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(54) **Title**: MICRO-RNA THAT REGULATES CARDIAC REMODELING

(57) **Abstract**: The present invention provides a method of treating or preventing pathologic cardiac hypertrophy, cardiac remodeling, myocardial infarction, or heart failure in a subject by inhibiting the expression or activity of miR-451 in heart cells of the subject. Methods of regulating cardiomyocyte survival and Akt signaling by modulating miR-451 expression or activity are also disclosed.

MICRO-RNA THAT REGULATES CARDIAC REMODELING

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims the benefit of priority of U.S. Provisional Application No. 61/176,698, filed May 8, 2009, which is herein incorporated by reference in its entirety.

STATEMENT OF GOVERNMENT SUPPORT

[002] This invention was made with government support under Grant Number HL093039 awarded by the National Institute of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

[003] The present invention relates to the treatment of cardiac disorders by administering agents that modulate the activity or expression of a microRNA (miRNA). In particular, the invention provides a method for treating or preventing cardiac disorders by inhibiting the expression or activity of miR-451 in the heart cells of a subject. In addition, the invention provides a method for regulating cardiomyocyte survival by contacting the cardiomyocyte with an inhibitor of miR-451.

BACKGROUND OF THE INVENTION

[004] 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.

[005] 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.

[006] 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%.

[007] 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.

[008] MicroRNAs have recently been implicated in a number of biological processes including regulation of developmental timing, apoptosis, fat metabolism, and hematopoietic cell differentiation among others. MicroRNAs (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.

[009] 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.

[0010] Recently, signature expression patterns of miRNAs associated with pathological cardiac hypertrophy, heart failure and myocardial infarction in humans and mouse models of heart disease have been identified (van Rooij *et al* (2006) Proc. Natl. Acad. Sci., Vol. 103(48):18255-60; van Rooij *et al.*, (2007) *Science*, Vol. 316: 575-579). Gain- and loss-of-function studies in mice have revealed profound and unexpected functions for these miRNAs in numerous facets of cardiac biology, including the control of myocyte growth, contractility, energy metabolism, fibrosis, and angiogenesis, providing glimpses of new regulatory mechanisms and potential therapeutic targets for heart disease. Remarkably, knockout mice lacking disease-inducing miRNAs are normal, but display aberrant responses to cardiac stress, suggesting the dedication of these miRNAs to disease-related processes rather than tissue homeostasis, and pointing to their potential as therapeutic targets. Thus, these identified miRNAs represent potential novel therapeutic targets for the development of treatments for a variety of cardiovascular diesases.

SUMMARY OF THE INVENTION

[0011] The present invention is based, in part, on the discovery that the expression of miR-451 is regulated in heart tissue following myocardial infarction and that overexpression of miR-451 in a cardiac-specific manner is sufficient to induce cardiac hypertrophy. The inventors have surprisingly found that miR-451 regulates cardiomyocyte survival and cardiogenesis. Accordingly, the present invention provides a method of treating or preventing pathologic cardiac hypertrophy, heart failure, cardiac remodeling, and myocardial infarction in a subject in need thereof. In one embodiment, the pathologic cardiac hypertrophy is associated with pulmonary arterial hypertension.

[0012] In one embodiment, the method comprises administering an inhibitor of miR-451 to the subject, wherein the expression or activity of miR-451 is reduced in the heart cells of the subject following administration. The inhibitor of miR-451 can be an antagomir or an antisense oligonucleotide. In some embodiments, the number of cardiomyocytes is increased

in the subject following administration of the miR-451 inhibitor as compared to a subject not receiving the miR-451 inhibitor.

[0013] The present invention also includes a method of regulating cardiomyocyte survival. In one embodiment, the method comprises contacting a cardiomyocyte with an inhibitor of miR-451. The cardiomyocyte can be *in vitro* or *in vivo*. In certain embodiments, cardiomyocyte survival is enhanced following contact with the miR-451 inhibitor. In other embodiments, Akt signaling is increased in the cardiomyocyte following contact with the miR-451 inhibitor. In still other embodiments, the expression of one or more genes regulated by miR-451 is increased in the cardiomyocyte following contact with the miR-451 inhibitor. Genes targeted by miR-451 can include AKTIP, OSR1, DKK1, and DKK3.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Figure 1. A. Northern blot analysis for miR-451 of RNA isolated from multiple mouse tissues. **B.** Real-time RT-PCR analysis for miR-451 of RNA harvested from neonatal rat cardiomyocytes or fibroblasts. **C.** Real-time RT-PCR analysis for miR-451 was performed on RNA from either whole animal or whole heart collected at embryonic days 12.5 and 15.5, and post-natal days 1, 3, and 5.

[0015] Figure 2. A. Rat vascular smooth muscle cells were differentiated for 6 days. Real-time RT-PCR analysis shows that miR-451 is induced during smooth muscle cell differentiation. **B.** C2C12 myoblasts were differentiated for 5 days. Real-time RT-PCR analysis shows that miR-451 levels are approximately 10-fold higher in differentiated versus undifferentiated myoblasts.

[0016] Figure 3. Real-time RT-PCR analysis of RNA from tissue harvested from the border zone of an infarct at 6- and 48-hours post-myocardial infarction. MiR-451 is significantly downregulated as compared to sham operated heart tissue.

[0017] Figure 4. Schematic of targeting strategy for generation of miR-451 conditional knockout mice.

[0018] Figure 5. Northern blot analysis (left panel) of cardiac tissue from a 6-week old transgenic mouse overexpressing miR-451 under the control of the α-MHC promoter. Densitometry analysis is shown in the right panel.

[0019] Figure 6. A. Absolute (left panel) and normalized (right panel) heart weight/body weight ratios from α-MHC-miR-451 transgenic animals and wild-type litter mates at 6 weeks

of age. **B.** Overexpression of miR-451 induces cardiac hypertrophy which progresses into dilated cardiomyopathy.

[0020] Figure 7. H & E stained (top panels) and Masson trichome stained (bottom panels) sections from wild-type littermates and α-MHC-miR-451 transgenic animals at 6 weeks of age and post mortem.

[0021] Figure 8. H & E stained (top panels) and Masson trichome stained (bottom panels) sections from wild-type littermates and α-MHC-miR-451 transgenic animals at 6 weeks of age and post mortem. Magnification-20x.

[0022] Figure 9. H & E stained section from an α-MHC-miR-451 transgenic animal post mortem showing loss of cadiomyoctyes.

[0023] Figure 10. H & E stained sections at 5x (top panels) and 20x (bottom panels) from wild-type littermates and α-MHC-miR-451 transgenic animals at 6 weeks of age and post mortem illustrating abnormalities in the atria of the hearts of the α-MHC-miR-451 transgenics.

[0024] Figure 11. A. Western blot analysis of cardiac tissue isolated from either transgenic mice overexpressing miR-451 or wild-type litter mates. Expression of GAPDH was used as a loading control. **B.** Schematic illustrating putative mechanism of miR-451 in cardiac remodeling.

[0025] Figure 12. GATA4 binds to both conserved GATA binding sequences (451-site1 and 451-site2) located in the enhancer region upstream of the miR-451 primary sequence as shown by gel shift.

[0026] Figure 13. GATA4 binding to the conserved sequences in the miR-451 enhancer region is specific. Cold wild-type oligo effectively competes for GATA binding to the labeled probe and mutant cold competitor (S1m and S2m) does not abolish the gel shift.

[0027] Figure 14. Relative luciferase activities of COS cells co-transfected with GATA4 and a miR-451 luciferase reporter construct having a wild-type (WT) GATA binding site or a mutation in site-1, site-2, or both sites (double mutant). GATA4 activation of the miR-451 luciferase reporter is dependent on both of the conserved GATA binding sites.

DETAILED DESCRIPTION OF THE INVENTION

[0028] Chronic and acute stress to the heart results in a pathological remodeling response accompanied by hypertrophy, fibrosis, myocyte apoptosis and eventual death from pump failure and arrhythmias. While classical pharmacological treatment strategies (*e.g.* beta-

blockers and ACE-inhibitors) can prolong survival in heart failure patients, these therapies are ultimately ineffective in preventing progression of the disease, underscoring the need for new mechanistic insights and therapeutic approaches.

[0029] MiRNAs act as negative regulators of gene expression by inhibiting the translation or promoting the degradation of target mRNAs. Because individual miRNAs often regulate the expression of multiple target genes with related functions, modulating the expression of a single miRNA can, in principle, influence an entire gene network and thereby modify complex disease phenotypes.

[0030] Thus, the present invention is based, in part, on the discovery of a miRNA that is highly expressed in the heart and is regulated in various cardiac disease states. In particular, the inventors have surprisingly discovered that overexpression of miR-451 under the control of the α-MHC promoter induces cardiac hypertrophy and leads to significant cardiac remodeling. Furthermore, overexpression of miR-451 causes abnormalities in the atria and cardiomyocyte loss. Accordingly, the present invention provides a method of treating various cardiac disorders in a subject by inhibiting the expression or activity of miR-451 in heart cells, particularly cardiomyocytes.

[0031] 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.

[0032] In humans, miR-451 is clustered with miR-144 in an intergenic region of chromosome 17. The mature miR-451 is processed from a bicistronic transcript also encoding miR-144. The mature human sequence for miR-451 is 5'-AAACCGUUACCAUUACUGAGUU-3' (SEQ ID NO: 1), while the human precursor sequence for miR-451 (pre-miR-451) is 5'-CUUGGGAAUGGCAAGGAAACCGUUACCAUUACUGAGUUUAGUAAUGGUAAUGGUUCUCUUGCUAUACCCAGA-3' (SEQ ID NO: 2).

[0033] In one embodiment, the present invention provides a method of treating or preventing pathologic cardiac hypertrophy, cardiac remodeling, myocardial infarction, or heart failure in a subject in need thereof comprising administering an inhibitor of miR-451 to the subject,

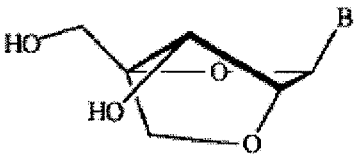
wherein the expression or activity of miR-451 is reduced in the heart cells of the subject following administration. "Heart cells" as used herein include cardiomyocytes, cardiac fibroblasts, and cardiac endothelial cells. In certain embodiments, the expression or activity of miR-451 is reduced in cardiomyocytes of the subject following administration of a miR-451 inhibitor. In some embodiments, the cardiac hypertrophy may be right ventricular hypertrophy associated with pulmonary arterial hypertension (PAH). Thus, the present invention includes a method of preventing or treating PAH in a subject by administering to the subject an inhibitor of miR-451. In another embodiment, the invention provides a method of preventing or delaying cardiac hypertrophy associated with PAH from progressing into heart failure by administering to the subject an inhibitor of miR-451.

[0034] In another embodiment, the subject in need thereof may be at risk for developing pathologic cardiac hypertrophy, cardiac remodeling, heart failure, or myocardial infarction. Such a subject may exhibit one or more risk factors including, but not limited to, long standing uncontrolled hypertension, pulmonary arterial hypertension, uncorrected valvular disease, chronic angina, recent myocardial infarction, congenital predisposition to heart disease or pathological hypertrophy. The subject at risk may be diagnosed as having a genetic predisposition to cardiac hypertrophy or may have a familial history of cardiac hypertrophy.

