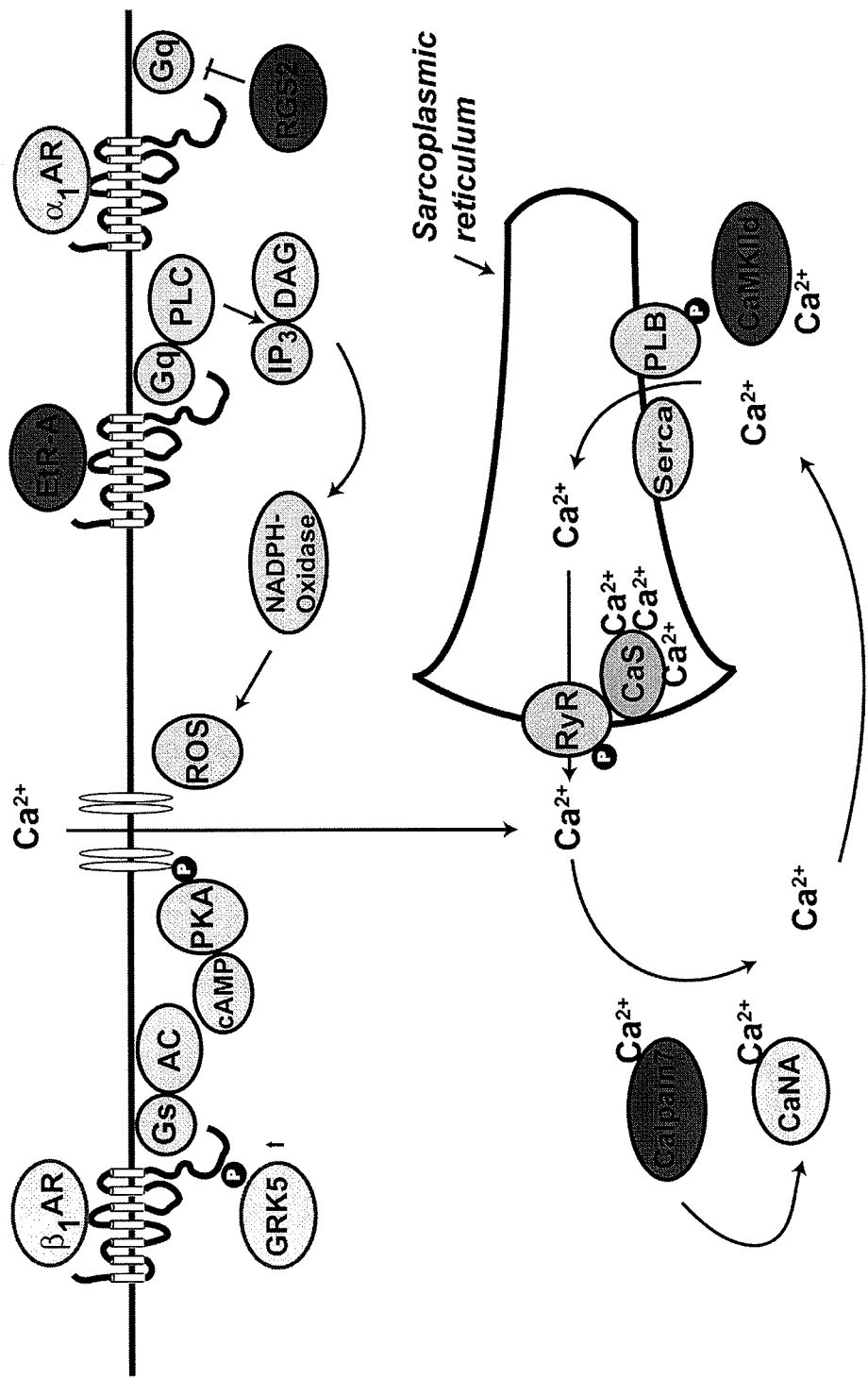
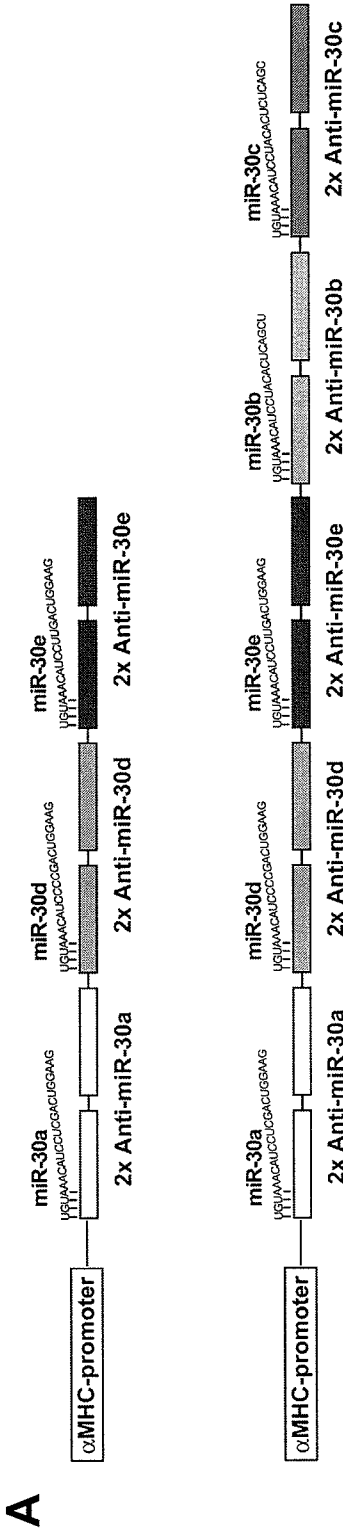


