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(54) Title: MICRORNA SIGNATURE TO PREDICT PROGNOSIS IN HEART FAILURE

(57) Abstract: Disclosed are methods and kits for determining prognosis of a subject with left ventricular dysfunction that involve determining expression level of one or more micro RNA ("miRNA") species selected from the group consisting of hsa-miR-367, hsa-miR-10a, hsa-miR-187, hsa-miR-452, hsa-miR-218, hsa-miR-10b, hsa-miR-214, hsa-miR-193a, and hsa-miR-565 in a sample from the subject and comparing the expression level to a reference level. Also disclosed are methods and compositions for treating left ventricular dysfunction that include an inhibitor of CMPXI.



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DESCRIPTION

MicroRNA SIGNATURE TO PREDICT PROGNOSIS IN HEART FAILURE

BACKGROUND OF THE INVENTION

This application claims priority to U.S. Provisional Patent Application serial number 61/167,028, filed April 6, 2009, which is herein specifically incorporated by reference in its entirety.

1. Field of the Invention

The present invention relates generally to the fields of heart disease, molecular biology, and pharmaceutical formulations. More particularly, it concerns methods for determining prognosis in a subject with left ventricular dysfunction that involve analysis of micro RNA ("miRNA") expression levels. The invention also concerns methods and compositions for treating left ventricular dysfunction involving inhibitors of CMPX1, a zinc finger transcription factor.

2. Description of Related Art

Congestive heart failure is one of the leading causes of human morbidity and mortality in the developed world. Numerous studies have led to a greater understanding of factors involved in disease development and progression. For example, the understanding that circulating neurohormones play a key role in the process of adverse cardiac remodeling and progression of heart failure has led to the widespread usage of beta-adrenergic blockers and antagonists of the renin-angiotensin system, such that these therapies are now first-line therapy for all patients with cardiomyopathy, regardless of cause or stage of disease (Hunt *et al.*, 2005). However, despite widespread usage of these therapies, congestive heart failure is one of the leading causes of morbidity and mortality in the western world. Current estimates indicate a prevalence of the disease of 2%, with an estimated 5 year mortality of close to 50% (Mosterd and Hoes, 2007), despite modern medical therapies.

While medical therapy has had an important, albeit modest effect on the outcomes of patients with heart failure, more advanced heart failure therapies have provided only incremental benefits. These therapies include placement of implantable cardioverter-defibrillators, placement of biventricular pacemakers, and implantation of left ventricular assist devices. In addition, cardiac transplantation, reserved for patients with end-stage heart

failure, is an effective treatment, although its usage is limited by organ supply. These advanced therapies have substantial costs, limiting their applicability to large numbers of patients afflicted by an epidemic of heart failure.

Among patients with advanced heart failure, the established risk factors for high mortality include advanced age, male sex, ventricular size, renal dysfunction, and low blood pressure. Prognostic models based upon clinical factors such as the Seattle Heart Failure Model (Levy *et al.*, 2006) have been shown to be somewhat effective in predicting mortality in patients with heart failure. However, such models are unlikely to capture the full biological variability of the disease, and may fall short in discriminating outcomes in patients with advanced heart failure, in whom the mortality curve can be quite steep. In addition, such models use clinical parameters with significant overlap with risk factors for poor outcomes due to advanced heart failure, suggesting that clinical risk predictors miss the optimal window for intervention for patients with advanced heart failure.

One of the major problems in the management and treatment of patients with advanced heart failure is the inability to predict which patients will survive with their disease for many years on a modern medical regimen, and which patients will progress despite treatment. Identifying such patients at an early stage of disease would be a very useful tool to help determine which patients represent a higher risk subset, and thus, which patients may benefit maximally from advanced heart failure therapy. This is particularly relevant to the usage of such advanced therapies as ventricular assist devices and cardiac transplantation, since these therapies have a substantial up-front morbidity and are thus often employed at a later stage of disease, only after a patient is deemed to have failed medical therapy. At such a point, patients are often very ill, increasing their procedural risk and decreasing their magnitude of benefit from device therapy or transplantation.

Currently, cardiologists use clinical parameters, echocardiographic parameters, and invasive hemodynamic studies to predict prognosis and to determine the treatment course for patients with cardiomyopathy. These parameters are limited in their ability to predict outcomes in such patients with advanced heart failure. Delayed referral for advanced heart failure therapies is strongly associated with poor outcome in patients with advanced heart failure. A diagnostic assay that would allow cardiologists to identify high-risk patients at an earlier time point and thus improve the outcomes in patients treated with advanced heart failure therapeutics would be of great utility. Thus, not only is there the need for a greater understanding of factors involved in the development and progression of heart failure, but

there is also a need to be able to accurately predict prognosis in patients with newly diagnosed heart failure.