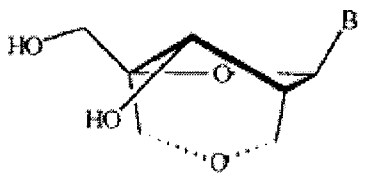
[0035] Preferably, administration of an inhibitor of miR-451 to the subject results in the improvement of one or more symptoms of cardiac hypertrophy, heart failure, or myocardial infarction in the subject, or in the delay in the transition from cardiac hypertrophy to heart failure. The one or more improved symptoms may 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 cardiac fibrosis, decreased collagen deposition in cardiac muscle, decreased left and right ventricular wall stress, decreased wall tension, increased quality of life, and decreased disease related morbidity or mortality. In certain embodiments, the number of cardiomyocytes is increased in the subject following administration of the miR-451 inhibitor as compared to a subject not receiving the miR-451 inhibitor.

[0036] In some embodiments, an inhibitor of miR-451 is an antisense oligonucleotide. The antisense oligonucleotides can include ribonucleotides or deoxyribonucleotides or a combination thereof. Preferably, the antisense oligonucleotides have at least one chemical modification (*e.g.*, sugar or backbone modification). For instance, suitable antisense

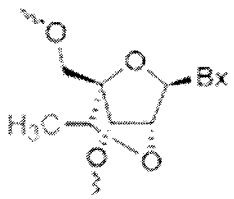
oligonucleotides may be comprised of one or more "conformationally constrained" or bicyclic sugar nucleoside modifications (BSN) that confer enhanced thermal stability to complexes formed between the oligonucleotide containing BSN and their complementary microRNA target strand. For example, in one embodiment, 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 miR-451 can contain combinations of BSN (LNA, CDNA and the like) or other modified nucleotides, and ribonucleotides or deoxyribonucleotides.



A



B



C

[0037] 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 can 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 miR-451 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.

[0038] Preferable antisense oligonucleotides useful for inhibiting the activity of miRNAs 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 miR-451 are about 8 to about 18 nucleotides in length, and in other embodiments about 12 to about 16 nucleotides in length. Any 8-mer or longer complementary to miR-451 may be used, *i.e.*, any antimiR complementary to the 5' end of the miRNA and progressing across the full complementary sequence of the miRNA. For instance, in one embodiment, the antisense oligonucleotide has a sequence of 5'-AACUCAGUAAUGGUAACGGUUU-3' (SEQ ID NO: 3). In another embodiment, the antisense oligonucleotide has a sequence of 5'-AACGGUUU-3' (SEQ ID NO: 4). In another embodiment, the antisense oligonucleotide has a sequence of 5'-GUAACGGUUU-3' (SEQ ID NO: 5). In another embodiment, the antisense oligonucleotide has a sequence of 5'-UGGUAACGGUUU-3' (SEQ ID NO: 6). In yet another embodiment, the antisense oligonucleotide has a sequence of 5'-AAUGGUAACGGUUU-3' (SEQ ID NO: 7). In still another embodiment, the antisense oligonucleotide has a sequence of 5'-GUAAUGGUAACGGUUU-3' (SEQ ID NO: 8). Antisense oligonucleotides can comprise a sequence that is at least partially complementary to a mature miR-451 sequence, *e.g.* at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% complementary to a mature miR-451 sequence. In some embodiments, the antisense oligonucleotide can be substantially complementary to a mature miR-451 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 100% complementary to a mature miR-451 sequence. In certain embodiments, the antisense oligonucleotide is at least partially complementary to SEQ ID NO: 1.

[0039] In some embodiments, the antisense oligonucleotides are antagomirs. "Antagomirs" are single-stranded, chemically-modified ribonucleotides that are at least partially complementary to a miR-451 sequence. Antagomirs may comprise one or more modified nucleotides, such as 2'-O-methyl-sugar modifications. In some embodiments, antagomirs comprise only modified nucleotides. Antagomirs 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 cholesterol or other moiety at its 3' end. Antagomirs suitable for inhibiting miR-451 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 miR-451 sequence. In some embodiments, the antagomir may be substantially complementary to a mature miR-451 sequence, that is at least about 95%, 96%, 97%, 98%, or 99% complementary to a target polynucleotide sequence. In other embodiments, the antagomirs are 100% complementary to a mature miR-451 sequence.

[0040] The inhibitory nucleotide molecules described herein preferably target the mature sequence of miR-451 (SEQ ID NO: 1). In some embodiments, inhibitors of miR-451 are antagomirs comprising a sequence that is perfectly complementary to the mature miR-451 sequence. In one embodiment, an inhibitor of miR-451 is an antagomir having a sequence that is partially or perfectly complementary to 5'-AAACCGUUACCAUUACUGAGUU-3' (SEQ ID NO: 1). In some embodiments, inhibitors of miR-451 are chemically-modified antisense oligonucleotides. In one embodiment, an inhibitor of miR-451 is a chemically-modified antisense oligonucleotide comprising a sequence substantially complementary to 5'-AAACCGUUACCAUUACUGAGUU-3' (SEQ ID NO: 1). 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 (*e.g.* mature or precursor miRNA sequence).

[0041] Antisense oligonucleotides may comprise a sequence that is substantially complementary to a precursor miRNA sequence (pre-miRNA) for miR-451. 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-miR-451 sequence. In one embodiment, an inhibitor of miR-451 function is an antisense oligonucleotide having a sequence that is substantially complementary to a pre-miR-451 sequence (SEQ ID NO: 2).

[0042] Any of the inhibitors of miR-451 described herein can be delivered to the target cell (*e.g.* heart cell) by delivering to the cell an expression vector encoding the miR-451 inhibitors. 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.

[0043] In one embodiment, an expression vector for expressing an inhibitor of miR-451 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 miR-451. 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.

[0044] 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-Takihara *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).

[0045] In certain embodiments, the promoter operably linked to a polynucleotide encoding a miR-451 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.

[0046] The present invention also includes methods for scavenging or clearing miR-451 inhibitors following treatment. The method may comprise overexpressing binding sites for the miR-451 inhibitors in cardiac tissue. The binding site regions preferably contain a sequence of the seed region for miR-451. The seed region is the 5' portion of a miRNA spanning bases 2-8, which is important for target recognition. In some embodiments, the binding site may contain a sequence from the 3'UTR of one or more targets of miR-451, such as AKTIP, DKK1, DKK3, or OSR1.

[0047] In another embodiment, the present invention provides a method of regulating cardiomyocyte survival comprising contacting a cardiomyocyte with an inhibitor of miR-451. In some embodiments, cardiomyocyte survival is enhanced following contact with the miR-451 inhibitor. In still other embodiments, Akt signaling is increased in the cardiomyocyte following contact with the miR-451 inhibitor. One or more genes regulated by miR-451 may be increased in the cardiomyocyte following contact with the inhibitor. Target genes of miR-451 include, but are not limited to, AKTIP, DKK1, DKK3, or OSR1. In one embodiment, the expression of AKTIP is increased in the cardiomyocyte following contact with the miR-451 inhibitor.

[0048] 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. The cardiomyocyte can be *in vitro* or *in vivo*.

[0049] The present invention also includes pharmaceutical compositions comprising an inhibitor of miR-451. 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.

[0050] In one embodiment, the pharmaceutical composition comprises an effective dose of a miR-451 inhibitor. For instance, the pharmaceutical composition comprises and effective dose of a modified antisense oligonucleotide targeting miR-451 as described herein. In some embodiments, the pharmaceutical composition comprises a modified antisense oligonucleotide having a sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, and SEQ ID NO: 8. 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 20 mg/kg to about 200 mg/kg, about 40 mg/kg to about 160 mg/kg, or about 80 mg/kg to about 100 mg/kg. In one embodiment, the inhibitor of miR-451 is administered at a dosage of about 20 mg/kg to about 200 mg/kg. In another embodiment, the inhibitor of miR-451 is administered at a dosage of about 80 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 (*e.g.* myocardial infarction, heart failure, or cardiac hypertrophy), and nature of inhibitor or agonist (*e.g.* antagomir, expression construct, 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.

[0051] 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 oligonucleotide inhibitors of miR-451 function or constructs expressing particular miR-451 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.

[0052] 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.

[0053] 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 or expression constructs comprising miRNA inhibitors 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.

[0054] 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.

[0055] 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 an antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

[0056] 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.

[0057] 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.

[0058] 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.

[0059] This invention is further illustrated by the following additional examples that should not be construed as limiting. Those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made to the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLES

Example 1. Expression of miR-451 correlates with muscle cell differentiation

[0060] To identify cardiac-specific microRNAs, northern blot analyses of various tissues isolated from mouse were performed. MiR-451 was found to be highly expressed in the heart, lung, and spleen (Figure 1A). To further examine the expression of miR-451 in the heart, real-time PCR analysis was performed for miR-451 in neonatal rat cardiomyocytes (NRVMs)

and fibroblasts. The results revealed that miR-451 is primarily expressed in cardiomyocytes (Figure 1B and C).

[0061] In addition, miR-451 expression appears to be developmentally regulated. Real-time RT-PCR analysis for miR-451 was performed on RNA from either whole animal or whole heart collected at embryonic days 12.5 and 15.5, and post-natal days 1, 3, and 5. MiR-451 expression was induced in the heart approximately 5-fold from E12.5 to E15.5 (Figure 1D). Expression is reduced during the early post-natal period and then increases again in the adult. These results suggest that miR-451 is expressed predominantly in cardiomyocytes and may play an important role in the developing heart as well as contribute to maintenance of the adult heart phenotype.

[0062] Interestingly, expression of miR-451 correlated with the differentiation of different types of muscle cells. For example, real-time RT-PCR analysis of rat vascular smooth muscle cells that had been differentiated for six days showed that miR-451 was induced during smooth muscle cell differentiation (Figure 2A). A similar result was obtained with C2C12 myoblasts. Real-time RT-PCR analysis of differentiating C2C12 myobalsts showed that miR-451 levels were approximately 10-fold higher in differentiated myoblasts as compared to undifferentiated myoblasts (Figure 2B). These findings indicate that miR-451 may play a role in muscle cell differentiation.

Example 2. MiR-451 is downregulated in the heart following myocardial infarction

[0063] To determine if miR-451 was regulated in the cardiac disease state, expression of miR-451 was measured following myocardial infarction (MI) in the mouse. Real-time RT-PCR analysis of RNA from tissue isolated from the border zone of the infarct at 6- and 48-hours post-MI revealed a significant downregulation of miR-451 when compared to sham operated heart tissue (Figure 3).

[0064] MiR-451 knockout mice were generated to further elucidate the role of miR-451 in cardiac disease (Figure 4). Specifically, a miR-451 targeting vector was generated by digesting a fragment (5' arm) extending upstream of the miR-451 coding region, which included the miR-144 coding region, and ligating the fragment into the pGKneoF2L2dta targeting plasmid upstream of the loxP sites and the Frt-flanked neomycin cassette. A second fragment (3' arm) was digested and ligated into the vector between the neomycin resistance and Dta negative selection cassettes. Targeted ES-cells carrying the disrupted allele were identified by Southern blot analysis with 5' and 3' probes. MiR-451 targeted ES clones were identified and used for blastocyst injection. The resulting chimeric mice will be bred to

C57BL/6 to obtain germline transmission of the mutant allele. MiR-451 knockout animals are expected to exhibit less cardiac hypertrophy in response to stress stimuli and more cell survival.