FIGURE 11A-B

Generation of miR-30 eraser



B De-repression of putative miR-30 target in eraser-mice:

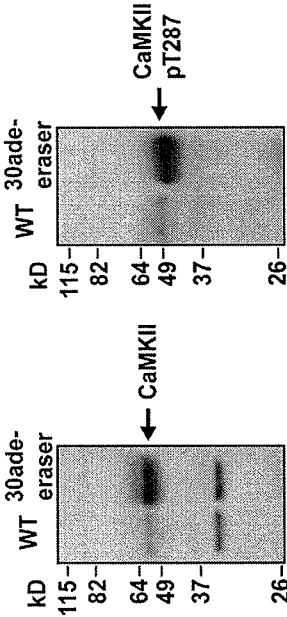


FIGURE 13A-B

Up-regulation of let-7 in cardiac disease

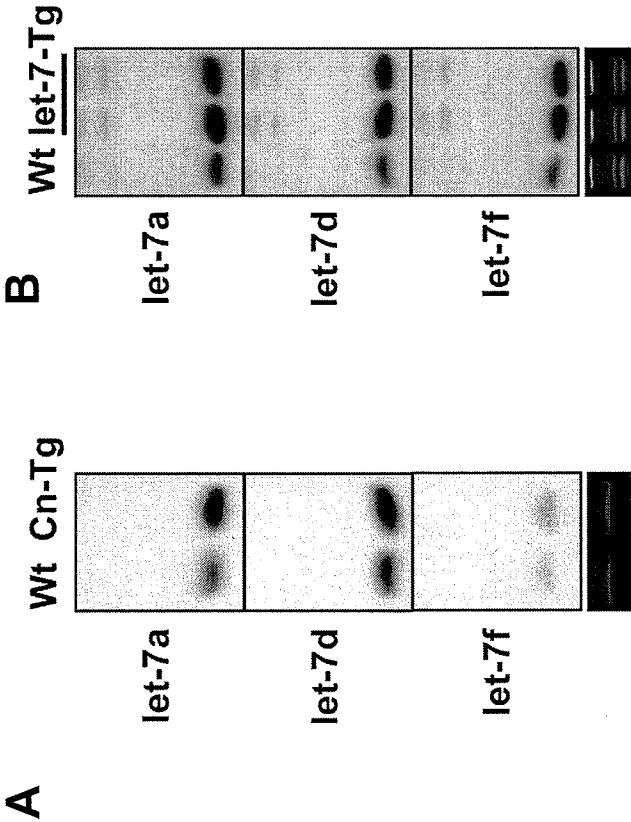
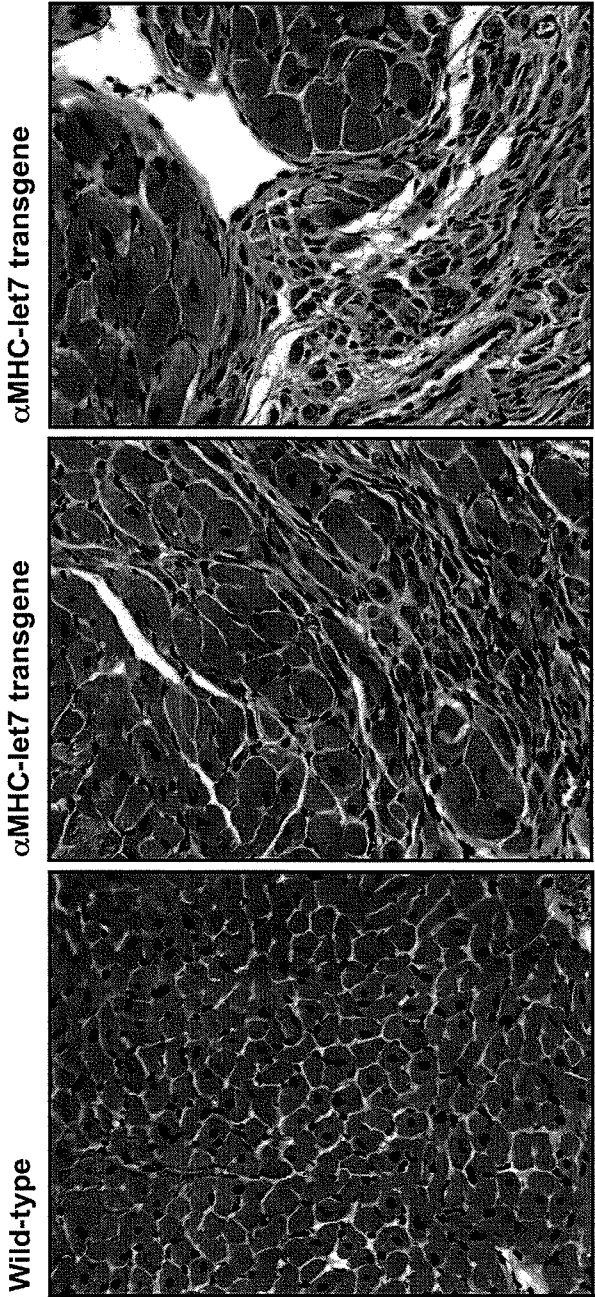