SUMMARY OF THE INVENTION

The present invention is in part based on the finding that patients with heart disease with divergent clinical outcomes have distinct micro RNA (“miRNA”) profiles. For example, the inventors have found that in patients with left ventricular dysfunction due to non-ischemic cardiomyopathy, a unique micro RNA signature can distinguish patients with poor outcomes and high mortality from those patients with better short-term outcomes, independent of any other clinical parameters. Furthermore, the present invention is in part based on the finding that the gene CMPX1, a zinc finger transcription factor of unknown function, is differentially expressed between patients with a poor short term outcome and patients with a better short term outcome, thus implicating CMPX1 as a target for therapeutic intervention in patients with left ventricular dysfunction.

In some aspects, the present invention concerns methods for determining prognosis in a subject with left ventricular dysfunction that involve determining expression level of one or more miRNA species selected from the group consisting of hsa-miR-367, hsa-miR-10a, hsa-miR-187, hsa-miR-452, hsa-miR-218, hsa-miR-10b, hsa-miR-214, hsa-miR-193a, and hsa-miR-565 in a sample from the subject, and comparing the expression level of the one or more miRNA species to one or more reference levels to determine prognosis. As used herein, “left ventricular dysfunction” refers to decreased contractility of the left ventricle of any cause. For example, the left ventricular dysfunction may result in heart failure. As used herein, the term “heart failure” is broadly used to mean any condition that reduces the ability of the heart to pump blood. “Prognosis” as used herein refers to a prediction of the course of a disease. For example, a prognosis may include a prediction as to whether a patient improves over time, or a prediction as to whether a patient will clinically deteriorate over time. A prognosis as used herein also refers to a prediction as to whether a patient will or will not respond to a particular therapy. The response may include, for example, improved left ventricular function or improvement in symptoms of left ventricular dysfunction.

The subject can be any subject with a heart. For example, the subject may be a human, a primate, a horse, a cow, a pig, a goat, a sheep, a dog, a cat, a mouse, a rat, or an avian species. In specific embodiments, the subject is a human. The human may be a subject that has previously been diagnosed with left ventricular dysfunction, or may be a subject that has not been previously diagnosed with left ventricular dysfunction. The subject may, at the

time of sampling, be known to have active left ventricular dysfunction or may be suspected of having left ventricular dysfunction. The subject may be a subject with a history of previously treated left ventricular dysfunction who is asymptomatic at the time the sample was obtained. The left ventricular dysfunction can be of any cause. For example, the left ventricular
5 dysfunction may be the result of ischemia (*e.g.*, ischemic cardiomyopathy), or may not be associated with ischemia (non-ischemic cardiomyopathy). In a specific embodiment, the subject has non-ischemic cardiomyopathy.

The expression level of one or more miRNA species can be determined using any method known to those of ordinary skill in the art. For example, the method may involve any
10 of a variety of techniques known to those of ordinary skill in the art. Examples of such techniques include reverse transcriptase (RT) PCR, PCR, allele specific oligonucleotide hybridization, size analysis, sequencing, hybridization, 5' nuclease digestion, single-stranded conformation polymorphism analysis, allele specific hybridization, primer specific extension, and oligonucleotide ligation assays.

15 The sample can be any tissue sample obtained from the subject. In particular embodiments, the sample is a heart tissue sample from the subject. For example, the sample may be endomyocardial tissue obtained by biopsy of the endomyocardium, or myocardial tissue obtained by biopsy.

The reference level is a reference level of miRNA expression level from a different
20 subject or group of subjects, wherein the level of miRNA expression level is the expression level of hsa-miR-367, hsa-miR-10a, hsa-miR-187, hsa-miR-452, hsa-miR-218, hsa-miR-10b, hsa-miR-214, hsa-miR-193a, or hsa-miR-565, or a combination thereof. For example, the reference level may be the expression level of one or more of the aforementioned miRNA species in one or more subjects with severe left ventricular dysfunction (positive control). In
25 other embodiments, the reference level is the expression level of one or more of the aforementioned miRNA species in one or more subjects without left ventricular dysfunction (negative control).