Example 3. Cardiac overexpression of miR-451 induces cardiac hypertrophy

[0065] To further examine the function of miR-451 in cardiac remodeling, transgenic mice overexpressing miR-451 under the control of the α-MHC promoter were generated. Cardiac specific overexpression resulted in an approximately 45-fold induction of miR-451 expression at six weeks of age (Figure 5). Overexpression of miR-451 resulted in a significant increase in heart weight/body weight ratio suggesting that miR-451 promotes cardiac hypertrophy (Figure 6A). Cardiac hypertrophy progressed into dilated cardiomyopathy resulting in earlier death in the α-MHC-miR-451 transgenic animals (Figure 6B). Hematoxylin and eosin and Masson trichome stained sections of heart tissue from the α-MHC-miR-451 transgenic mice exhibit substantial cardiac remodeling when compared to sections from wild-type hearts (Figures 7 and 8). A significant decrease in the number of cardiomyocytes was also observed in the α-MHC-miR-451 transgenic animals (Figure 9). In addition, atrial abnormalities were present in the miR-451 transgenics (Figure 10). These findings indicate that miR-451 is regulated and actively involved in the process of pathological remodeling of the heart and may represent a novel therapeutic target for treatment of cardiac disease.

Example 4. MiR-451 targets genes involved in cell survival and cardiogenesis

[0066] Using target prediction software, miR-451 is predicted to target genes involved in cell survival and cardiogenesis, including DKK1, DKK3, Akt interacting protein (AKTIP), and odd-skipped related 1 (OSR) (Table 1). Western blot analysis of heart tissue isolated from transgenic mice overexpressing miR-451 showed a downregulation of both DKK1 and AKTIP (Figure 11A) suggesting that these two proteins are *in vivo* targets of miR-451.

[0067] The current data suggest that miR-451 influences cardiac remodeling by reducing cell survival and cardiogenesis, including atrial development, by repressing its targets AKTIP, DKK1, DKK3, and OSR1. Repression of AKTIP results in a reduction of AKT activation, which in turn leads to a decrease in cell survival, while repression of DKK1 and DKK3 activates Wnt signaling and represses cardiogenesis (Figure 11B). The OSR1 transcription factor is required for normal atrial septum development.

Table 1. Predicted targets of miR-451

Target gene	Gene name
OSR1	odd-skipped related 1 (Drosophila)
TTN	titin
C11orf30	chromosome 11 open reading frame 30
RKHD2	ring finger and KH domain containing 2
SAMD4B	sterile alpha motif domain containing 4B
CAB39	calcium binding protein 39
ZNF644	zinc finger protein 644
AKTIP	AKT interacting protein
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide
VAPA	VAMP (vesicle-associated membrane protein)-associated protein A, 33kDa
CUGBP2	CUG triplet repeat, RNA binding protein 2
FBXO33	F-box protein 33
YTHDF2	YTH domain family, member 2
FMNL3	formin-like 3

Example 5. GATA-4 regulates miR-451 expression

[0068] Expression of the primary miRNA transcript encoding miR-451 was reported to be regulated by the GATA-1 transcription factor in erythroid precursor cells (Dore *et al.* (2008) Proc. Natl. Acad. Sci. USA, Vol. 105: 3333-3338). MiR-451 is encoded on a polycistronic primary transcript with miR-144 and the enhancer region upstream of the common precursor sequence is conserved across several mammalian species (See Dore *et al.*). The enhancer region upstream of the miR-451 primary sequence contains two independent GATA binding sequences (Dore *et al.*). To determine if miR-451 expression is regulated by a GATA transcription factor in murine heart, gel shift assays were performed with separate probes comprising the sequences from each of the two GATA binding sites in the miR-451 enhancer region. As shown in Figure 12, GATA4 recognized and bound to each of the GATA sequences from the miR-451 enhancer region. The specificity of GATA4 binding was demonstrated with addition of unlabeled probes. Probe sequence comprising either the site 1 or site 2 GATA sequence from the miR-451 enhancer region decreased GATA4 binding to the labeled probe (Figure 13). However, probes comprising a mutated GATA binding sequence had no effect. These results suggest that GATA4 can recognize the GATA binding sequences present in the miR-451 enhancer region.

[0069] To examine the regulation of miR-451 at the molecular level, luciferase constructs consisting of the enhancer region upstream of the pri-miR-451 sequence were generated. The responsiveness of the miR-451 enhancer region to GATA4 in COS cells was examined

(Figure 14). GATA4 activation of the miR-451 luciferase reporter was dependent on both GATA binding sequences in the enhancer region as demonstrated by mutational analysis in transfected COS cells (Figure 14). These findings indicate that GATA4 can regulate expression of miR-451.

[0070] All publications, patents and patent applications discussed and cited herein are incorporated herein by reference in their entireties. 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.

[0071] 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 treating or preventing pathologic cardiac hypertrophy, cardiac remodeling, myocardial infarction, or heart failure in a subject in need thereof comprising administering an inhibitor of miR-451 to the subject, wherein the expression or activity of miR-451 is reduced in the heart cells of the subject following administration.

2. The method of claim 1, wherein the inhibitor of miR-451 is an antisense oligonucleotide.

3. The method of claim 2, wherein the antisense oligonucleotide comprises a sequence that is at least partially complementary to a mature sequence of miR-451.

4. The method of claim 3, wherein the antisense oligonucleotide comprises a sequence that is at least partially complementary to SEQ ID NO: 1.

5. The method of claim 3, wherein the antisense oligonucleotide comprises at least one sugar and/or backbone modification.

6. The method of claim 3, wherein the antisense oligonucleotide is about 8 to about 18 nucleotides in length.

7. The method of claim 3, wherein the antisense oligonucleotide has a sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, and SEQ ID NO: 8.

8. The method of claim 1, wherein the number of cardiomyocytes is increased in the subject following administration of the miR-451 inhibitor as compared to a subject not receiving the miR-451 inhibitor.

9. The method of claim 1, wherein the subject is human.

10. The method of claim 9, wherein the human is diagnosed with pulmonary arterial hypertension.

11. The method of claim 1, wherein the pathologic cardiac hypertrophy is right ventricular hypertrophy associated with pulmonary arterial hypertension.

12. A method of regulating cardiomyocyte survival comprising contacting a cardiomyocyte with an inhibitor of miR-451.

13. The method of claim 12, wherein cardiomyocyte survival is enhanced following contact with the miR-451 inhibitor.

14. The method of claim 12, wherein Akt signaling is increased in the cardiomyocyte following contact with the miR-451 inhibitor.

15. The method of claim 12, wherein the expression of Aktip is increased in the cardiomyocyte following contact with the miR-451 inhibitor.

16. The method of claim 12, wherein inhibitor of miR-451 is an antisense oligonucleotide.

17. The method of claim 16, wherein the antisense oligonucleotide comprises a sequence that is at least partially complementary to a mature sequence of miR-451.

18. The method of claim 17, wherein the antisense oligonucleotide comprises a sequence that is at least partially complementary to SEQ ID NO: 1.

19. The method of claim 17, wherein the antisense oligonucleotide comprises at least one sugar and/or backbone modification.

20. The method of claim 17, wherein the antisense oligonucleotide is about 8 to about 18 nucleotides in length.

21. The method of claim 17, wherein the antisense oligonucleotide has a sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, and SEQ ID NO: 8.

22. The method of claim 12, wherein the cardiomyocyte is *in vitro* or *in vivo*.

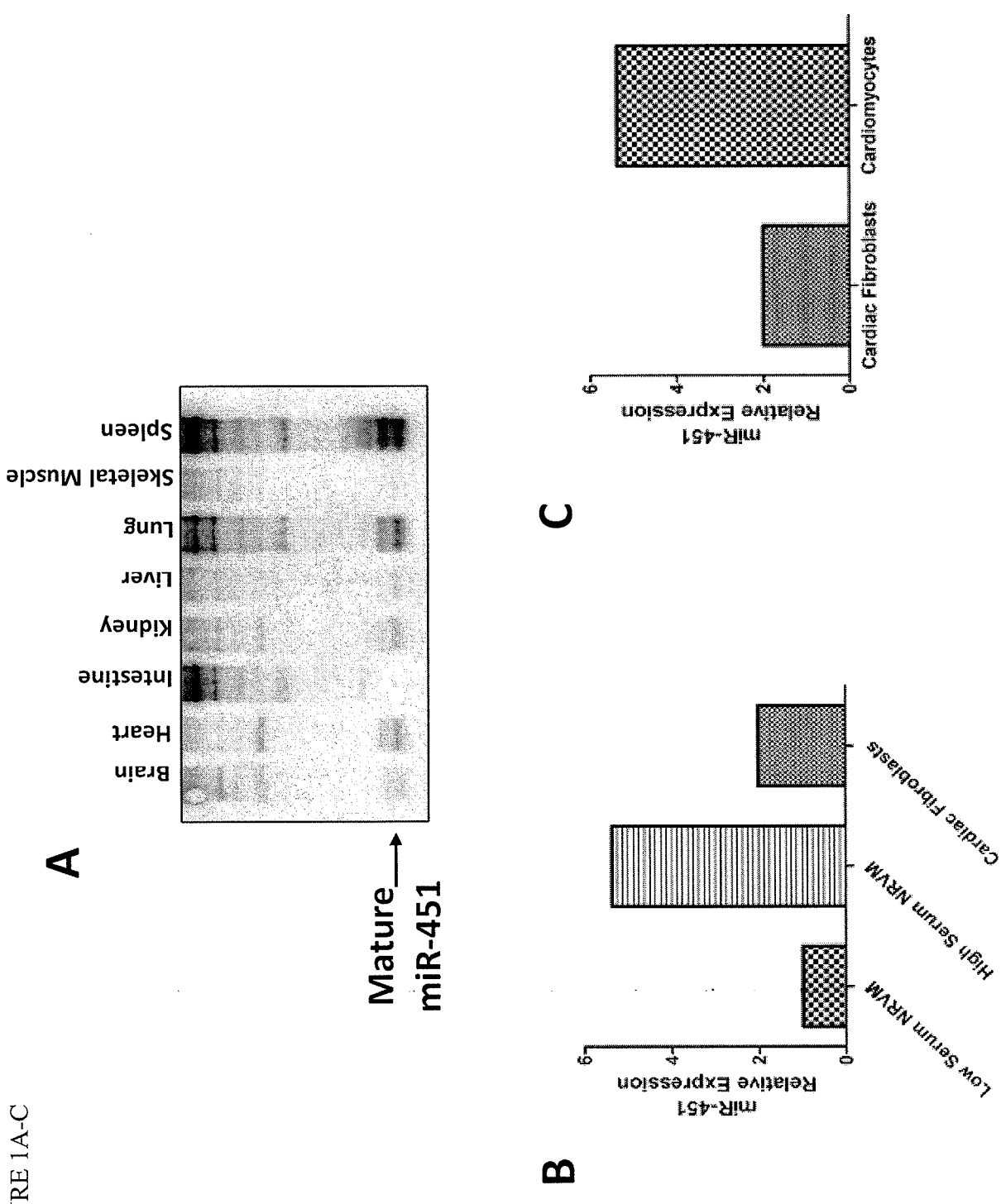

A
Brain
Heart
Intestine
Kidney
Liver
Lung
Skeletal Muscle
Spleen
Mature
miR-451
B
miR-451
Relative Expression
0
2
4
6
Low Serum NRVM
High Serum NRVM
Cardiac Fibroblasts
C
miR-451
Relative Expression
0
2
4
6
Cardiac Fibroblasts
Cardiomyocytes