FIGURE 14

High variability in cardiomyocyte size in let-7 transgenic mice



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 10/31147

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/70; C12N 15/00 (2010.01)

USPC - 514/44A; 435/455

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)

IPC(8) - A61K 31/70; C12N 15/00 (2010.01)

USPC - 514/44A; 435/455

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
 PubWEST(USPT,PGPB,EPAB,JPAB); Medline, Google: miRNA, microRNA, family, inhibitor, two, microRNA sponge, miR-30, let-7?, antisense, oligonucleotide, chemical modification, bicyclic sugar, nucleoside, locked nucleic acid, LNA, partially complementary, binding, reduce, activity, expression, linked, in vitro, in vivo, cardiomyocyte, pharmaceutical. Ge

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	EBERT et al. MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. Nat Methods. 2007, 4(9):721-726; Abstract; pg 722, col 2, para 1; pg 723, Fig 3; pg 724, col 1, top para; and pg 725, col 1, last para, and col 2, para 1	1-3, 5-9, 22-23 ----- 4, 10-12, 14, 16-18, 20, 24, 45-51, 53, 55, 57, 59-67
Y	US 2008/0176766 A1 (BROWN et al.) 24 July 2008 (24.07.2008) Abstract, para [[0035], [0047], [0050], [0061], [0108], [0208], [0288], [0296]-[0297], [0304], [0306], and Table 1	4, 10-12, 14, 16-18, 20, 24, 45-51, 53, 55, 57, 59-67
Y	US 2006/0292616 A1 (NEELY et al.) 28 December 2006 (28.12.2006) para [0081] and SEQ ID NO: 7	11, 50
A	US 2008/0119427 A1 (BHAT et al.) 22 May 2008 (22.05.2008) para [0021]	7, 65

☐ 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

30 August 2010 (30.08.2010)

Date of mailing of the international search report

14 SEP 2010

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

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 10/31147

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

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

a. (means)

☐

on paper

☒

in electronic form

b. (time)

☐

in the international application as filed

☒

together with the international application in electronic form

☐

subsequently to this Authority for the purposes of search

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

3. Additional comments:

GenCore 6.3: SEQ ID NO:18

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 10/31147

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.:
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:

Group I+: claims 1-24, 45-67, drawn to a method of reducing expression of two or more members of a miRNA family in a cell comprising contacting the cell with an inhibitor, wherein the inhibitor targets two or more members of the miRNA family, and wherein the expression or activity of the two or more miRNA family members is reduced in the cell following contact with the inhibitor. The first invention encompasses an inhibitor of SEQ ID NO:18. Should an additional fee(s) be paid, Applicant is invited to elect an additional SEQ ID NO(s) to be searched. The exact claims searched will depend on the specifically elected SEQ ID NO(s).