The reference level can be obtained from a single subject or from a group of subjects. The reference level of miRNA expression can be determined using any method known to
30 those of ordinary skill in the art, such as any of the methods discussed above and elsewhere in this description. In some embodiments, the reference level is an average level of expression of hsa-miR-367, hsa-miR-10a, hsa-miR-187, hsa-miR-452, hsa-miR-218, hsa-miR-10b, hsa-miR-214, hsa-miR-193a, or hsa-miR-565 obtained from a cohort of subjects with left ventricular dysfunction with a known poor outcome following a therapeutic intervention. In

other embodiments, the reference level is an average level of expression of hsa-miR-367, hsa-miR-10a, hsa-miR-187, hsa-miR-452, hsa-miR-218, hsa-miR-10b, hsa-miR-214, hsa-miR-193a, or hsa-miR-565 obtained from a cohort of subjects with left ventricular dysfunction with a known good outcome following a therapeutic intervention. Good outcome can be measured by any method known to those of ordinary skill in the art. For example, good outcome can be assessed as improvement in signs or symptoms of left ventricular dysfunction or prolonged survival compared to another cohort of subjects. The reference level may be a single value of miRNA expression level, or it may be a range of values of miRNA expression level. The reference level may also be depicted graphically as an area on a graph.

In non-limiting examples, normalized cycle thresholds derived from quantitative RT-PCT using levels of RNU44 and RNU48 for data normalization are used as reference levels. Values set forth in Table 1 are associated with a poor prognosis:

Table 1.

miRNA	Reference Level (Normalized Cycle Thresholds)
hsa-miR-10a	>5.5
hsa-miR-367	<7.1
hsa-miR-187	<9.9
hsa-miR-452	<7.0
hsa-miR-218	<4.9
hsa-miR-10b	>7.1
hsa-miR-214	>1.8
hsa-miR-193a	>6.0
hsa-miR-565	>4.0

In some embodiments, the distribution of expression of hsa-miR-367, hsa-miR-10a, hsa-miR-187, hsa-miR-452, hsa-miR-218, hsa-miR-10b, hsa-miR-214, hsa-miR-193a, and/or hsa-miR-565 in a sample from the subject is determined, and compared with the distribution of hsa-miR-367, hsa-miR-10a, hsa-miR-187, hsa-miR-452, hsa-miR-218, hsa-miR-10b, hsa-miR-214, hsa-miR-193a, and/or hsa-miR-565 in samples from a cohort of subjects with left ventricular dysfunction that had a particular outcome. In this manner, it may be possible to establish statistically significant correlations between particular levels of miRNA expression and prognosis.

In some embodiments, reduced expression level of hsa-miR-10a, hsa-miR-187, hsa-miR-452, or hsa-miR-218 relative to a reference level is indicative of left ventricular dysfunction that is not associated with a high mortality rate. For example, reduced expression level of hsa-miR-10a, hsa-miR-187, hsa-miR-452, or hsa-miR-218 in the sample from the
5 subject relative to expression level of hsa-miR-10a, hsa-miR-187, hsa-miR-452, or hsa-miR-218 in a reference set of samples obtained from subjects with severe left ventricular dysfunction known to be associated with a poor prognosis, indicates a reduced likelihood that the subject has severe left ventricular dysfunction associated with a poor prognosis.

In other embodiments, increased expression level of hsa-miR-367, hsa-miR-10b, hsa-miR-214, hsa-miR-193a, or hsa-miR-565 relative to a reference level is indicative of left
10 ventricular dysfunction that is not associated with a high mortality rate. For example, increased expression level of hsa-miR-367, hsa-miR-10b, hsa-miR-214, hsa-miR-193a, or hsa-miR-565 in the sample from the subject relative to expression level of hsa-miR-367, hsa-miR-10b, hsa-miR-214, hsa-miR-193a, or hsa-miR-565 in a reference set of samples obtained
15 from subjects with severe left ventricular dysfunction known to be associated with a poor prognosis, indicates a reduced likelihood that the subject has severe left ventricular dysfunction associated with a poor prognosis.

In some embodiments of the methods set forth herein, the method further comprises obtaining a sample from the subject. The sample may be any sample as discussed above, but
20 in particular embodiments the sample is endomyocardial tissue.

Any of the foregoing methods may optionally include assessment of one or more additional factors for evaluating a subject with left ventricular dysfunction. Examples of such assessments include measurement of left ventricular contractility, measurement of left ventricular ejection fraction, and so forth. The method may further comprise performing
25 echocardiography, performing cardiac catheterization, or performing open heart surgery.

In some embodiments, the method further comprises determining expression level of CMPX1 in a sample from the subject and comparing the expression level of CMPX1, a zinc finger transcription factor, to a reference level. Determination of level of expression of CMPX1 may be performed by measuring mRNA or by directly measuring CMPX1 protein
30 levels. The amino acid sequence of CMPX1 is provided as SEQ ID NO:1, and the nucleotide sequence of the gene encoding CMPX1 is provided as SEQ ID NO:2. Any technique known to those of ordinary skill in the art may be employed, and examples of such techniques are discussed in the specification below.