FIGURE 1A-C

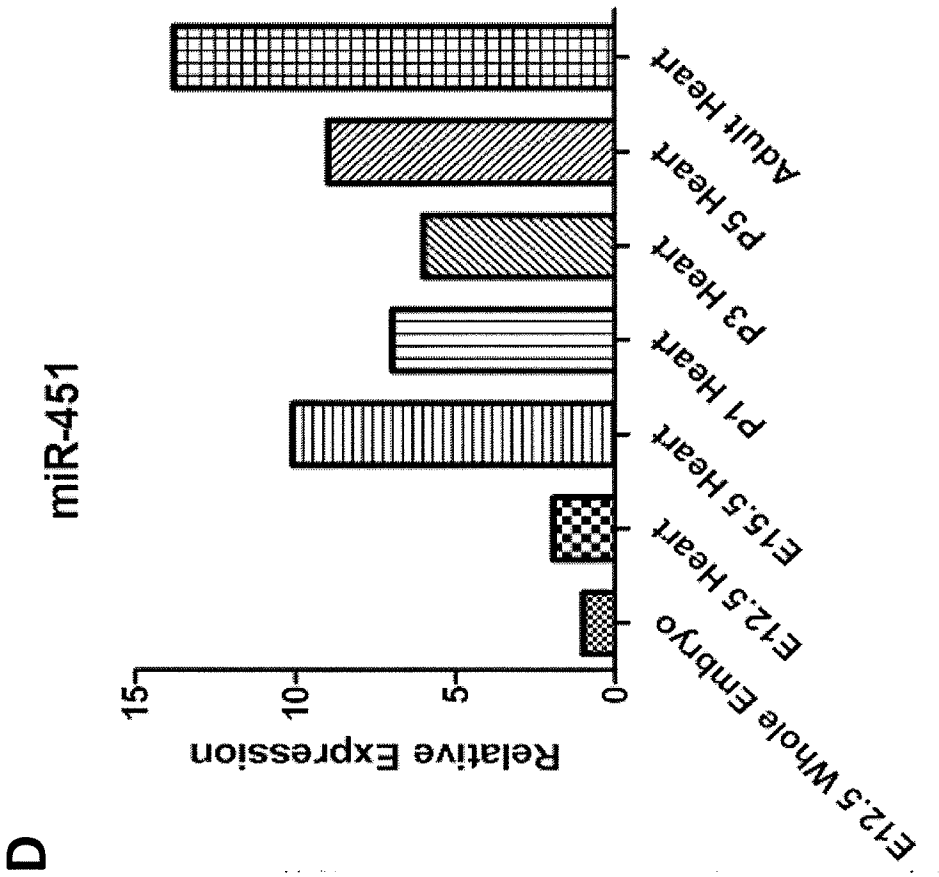
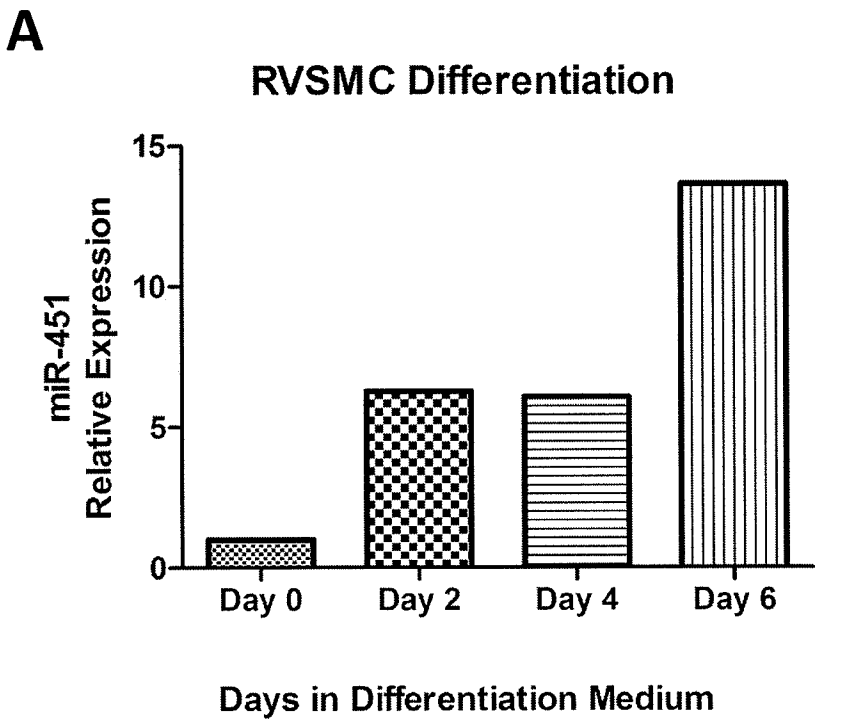

D
miR-451
Relative Expression
0
5
10
15
E12.5 Whole Embryo
E12.5 Heart
E15.5 Heart
P1 Heart
P3 Heart
P5 Heart
Adult Heart

FIGURE 1D

FIGURE 2A-B


A
RVSMC Differentiation
miR-451
Relative Expression
15
10
5
0
Day 0
Day 2
Day 4
Day 6
Days in Differentiation Medium

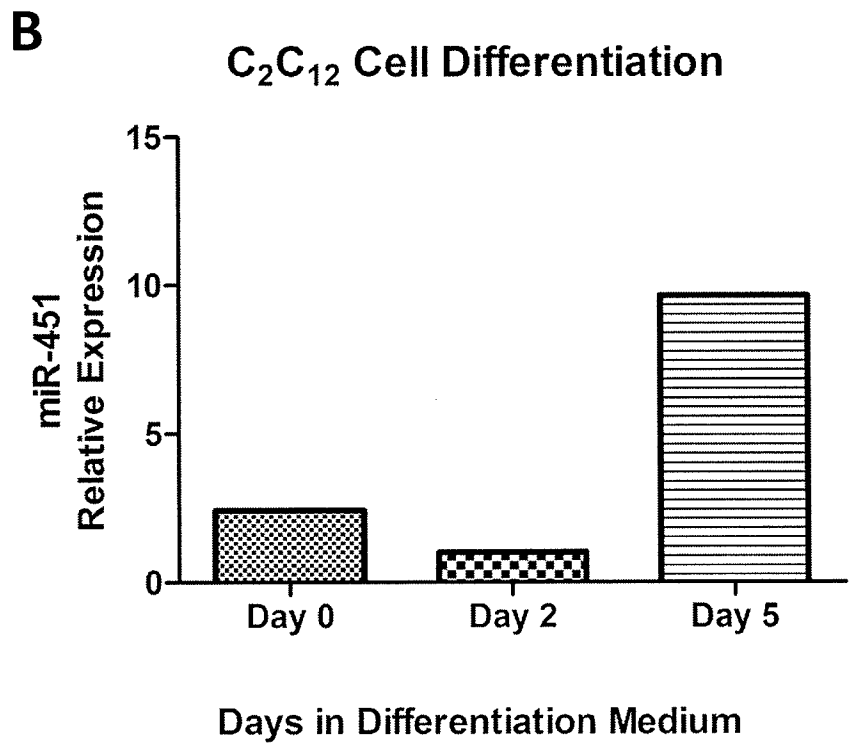
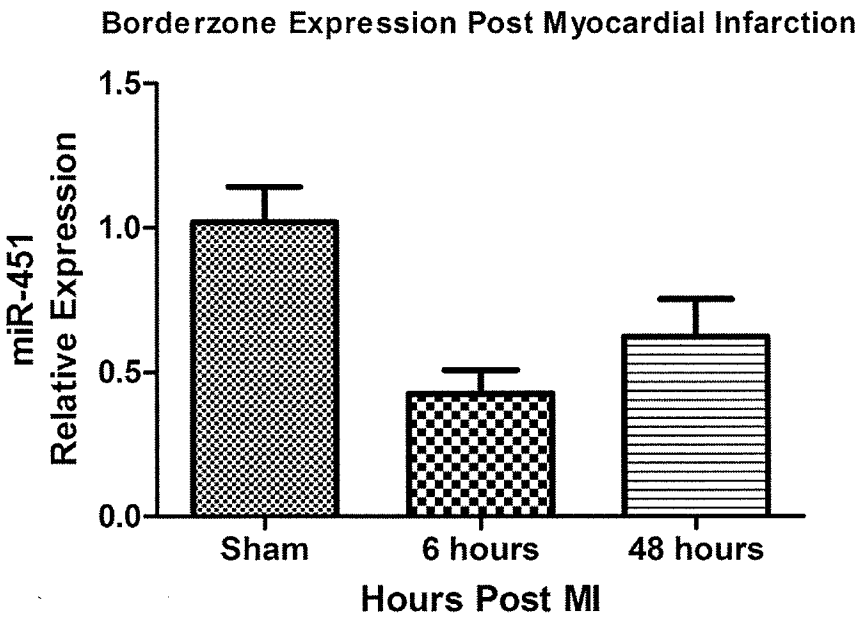

B
C2C12 Cell Differentiation
miR-451
Relative Expression
15
10
5
0
Day 0
Day 2
Day 5
Days in Differentiation Medium

FIGURE 3


Borderzone Expression Post Myocardial Infarction
miR-451
Relative Expression
1.5
1.0
0.5
0.0
Sham
6 hours
48 hours
Hours Post MI

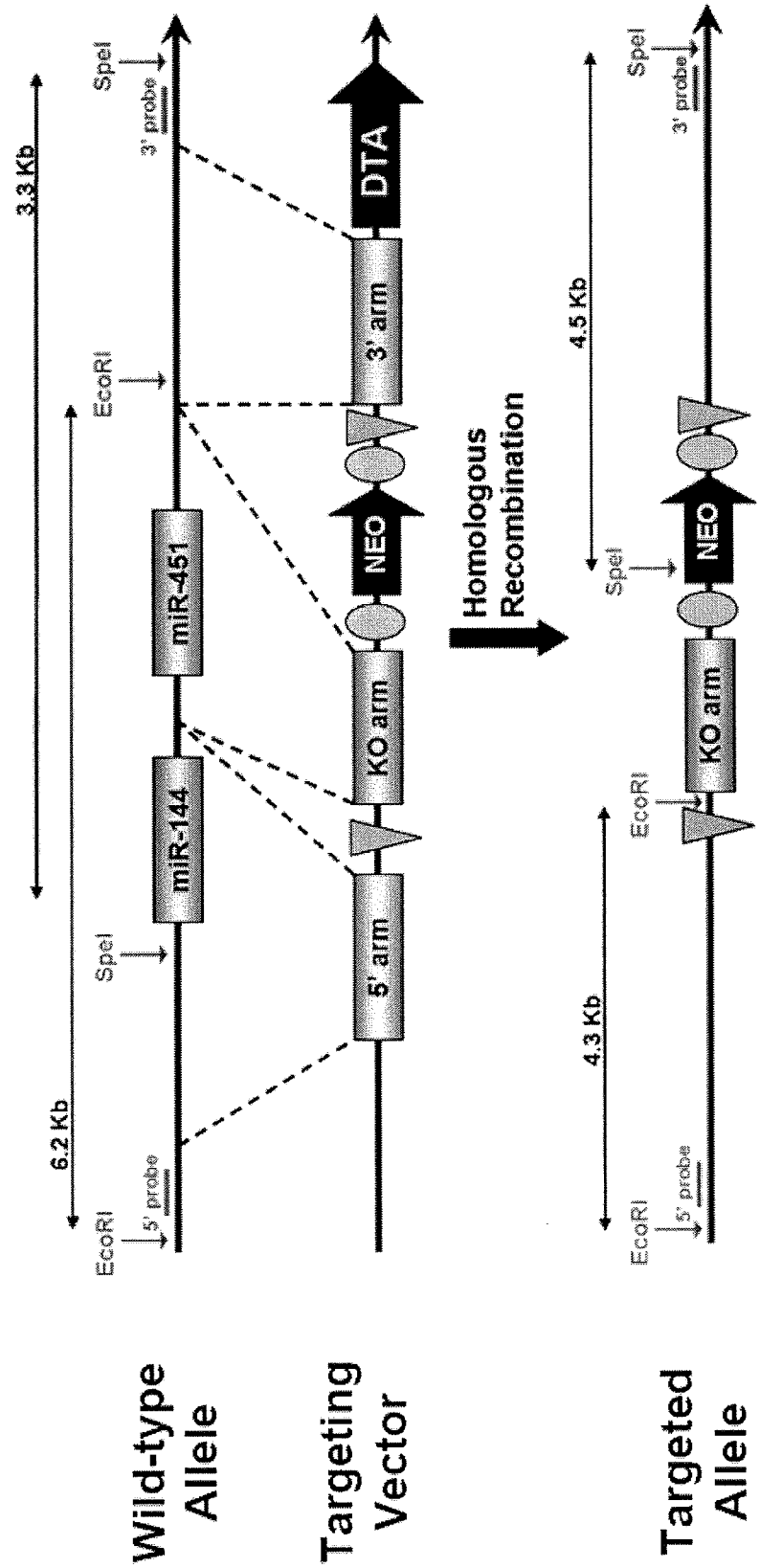

Wild-type Allele
Targeting Vector
Targeted Allele
Homologous Recombination
3.3 Kb
6.2 Kb
4.3 Kb
4.5 Kb
EcoRI
SpeI
5' probe
3' probe
miR-144
miR-451
5' arm
KO arm
3' arm
NEO
DTA