[NOTE: Claims 13, 15, 19, 21, 52, 54, 56, and 58 were excluded from Group I because they are drawn to a nonelected subject matter.]

Group II, claims 25-26, drawn to a method of designing a nucleic acid for inhibiting the expression or activity of some or all members of a miRNA family.

*****Continued in the extra sheet*****

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.:
1-12, 14, 16-18, 20, 22-24, 45-51, 53, 55, 57, and 59-67, limited to SEQ ID NO: 18

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.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 10/31147

***** Supplemental Box *****

Continuation of: Box No. III (unity of invention is lacking)

Group III, claims 27-29, 33-44, drawn to a method comprising administering to the subject an inhibitor of at least one miR-30 family member, wherein the expression or activity of the at least one miR-30 family member is reduced in heart cells of the subject following administration of the inhibitor.

Group IV, claims 30-32, drawn to a method comprising administering to the subject an inhibitor of at least one let-7 family member, wherein the expression or activity of the at least one let-7 family member is reduced in heart cells of the subject following administration of the inhibitor.

The inventions listed as Groups I+ through 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 inventions of Groups III-IV do not include the inventive concept of an inhibitor that targets two or more members of the miRNA family, as required by Groups I+ and II.

The inventions of Group I+ do not include the inventive concept of designing a nucleic acid for inhibiting the expression or activity of some or all members of a miRNA family, as required by Group II.

The inventions of Group III do not include the inventive concept of administering to the subject an inhibitor of at least one let-7 family member, as required by Group IV

The inventions of Group IV do not include the inventive concept of administering to the subject an inhibitor of at least one miR-30 family member, as required by Group III.

The inventions of Groups I+ and II share the technical feature of an inhibitor that targets two or more members of the miRNA family. However, this shared technical feature does not represent a contribution over prior art. Specifically, a paper titled "MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells" by EBERT et al. (Nat Methods 2007, 4(9):721-726) discloses an inhibitor (pg 723, col 2, lower para - 'HeLa cells with target reporters and sponges against two micro-RNAs with different seeds: ...In HeLa cells, microRNA expression profiling detects high levels of miR-30c and miR-30d, and a much lower level of miR-30e') ---wherein the inhibitor targets two or more members of the miRNA family (Abstract - 'we developed microRNA inhibitors that can be expressed in cells, ... Termed "microRNA sponges".... such that a single sponge can be used to block an entire microRNA seed family'; pg 724, col 1, top para - 'the spongedesigned to miR-30e derepressed the target by over fourfold, suggesting cross-reactivity with the more abundant miR-30 family members'), and ---wherein the expression or activity of the two or more miRNA family members is reduced in the cell following contact with the inhibitor (Abstract - 'we developed microRNA inhibitors that can be expressed in cells, ... Termed "microRNA sponges".... such that a single sponge can be used to block an entire microRNA seed family'; pg 723, col 2, lower para - 'we transfected HeLa cells with target reporters and sponges against two micro- RNAs with different seeds: ...Dose-dependent derepression was apparent in samples'; Fig 3). As said inhibitor targeting two or more members of the miRNA family was known at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups.

The inventions of Group I+ share the technical feature of a method of reducing expression of two or more members of a miRNA family in a cell comprising contacting the cell with an inhibitor that targets two or more members of the miRNA family. However, this shared technical feature does not represent a contribution over EBERT et al. that discloses "microRNA inhibitors that can be expressed in cells, ... Termed "microRNA sponges", these competitive inhibitors are transcripts expressed from strong promoters, containing multiple, tandem binding sites to a microRNA of interest.... such that a single sponge can be used to block an entire microRNA seed family' (Abstract).

Another technical feature of the inventions listed as Group I+ is the specific nucleic acid sequence recited therein. The inventions do not share a special technical feature, because US 2006/0292616 A1 to Neely, et al. teaches the corresponding DNA sequence (SEQ ID NO 7). In addition, US 2004/0053411 A1 to Cullen, et al. discloses 22-nucleotide-long siRNA comprising 1-21 nucleotides of the claimed SEQ ID NO:18. Without a shared special technical feature, the inventions lack unity with one another.

Groups I+ through IV therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.