The present invention also concerns methods for determining prognosis in a subject with left ventricular dysfunction, involving determining expression level of CMPX1, a zinc finger transcription factor, in a sample from the subject and comparing the expression level of CMPX1 in the sample from the subject to a reference level to determine prognosis. The sample may be any such sample as discussed above, but in particular embodiments the sample is a heart tissue sample obtained by endomyocardial biopsy. In some embodiments, an increased expression level of CMPX1 relative to the reference level is indicative of left ventricular dysfunction that is associated with a high mortality rate. The reference level may be the level of CMPX1 in a subject (or mean level from a group of subjects) with left ventricular dysfunction that is not associated with a high mortality rate, or may be the level of CMPX1 in a subject (or mean level from a group of subjects) that do not have left ventricular dysfunction. In some non-limiting examples, the reference level of CMPX1 that is associated with poor prognosis is a normalized intensity value (arbitrary units) of greater than 150. This reference level was obtained using probe set 228988_at from the Human Genome U133 Plus 2.0 array (Affymetrix).

Also disclosed are methods of treating left ventricular dysfunction in a subject that involve administering to the subject a pharmaceutically effective amount of a composition comprising an inhibitor of CMPX1. The inhibitor of CMPX1 may be a compound that inhibits the expression level of a gene encoding CMPX1, or may be a compound that acts to inhibit function of the CMPX1 protein. The inhibitor may be any inhibitor, such as a polynucleotide, a protein, a polypeptide, a peptide, an antibody, an antibody fragment, or a small molecule. In a particular embodiment, the inhibitor of CMPX1 is a polynucleotide. In more particular embodiments, the inhibitor of CMPX1 is a RNA. In a specific embodiment, the inhibitor of CMPX1 is a miRNA, or a nucleic acid that encodes a miRNA. The polynucleotide may be a polynucleotide that hybridizes to a nucleic acid that encodes CMPX1.

The left ventricular dysfunction may be left ventricular dysfunction of any cause. For example, the left ventricular dysfunction may be associated with ischemic cardiomyopathy or non-ischemic cardiomyopathy. In a particular embodiment, the left ventricular dysfunction of the subject is associated with non-ischemic cardiomyopathy. The subject may be a subject with congestive heart failure.

In some embodiments, the methods set forth herein further include administering to the subject a secondary form of therapy for the treatment of left ventricular dysfunction. Non-limiting examples of the secondary form of therapy include a pharmaceutical agent, a

left ventricular assist device, or cardiac transplantation. Non-limiting examples of pharmaceutical agents include beta-blockers, calcium channel blockers, and inotropic agents. Examples of specific agents are set forth in the specification below.

Aspects of the present invention also include kits for determining prognosis of a subject with left ventricular dysfunction that include one or more polynucleotides for analysis of at least one miRNA species selected from the group consisting of hsa-miR-367, hsa-miR-10a, hsa-miR-187, hsa-miR-452, hsa-miR-218, hsa-miR-10b, hsa-miR-214, hsa-miR-193a, and hsa-miR-565, wherein each polynucleotide specifically hybridizes to at least one miRNA species selected from the group consisting of hsa-miR-367, hsa-miR-10a, hsa-miR-187, hsa-miR-452, hsa-miR-218, hsa-miR-10b, hsa-miR-214, hsa-miR-193a, and hsa-miR-565. In some embodiments, the kit further includes a set of primers specific for transcription or reverse transcription of one or more miRNA species selected from the group consisting of hsa-miR-367, hsa-miR-10a, hsa-miR-187, hsa-miR-452, hsa-miR-218, hsa-miR-10b, hsa-miR-214, hsa-miR-193a, and hsa-miR-565. The kit may optionally include a miRNA array card, wherein the one or more polynucleotides are arrayed on said card. In some embodiments, the kit further includes a software package for statistical analysis of miRNA expression level of the one or more miRNA species selected from the group consisting of hsa-miR-367, hsa-miR-10a, hsa-miR-187, hsa-miR-452, hsa-miR-218, hsa-miR-10b, hsa-miR-214, hsa-miR-193a, and hsa-miR-565 relative to a reference level, as discussed above. The kit may optionally include instructions for use of the kit components.

The present invention also concerns kits for treating left ventricular dysfunction of a subject, the kit comprising an inhibitor of CMPX1 as set forth above. The kit may optionally include a secondary pharmaceutical agent that can be applied in the treatment of left ventricular dysfunction in the subject. The secondary pharmaceutical agent may be comprised in the same container as the inhibitor of CMPX1, or may be comprised in a separate container.