FIGURE 4

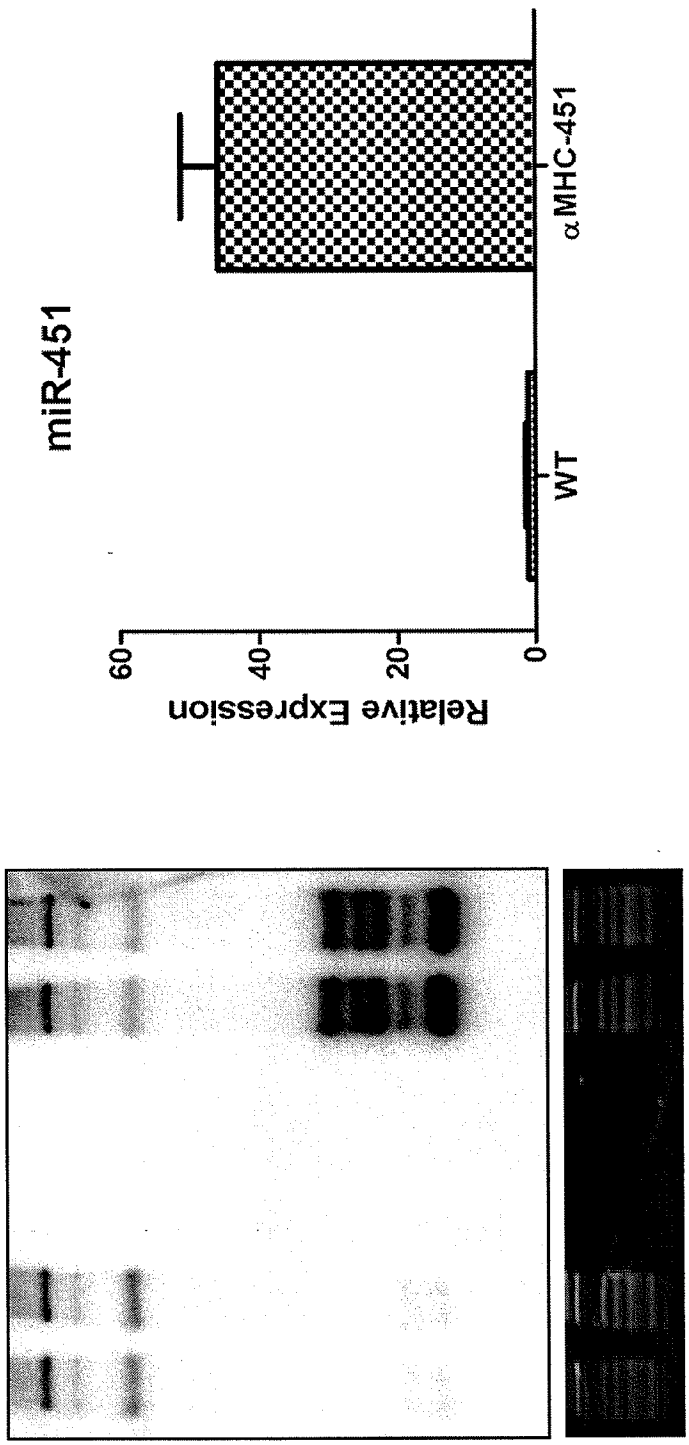


FIGURE 5

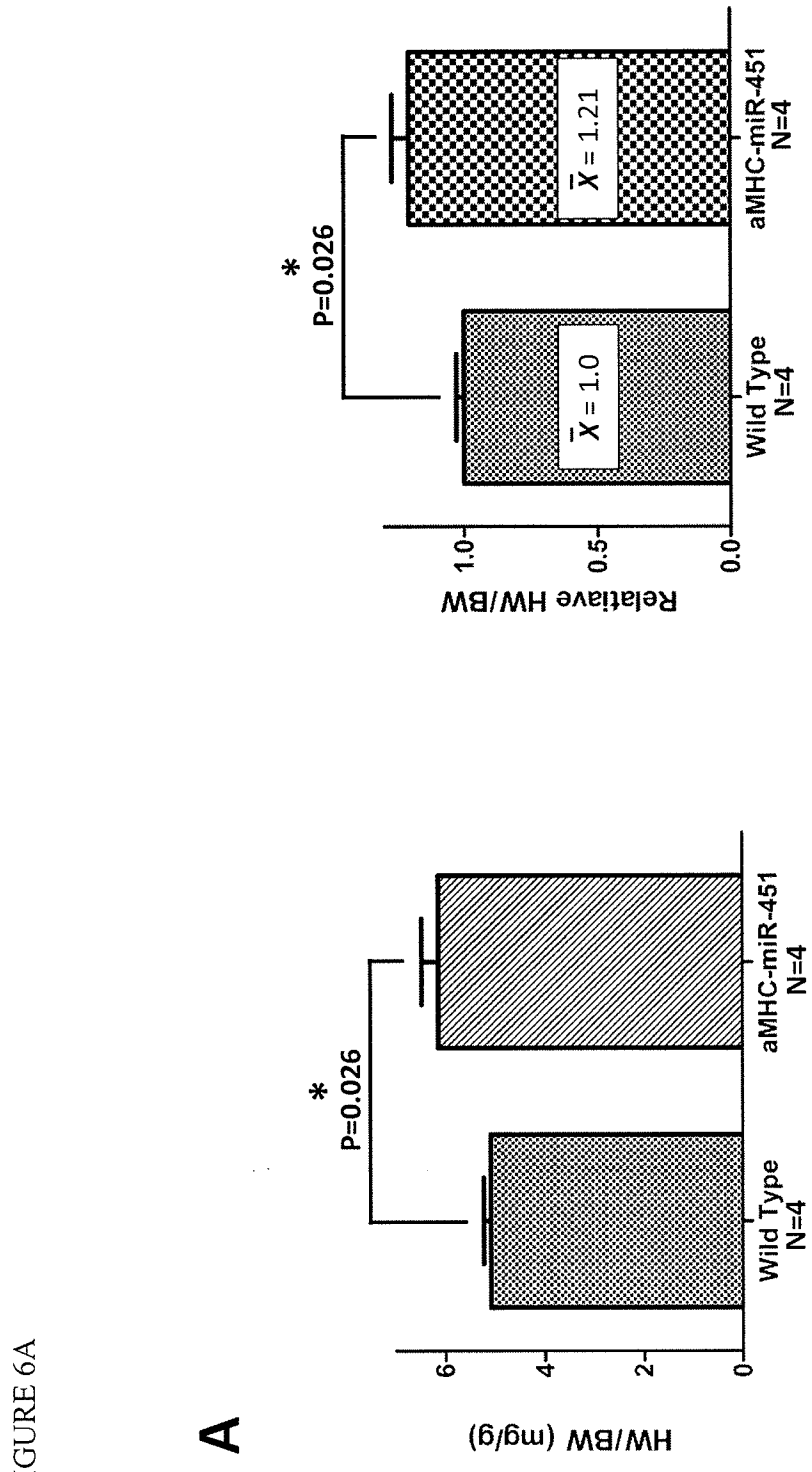

A
HW/BW (mg/g)
0
2
4
6
*
P=0.026
Wild Type
N=4
aMHC-miR-451
N=4
Relative HW/BW
0.0
0.5
1.0
$\bar{X}$ = 1.0
$\bar{X}$ = 1.21