The present invention also includes pharmaceutical compositions that include an inhibitor of CMPX1 and a secondary compound that can be applied in the treatment of left ventricular dysfunction in a subject. Non-limiting examples of secondary compounds that can be applied in the treatment of left ventricular dysfunction are discussed above and elsewhere in this specification. Some examples include a beta-blocker, an angiotensin converting enzyme inhibitor, hydralazine, an aldosterone antagonist, a diuretic, or an inotrope. In some embodiments, the inhibitor is CMPX1 is hsa-miR-367, a nucleic acid that encodes miR-367, or a nucleic acid that hybridizes to a gene that encodes CMPX1.

It is specifically contemplated that any limitation discussed with respect to one embodiment of the invention may apply to any other embodiment of the invention. Furthermore, any composition of the invention may be used in any method of the invention, and any method of the invention may be used to produce or to utilize any composition of the invention.

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

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

As used herein the specification, “a” or “an” may mean one or more, unless clearly indicated otherwise. As used herein in the claim(s), when used in conjunction with the word “comprising,” the words “a” or “an” may mean one or more than one. As used herein “another” may mean at least a second or more.

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

BRIEF DESCRIPTION OF THE FIGURES

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

FIG. 1. Principal component analysis mapping based upon microRNA profiles of heart failure patients with divergent outcomes. Principal component analysis of microRNAs array data derived from endomyocardial biopsy samples from heart failure patients with poor outcomes (Group 2, lower spheres) or better outcomes (Group 1, upper spheres) demonstrates distinct clustering according to clinical outcome.

FIG. 2. Dendrogram derived from hierarchical clustering analysis of microRNA profiles derived from endomyocardial biopsy samples from heart failure patients. Unbiased

hierarchical clustering analysis of microRNA profiles of patients with non-ischemic cardiomyopathy reveals hierarchical clustering into two distinct groups according to clinical outcome.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Currently, cardiologists use clinical parameters, echocardiographic parameters, and invasive hemodynamic studies to predict prognosis and to determine the treatment course for patients with left ventricular dysfunction, such as non-ischemic cardiomyopathy. These parameters are limited in their ability to predict outcomes in such patients with advanced heart failure. Delayed referral for advanced heart failure therapies is strongly associated with poor outcome in patients with advanced heart failure. A diagnostic assay would allow cardiologists to identify high-risk patients at an earlier time point and thus improve the outcomes in patients treated with advanced heart failure. The present invention is in part based on the finding that heart failure patients with divergent outcomes have unique micro RNA (“miRNA”) signatures. The present invention is also in part based on the finding that an individual gene (CMPX1) of unknown function is significantly differentially expressed between groups of heart failure patients with divergent clinical outcomes. These findings implicate CMPX1 as a target for therapeutic intervention in patients with left ventricular dysfunction.

A. miRNAs

1. Background

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

miRNAs thus far observed have been approximately 21-22 nucleotides in length and they arise from longer precursors, which are transcribed from non-protein-encoding genes. See review of Carrington *et al.* (2003). The precursors form structures that fold back on each other in self-complementary regions; they are then processed by the nuclease Dicer in animals or DCL1 in plants. miRNA molecules interrupt translation through precise or imprecise base-pairing with their targets.

miRNAs are involved in gene regulation. Some miRNAs, including *lin-4* and *let-7*, inhibit protein synthesis by binding to partially complementary 3' untranslated regions (3' UTRs) of target mRNAs. Others function like siRNA and bind to perfectly complementary mRNA sequences to destroy the target transcript.

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

There are a few examples of miRNAs that play critical roles in cell differentiation, early development, and cellular processes like apoptosis. *lin-4* and *let-7* both regulate passage from one larval state to another during *C. elegans* development (Ambros, 2003). *mir-14* and *bantam* are *drosophila* miRNAs that regulate cell death, apparently by regulating the expression of genes involved in apoptosis (Brennecke *et al.*, 2003, Xu *et al.*, 2003). miR-181 guides hematopoietic cell differentiation (Chen *et al.*, 2004). Enhanced understanding of the functions of miRNAs will undoubtedly reveal regulatory networks that contribute to normal development, differentiation, inter- and intra-cellular communication, cell cycle, angiogenesis, apoptosis, and many other cellular processes.

2. miRNA Sequence Information

Certain embodiments of the present invention involve methods for determining prognosis in a subject with left ventricular dysfunction that involve determining expression level of one or more miRNA species in a sample from the subject. The miRNA species that are analyzed include species selected from the group shown in Table 2 below.

Table 2. Selected miRNA

miRNA Designation	Sequence	Sequence Identifier
hsa-miR-367	acuguugcuaauaugcaacucu	SEQ ID NO:3
hsa-miR-10a	uaccuguagaucggaauuugug	SEQ ID NO:4
hsa-miR-187	ucgugucuuguguugcagccgg	SEQ ID NO:5
hsa-miR-452	aacuguuugcagaggaaacuga	SEQ ID NO:6
hsa-miR-218	uugugcuugaucuaaccaugu	SEQ ID NO:7
hsa-miR-10b	uaccuguagaaccgaauuugug	SEQ ID NO:8
hsa-miR-214	ugccugucuacacuugcugugc	SEQ ID NO:9
hsa-miR-193a	ugggucuuugcgggcgagauga	SEQ ID NO:10
hsa-miR-565	ggcuggcucgcgaugucuguuu	SEQ ID NO:11

B. Methods for Analyzing Expression Level of miRNA and Gene Expression

Some embodiments of the methods of the present invention involve analysis of miRNA expression level or gene expression level. Methods for analyzing gene expression or expression of miRNA include, but are not limited to, methods based on hybridization analysis of polynucleotides, sequencing of polynucleotides, and analysis of protein expression such as proteomics-based methods. Commonly used methods for the quantification of mRNA expression level in a sample include northern blotting and in situ hybridization (Parker & Barnes, 1999), RNase protection assays (Hod, 1992), and PCR-based methods, such as reverse transcription polymerase chain reaction (RT-PCR) (Weis *et al.*, 1992). In some embodiments, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. Representative methods for sequencing-based gene expression analysis include Serial Analysis of Gene Expression (SAGE), and gene expression analysis by massively parallel signature sequencing (MPSS).

1. PCR-Based Methods

Gene expression level or miRNA expression level can be analyzed using techniques that employ PCR. PCR is useful to amplify and detect transcripts from a sample. Examples of PCT methodologies are discussed below.

RT-PCR is a sensitive quantitative method that can be used to compare mRNA levels in different samples (*e.g.*, endomyocardial biopsy samples) to examine gene expression signatures.

To perform RT-PCR, mRNA is isolated from a sample. For example, total RNA may be isolated from a sample of heart tissue. mRNA may also be extracted, for example, from frozen or archived paraffin-embedded and fixed tissue samples. Methods for mRNA extraction are known in the art. See, *e.g.*, Ausubel *et al.* (1997). Methods for RNA extraction from paraffin embedded tissues are disclosed, for example, in Rupp and Locker, 1987, and De Andres *et al.*, 1995. Purification kits for RNA isolation from commercial manufacturers, such as Qiagen, can be used. For example, total RNA from a sample can be isolated using Qiagen RNeasy mini-columns. Other commercially available RNA isolation kits include MasterPure.TM. Complete DNA and RNA Purification Kit (EPICENTRE.TM., Madison, Wis.), and, Paraffin Block RNA Isolation Kit (Ambion, Inc.). Total RNA from tissue samples can be also isolated using RNA Stat-60 (Tel-Test) or by cesium chloride density gradient centrifugation.

RNA is then reverse transcribed into cDNA. The cDNA is amplified in a PCR reaction. A variety of reverse transcriptases are known in the art. The reverse transcription step is typically primed using specific primers, random hexamers, or oligo-dT primers, depending on the conditions and desired readout. For example, extracted RNA can be reverse-transcribed using a GeneAmp RNA PCR kit (Perkin Elmer, Calif., USA), following the manufacturer's instructions. The derived cDNA can then be used as a template in the subsequent PCR reaction. The PCR reaction may employ the Taq DNA polymerase, which has a 5'-3' nuclease activity but lacks a 3'-5' proofreading endonuclease-activity. Two oligonucleotide primers are used to generate an amplicon in the PCR reaction.

For quantitative PCR, a third oligonucleotide, or probe, is used to detect nucleotide sequence located between the two PCR primers. The probe is non-extendible by Taq DNA polymerase enzyme, and typically is labeled with a reporter fluorescent dye and a quencher fluorescent dye. Any laser-induced emission from the reporter dye is quenched by the quenching dye when the two dyes are located close together as they are on the probe. During the amplification reaction, the Taq DNA polymerase enzyme cleaves the probe in a template-dependent manner. The resultant probe fragments disassociate in solution, and signal from the released reporter dye is free from the quenching effect of the second fluorophore. One molecule of reporter dye is liberated for each new molecule synthesized, and detection of the unquenched reporter dye provides the basis for quantitative analysis.

RT-PCR can be performed using commercially available equipment, such as an ABI PRISM 7700.TM. Sequence Detection System (Perkin-Elmer-Applied Biosystems, Foster City, Calif., USA), or Lightcycler.RTM. (Roche Molecular Biochemicals, Mannheim,

Germany). Samples can be analyzed using a real-time quantitative PCR device such as the ABI PRISM 7700.TM. Sequence Detection System.TM.