FIGURE 6A

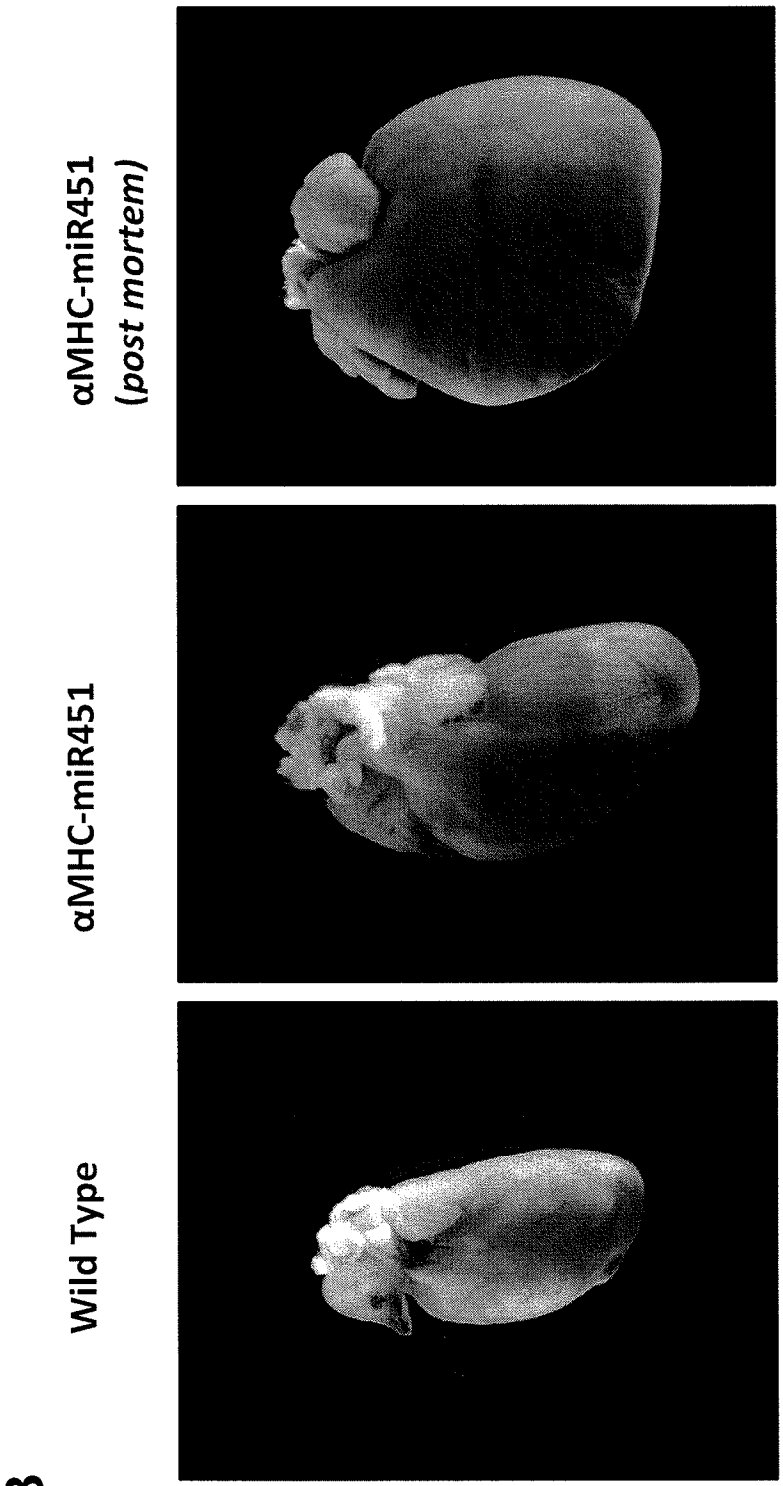


FIGURE 6B

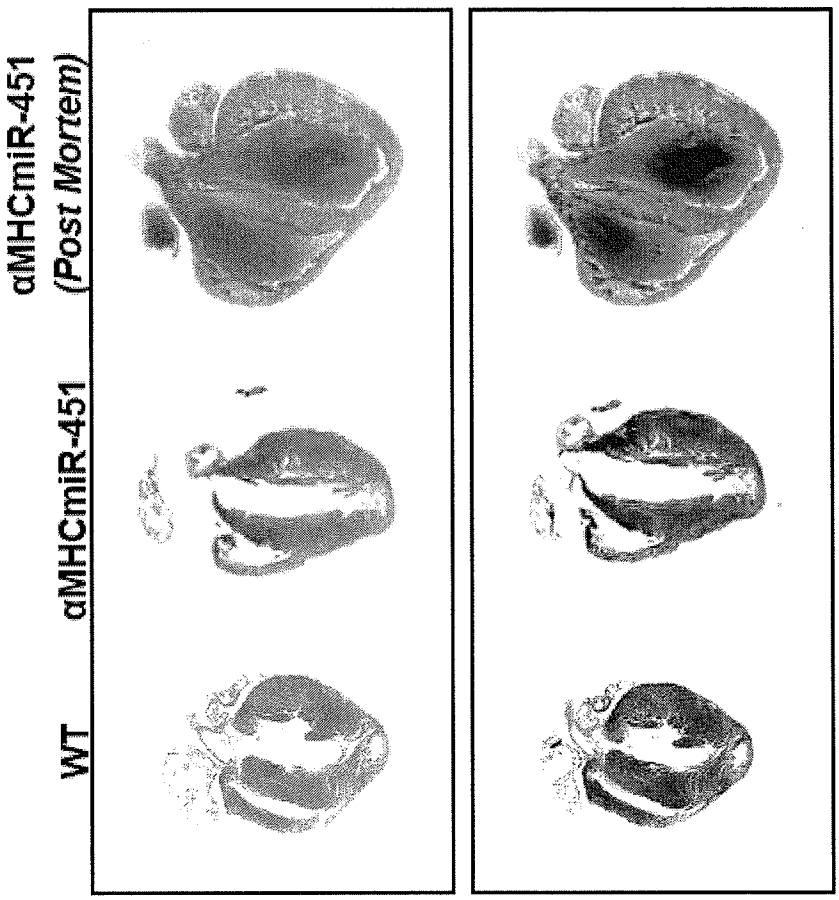


FIGURE 7

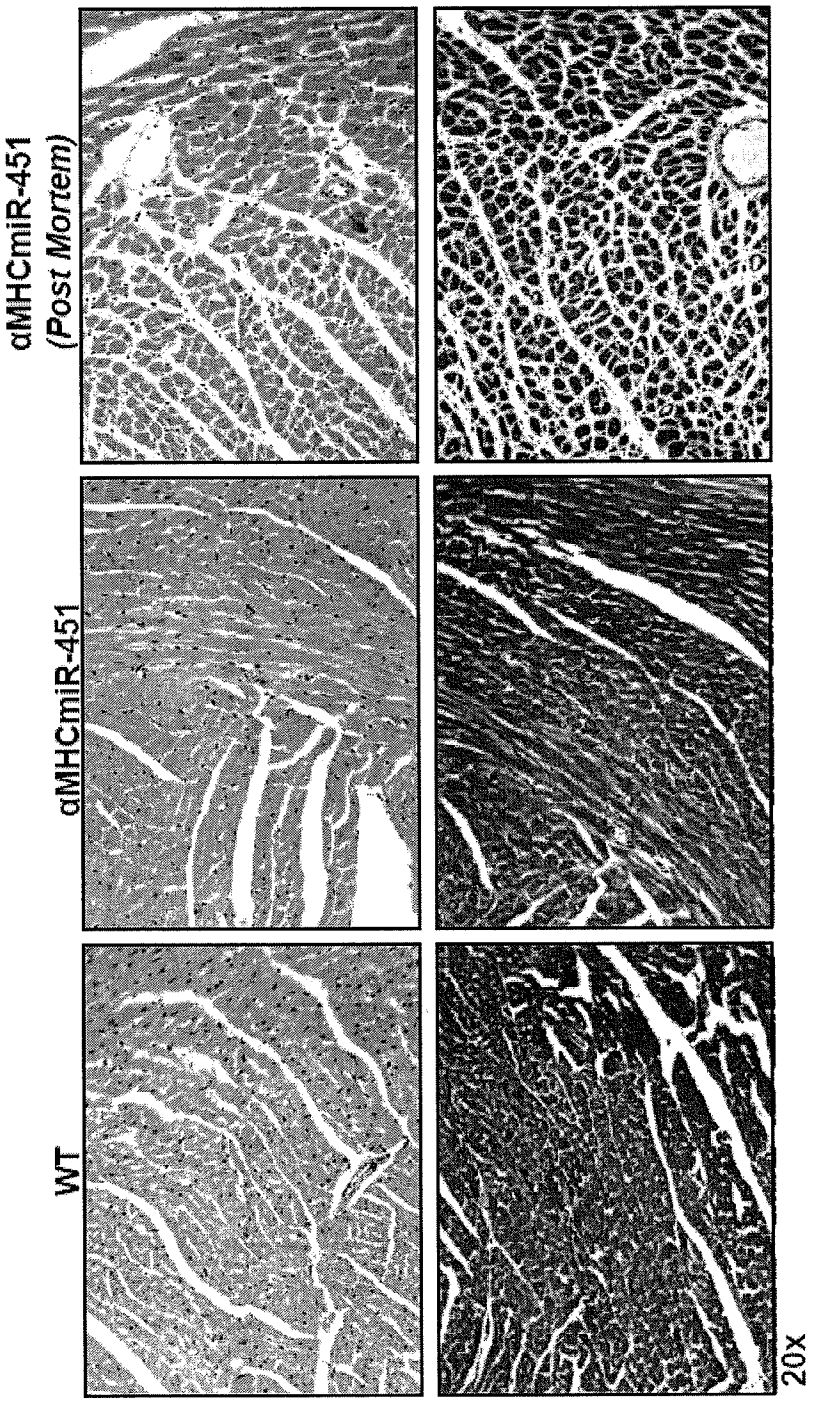

WT
αMHCmiR-451
αMHCmiR-451
(Post Mortem)
H&E
Masson's
Trichrome
20x

FIGURE 8

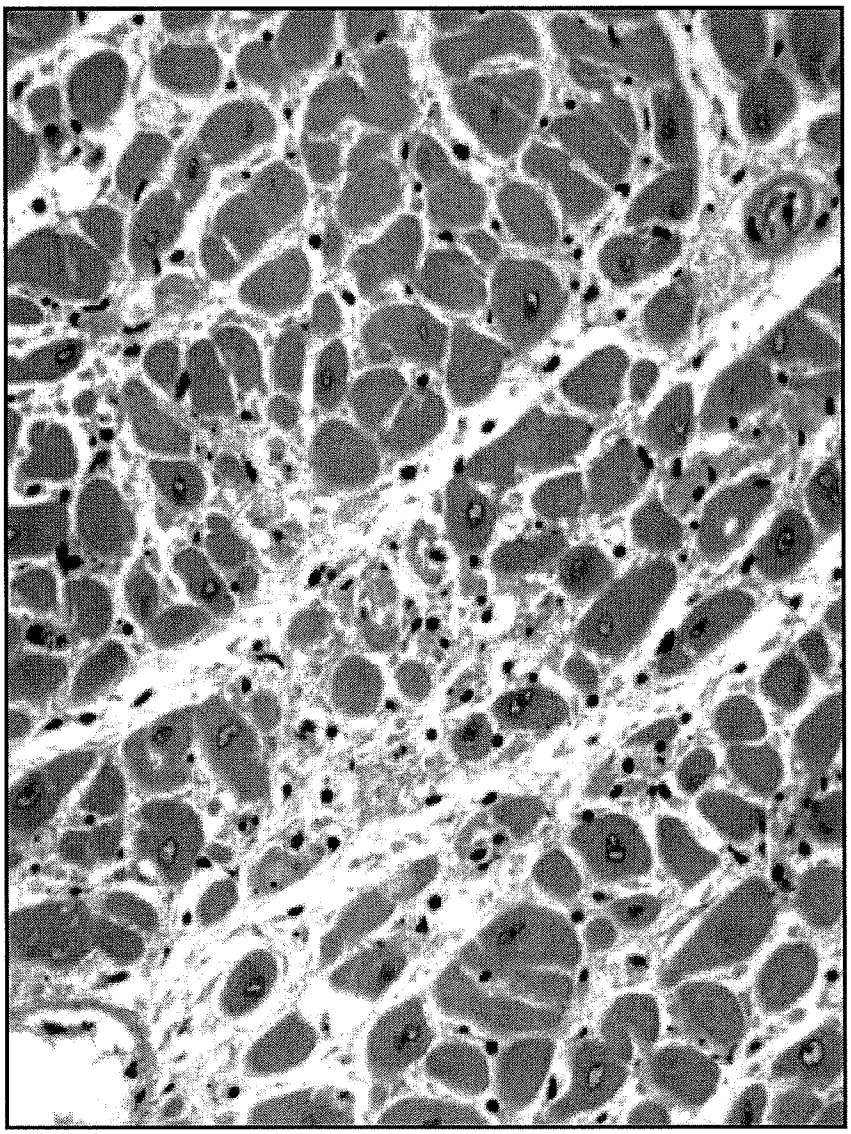


FIGURE 9

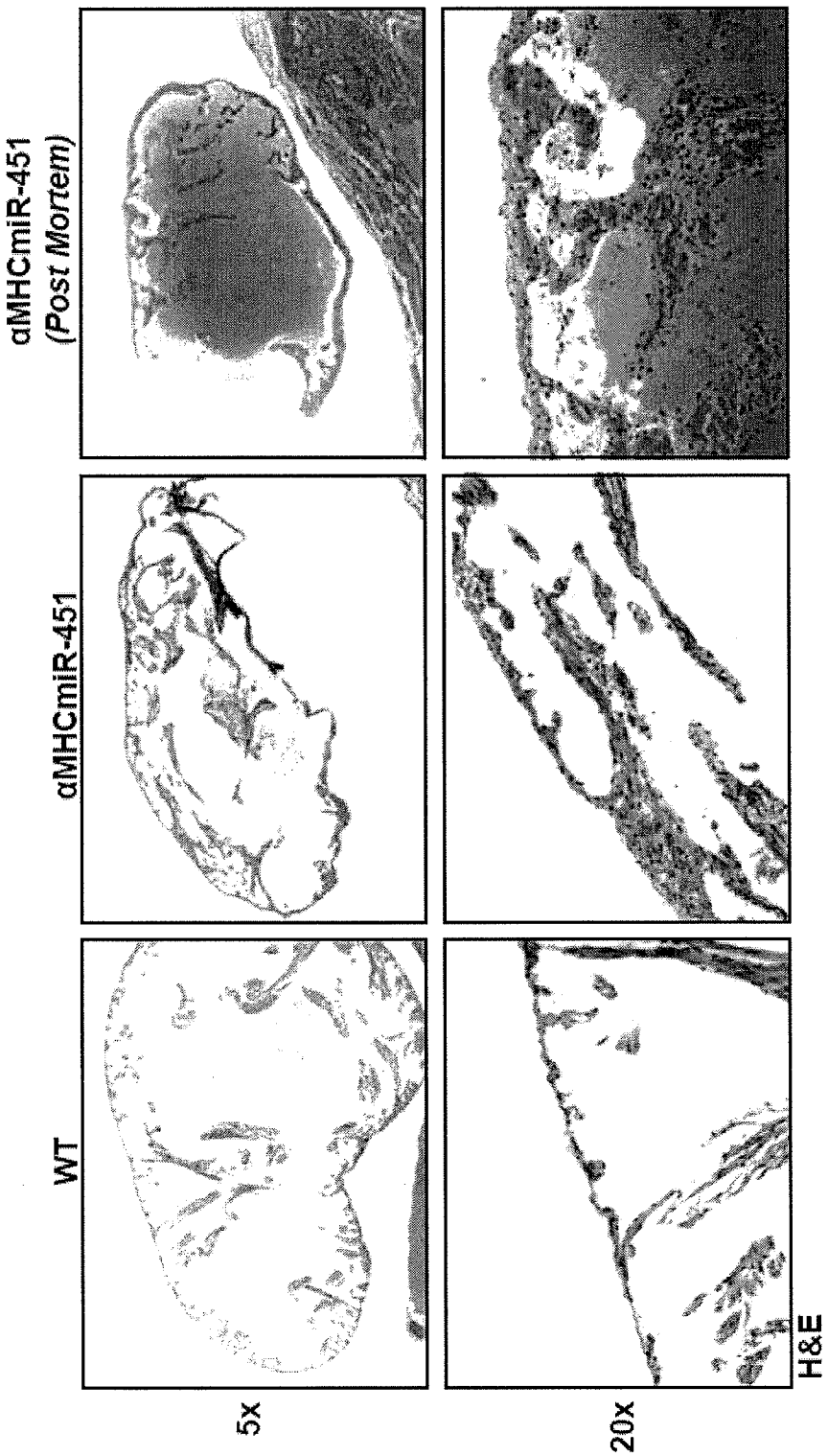
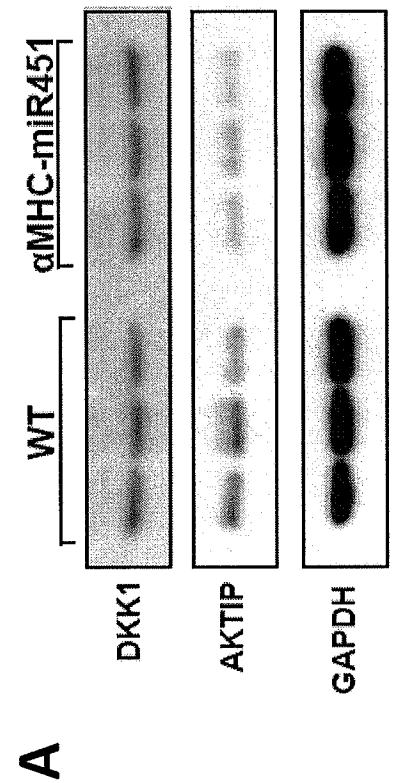


FIGURE 10


A
WT
αMHC-miR451
DKK1
AKTIP
GAPDH

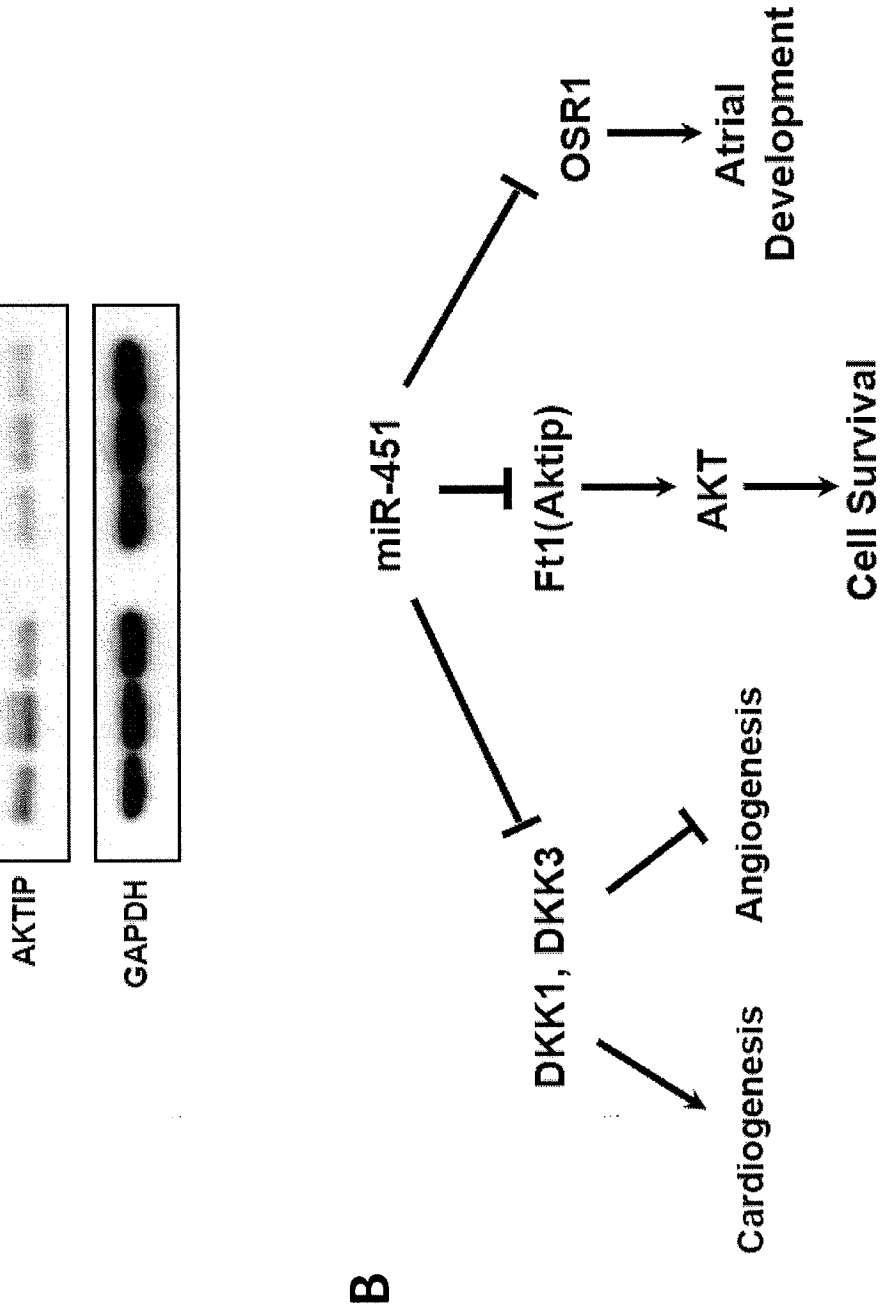

B
miR-451
DKK1, DKK3
Cardiogenesis
Angiogenesis
Ft1(Aktip)
AKT
Cell Survival
OSR1
Atrial
Development

FIGURE 11A-B

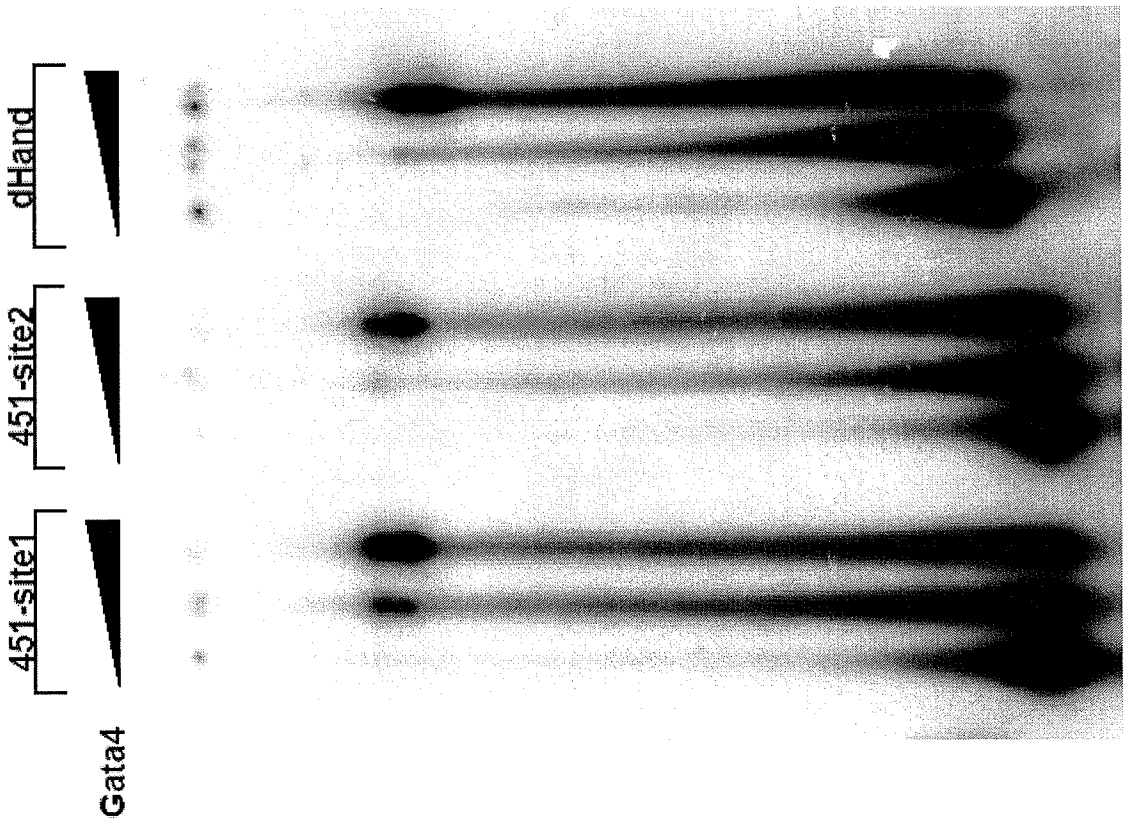
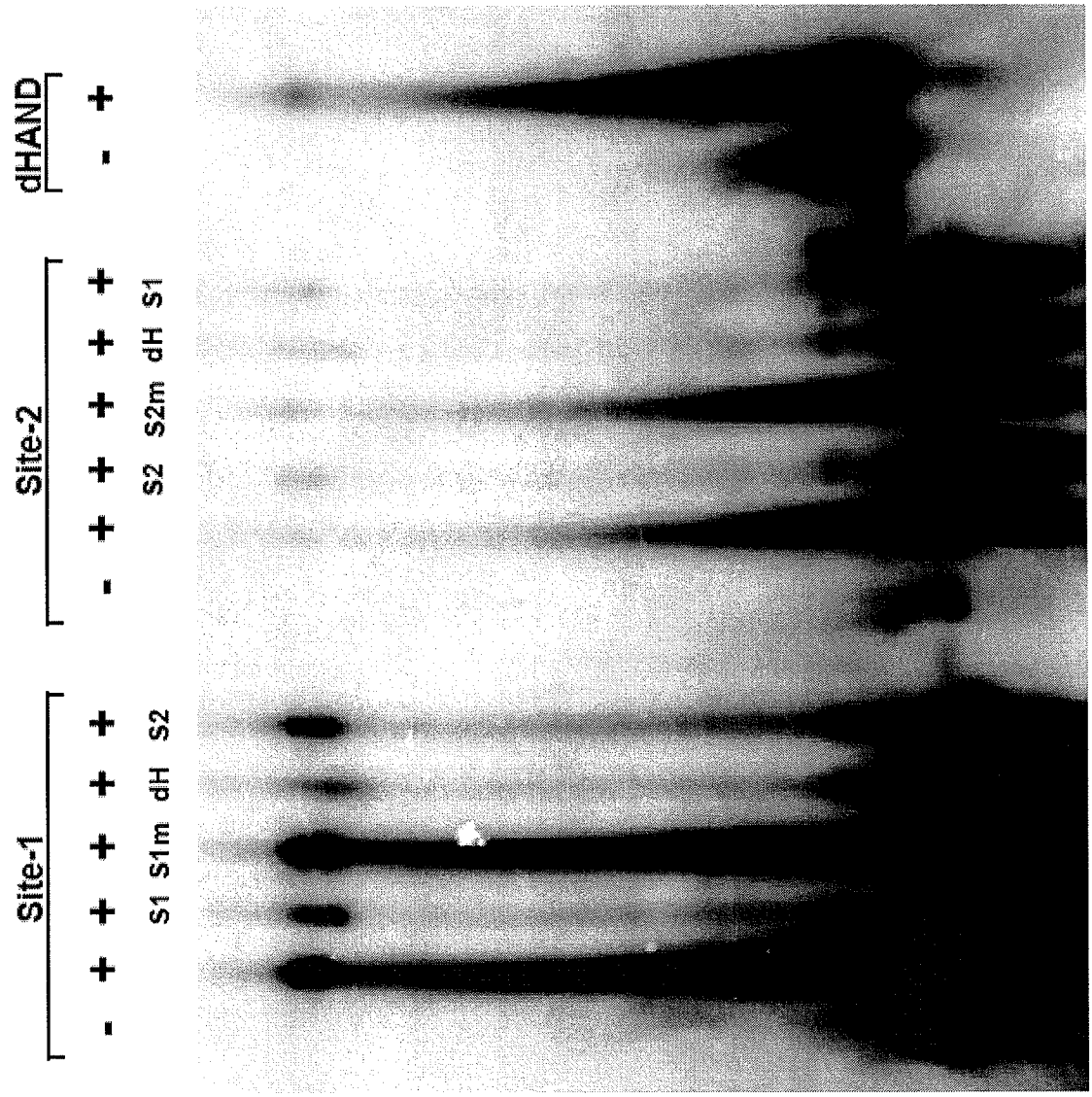