To minimize errors and the effect of sample-to-sample variation, RT-PCR is usually performed using an internal standard. A suitable internal standard is expressed at a constant level among different tissues, and is unaffected by the experimental variable. RNAs frequently used to normalize patterns of gene expression are mRNAs for the housekeeping genes glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and .beta.-actin.

A variation of the RT-PCR technique is real time quantitative PCR, which measures PCR product accumulation through a dual-labeled fluorogenic probe, such as a TaqMan.TM. probe. Real time PCR is compatible both with quantitative competitive PCR, where internal competitor for each target sequence is used for normalization, and with quantitative comparative PCR using a normalization gene contained within the sample, or a housekeeping gene for RT-PCR.

Gene expression level may be examined using fixed, paraffin-embedded tissues as the RNA source. Briefly, in one exemplary method, sections of paraffin-embedded tissue samples are cut. RNA is extracted, and protein and DNA are removed. After analysis of the RNA concentration, RNA repair and/or amplification steps may be performed, if necessary, and RNA is reverse transcribed using gene specific promoters followed by RT-PCR. Methods of examining expression level in fixed, paraffin-embedded tissues, are described, for example, in Godfrey *et al.*, 2000; and Specht *et. al.*, 2001.

Another approach for gene expression analysis employs competitive PCR design and automated, high-throughput matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS detection and quantification of oligonucleotides. This method is described by Ding and Cantor, 2003. See also the MassARRAY-based gene expression profiling method, developed by Sequenom, Inc. (San Diego, Calif.).

Additional PCR-based techniques for gene expression analysis include, *e.g.*, differential display (Liang and Pardee, 1992); amplified fragment length polymorphism (iAFLP) (Kawamoto *et al.*, 1999); BeadArray.TM. technology (Illumina, San Diego, Calif.; Oliphant *et al.*, 2002; Ferguson *et al.*, 2000); BeadsArray for Detection of Gene Expression (BADGE), using the commercially available Luminex100 LabMAP system and multiple color-coded microspheres (Luminex Corp., Austin, Tex.) in a rapid assay for gene expression (Yang *et al.*, 2001); and high coverage expression profiling (HiCEP) analysis (Fukumura *et al.*, 2003).

2. Microarrays

Evaluating gene expression level in a sample can also be performed with microarrays. Microarrays permit simultaneous analysis of a large number of gene expression products. Typically, polynucleotides of interest are plated, or arrayed, on a microchip substrate. The arrayed sequences are then hybridized with nucleic acids (*e.g.*, DNA or RNA) from cells or tissues of interest. The source of mRNA typically is total RNA. If the source of mRNA is endomyocardial tissue, mRNA can be extracted.

In various embodiments of the microarray technique, probes to at least 10, 25, 50, 100, 200, 500, 1000, 1250, 1500, or 1600 polynucleotides are immobilized on an array substrate (for example, a porous or nonporous solid support, such as a glass, plastic, or gel surface). The probes can include DNA, RNA, copolymer sequences of DNA and RNA, DNA and/or RNA analogues, or combinations thereof.

In some embodiments, a microarray includes a support with an ordered array of binding (*e.g.*, hybridization) sites for each individual polynucleotide of interest. The microarrays can be addressable arrays, such as positionally addressable arrays where each probe of the array is located at a known, predetermined position on the solid support such that the identity of each probe can be determined from its position in the array.

Each probe on the microarray can be between about 10-50,000 nucleotides in length. The probes of the microarray can consist of nucleotide sequences with lengths: less than 1,000 nucleotides, such as sequences 10-1,000, or 10-500, or 10-200 nucleotides in length. An array can include positive control probes, such as probes known to be complementary and hybridizable to sequences in the test sample, and negative control probes such as probes known to not be complementary and hybridizable to sequences in the test sample.

Methods for attaching nucleic acids to a surface are well-known in the art. Methods for immobilizing nucleic acids on glass are described in Schena *et al.*, 1995; DeRisi *et al.*, 1996; Shalon *et al.*, 1996; and Schena *et al.*, 1995). Techniques are known for producing arrays with thousands of oligonucleotides at defined locations using photolithographic techniques are described by Fodor *et al.*, 1991; Pease *et al.*, 1994; Lockhart *et al.*, 1996; U.S. Pat. Nos. 5,578,832; 5,556,752; and 5,510,270). Other methods for making microarrays have been described. See, *e.g.*, Maskos and Southern, 1992. Any type of array may be used in the context of the present invention.