FIGURE 12

FIGURE 13


Probe
GATA4
Cold Competitor
Site-1
- + + + +
S1 S1m dH S2
Site-2
- + + + + +
S2 S2m dH S1
dHAND
- +

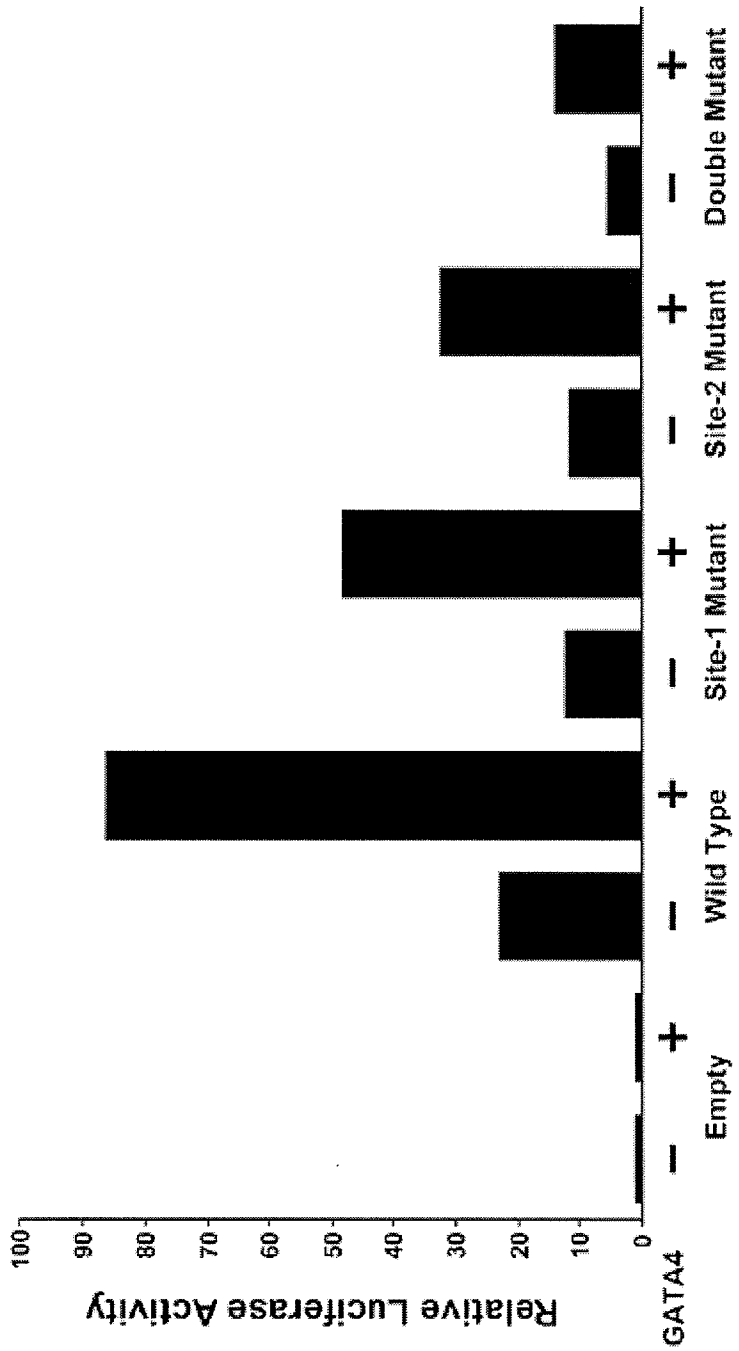

miR-451 Enhancer
Relative Luciferase Activity
100
90
80
70
60
50
40
30
20
10
0
GATA4
– + – + – + – +
Empty
Wild Type
Site-1 Mutant
Site-2 Mutant
Double Mutant

FIGURE 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/34227

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 31/7088, C07H 21/02 (2010.01)
USPC - 514/44A, 435/375, 536/24.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC: 514/44A, 435/375, 536/24.5

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)
Databases: PubWEST (PGPB,USPT,USOC,EPAB,JPAB; PLUR=NO; OP=ADJ) Google Scholar, Google Patents.
Search terms used: mir_451, microRNA, cardiomyocyte$, myocardial infarction, cardiac hypertrophy, heart failure, heart attack, *cardiac remodeling, arterial hypertension, Akt signaling, AKTIP, OSRI, DKKI, DKK3

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2008/061537 (MOLLER et al.) 29 May 2008 (29.05.2008) esp: pg 10 ln 17-19; pg 18 ln 4-5; pg 19 ln 9; pg 18 ln 21 and ln 33; pg 42 ln 11-13, pg 59 ln 15-31, pg 57 ln 25-28; SEQ ID NO: 363, SEQ ID NO: 376, Table 2.	1-22
A	CHENG et al., MicroRNAs Are Aberrantly Expressed in Hypertrophic Heart: Do They Play a Role in Cardiac Hypertrophy? Am J Pathol, Vol. 170, No. 6, June 2007, Vol 170, No 6, pg 1831 -1840 (plus Supplementary Table uploaded in support of document). esp: pg 1835 second column, last four lines; Figure 2B and Figure 2C.	1-22
A	ZHAN et al., MicroRNA expression dynamics during murine and human erythroid differentiation. Experimental Hematology, July 2007, Vol 35, No 7, pp 1015-1025. abstract.	1-22
A	MASAKI et al., Expression patterns of microRNAs 155 and 451 during normal human erythropoiesis. Biochemical and Biophysical Research Communications, 21 December 2007, Vol 364, No 3, pp 509-514. abstract.	1-22
A,P	US 2009/0124566 A1 (CHI et al.) 14 May 2009 (14.05.2009) entire document.	1-22
A,P	US 2009/0306181 A1 (IKEDA et al.) 10 December 2009 (10.12.2009) entire document.	1-22
A,P	US 2009/0226375 A1 (OLSON et al.) 10 September 2009 (10.09.2009) entire document.	1-22
A,P	US 2009/0281167 A1 (SHEN et al.) 12 November 2009 (12.11.2009) entire document.	1-22
A,P	US 2010/0035963 A1 (CHAJUT et al.) 11 February 2010 (11.02.2010) entire document.	1-22

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
03 August 2010 (03.08.2010)	20 AUG 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 10/34227

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FU et al., Mir-144 selectively regulates embryonic alpha-hemoglobin synthesis during primitive erythropoiesis. Blood, 5 February 2009, Vol 113, No 6, pg 1340-1349.	1-22
A,P	ZHANG et al., Abstract 3026: MicroRNA Expression Profiling in the Ischemic Preconditioned Heart: Functional Role of a MiR-1/GATA-4/MiR-144, -451, and -762 Circuit, Circulation, 3 November 2009, Vol 120, No 18 Supplement; Abstracts From Scientific Sessions 2009.	1-22
A,P	WANG et al., Abstract 5016: Circulating MicroRNA: A Novel Potential Biomarker for Early Detection of Acute Myocardial Infarction. Circulation, 3 November 2009, Vol 120, No 18 Supplement; Abstracts From Scientific Sessions 2009.	1-22
A	DA COSTA MARTINS et al., Conditional Dicer Gene Deletion in the Postnatal Myocardium Provokes Spontaneous Cardiac Remodeling. Circulation, 7 October 2008, Vol 118, No 15, pp 1567-1576.	1-22
A	DIVAKARAN et al., The Emerging Role of MicroRNAs in Cardiac Remodeling and Heart Failure. Circ. Res., 7 November 2008, Vol 103, No 10, pp1072-1083.	1-22
A	DORE et al., A GATA-1-regulated microRNA locus essential for erythropoiesis. PNAS, 4 March 2008, Vol 105, No 9, pg 3333-3338.	1-22
A	FONTANA et al., Role of microRNAs in haemopoiesis, heart hypertrophy and cancer. Biochemical Society Transactions, December 2008, Vol 36, Pt 6, pp 1206-1210.	1-22
A,T	IKEDA et al., Expression and Function of MicroRNAs in Heart Disease. Current Drug Targets, August 2010, Vol 11, No 8 pg 913-925.	1-22
A	VAN ROOIJ et al., A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. PNAS, 28 November 2006, Vol 103, No 48, pp 18255-18260.	1-22
A,P	SHI et al., Altered expression of microRNAs in the myocardium of rats with acute myocardial infarction. BMC Cardiovascular Disorders 2010, Vol 10, pp 11 (1 March 2010).	1-22
A	US 2007/0050146 A1 (BENTWICH et al.) 1 March 2007 (01.03.2007) entire document.	1-22