3. Serial Analysis of Gene Expression (SAGE)

Gene expression level or miRNA expression level in samples may also be determined by serial analysis of gene expression (SAGE), which is a method that allows the simultaneous and quantitative analysis of a large number of gene transcripts, without the need of providing an individual hybridization probe for each transcript (see Velculescu *et al.*, 1995; and Velculescu *et al.*, 1997). Briefly, a short sequence tag (about 10-14 nucleotides) is generated that contains sufficient information to uniquely identify a transcript, provided that the tag is obtained from a unique position within each transcript. Then, many transcripts are linked together to form long serial molecules, that can be sequenced, revealing the identity of the multiple tags simultaneously. The expression pattern of a population of transcripts can be quantitatively evaluated by determining the abundance of individual tags, and identifying the gene corresponding to each tag.

4. Protein Detection Methodologies

Immunohistochemical methods are also suitable for detecting the expression of the genes such as CMPX1. Antibodies, most preferably monoclonal antibodies, specific for a gene product are used to detect expression. The antibodies can be detected by direct labeling of the antibodies themselves, for example, with radioactive labels, fluorescent labels, hapten labels such as, biotin, or an enzyme such as horse radish peroxidase or alkaline phosphatase. Alternatively, unlabeled primary antibody is used in conjunction with a labeled secondary antibody, comprising antisera, polyclonal antisera or a monoclonal antibody specific for the primary antibody. Immunohistochemistry protocols and kits are well known in the art and are commercially available.

Proteomic methods can allow examination of global changes in protein expression in a sample. Proteomic analysis may involve separation of individual proteins in a sample by 2-D gel electrophoresis (2-D PAGE), and identification of individual proteins recovered from the gel, such as by mass spectrometry or N-terminal sequencing, and analysis of the data using bioinformatics. Proteomics methods can be used alone or in combination with other methods for evaluating gene expression.

In various aspects, the expression of certain genes in a sample is detected to provide clinical information, such as information regarding prognosis. Thus, gene expression assays include measures to correct for differences in RNA variability and quality. For example, an assay typically measures and incorporates the expression of certain normalizing genes, such known housekeeping genes. Alternatively, normalization can be based on the mean or

median signal (Ct) of all of the assayed genes or a large subset thereof (global normalization approach). In some embodiments, a normalized test RNA (e.g., from a patient sample) is compared to the amount found in a sample from a patient with left ventricular dysfunction. The level of expression measured in a particular test sample can be determined to fall at some percentile within a range observed in reference sets.

C. Kits

The technology herein includes kits for evaluating miRNA or gene expression in samples. A "kit" refers to a combination of physical elements. For example, a kit may include, for example, one or more components such as probes, including without limitation specific primers, antibodies, a protein-capture agent, a reagent, an instruction sheet, and other elements useful to practice the technology described herein. These physical elements can be arranged in any way suitable for carrying out the invention.

Kits for analyzing RNA expression may include, for example, a set of oligonucleotide probes for detecting expression of a gene such as CHXP1 or a miRNA. The probes can be provided on a solid support, as in an array (e.g., a microarray), or in separate containers. The kits can include a set of oligonucleotide primers useful for amplifying a set of genes described herein, such as to perform PCR analysis. Kits can include further buffers, enzymes, labeling compounds, and the like. Any of the compositions described herein may be comprised in a kit. In a non-limiting example, an individual miRNA is included in a kit. The kit may further include water and hybridization buffer to facilitate hybridization of the two strands of the miRNAs. The kit may also include one or more transfection reagents to facilitate delivery of the miRNA to cells.

A kit for analyzing protein expression can include specific binding agents, such as immunological reagents (e.g., an antibody) for detecting protein expression of a gene of interest. For example, the kit can include an antibody that detects expression of CHXP1 expression in a tissue sample.

The components of the kits may be packaged either in aqueous media or in lyophilized form. The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which a component may be placed, and preferably, suitably aliquoted. Where there is more than one component in the kit, the kit also will generally contain a second, third or other additional container into which the additional components may be separately placed. However, various combinations of components may be comprised in a single vial. The kits of the present invention also will typically include a means for containing the nucleic acids, and any other reagent containers in

close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained.

When the components of the kit are provided in one and/or more liquid solutions, the liquid solution is an aqueous solution, such as a sterile aqueous solution.

5 However, the components of the kit may be provided as dried powder(s). When reagents and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means.

10 The container means will generally include at least one vial, test tube, flask, bottle, syringe and/or other container means, into which the nucleic acid formulations are placed, preferably, suitably allocated. The kits may also comprise a second container means for containing a sterile, pharmaceutically acceptable buffer and/or other diluent.

The kits of the present invention will also typically include a means for containing the vials in close confinement for commercial sale.

15 Such kits may also include components that preserve or maintain the miRNA or that protect against its degradation. Such components may be RNase-free or protect against RNases. Such kits generally will comprise, in suitable means, distinct containers for each individual reagent or solution.

20 A kit will also include instructions for employing the kit components as well the use of any other reagent not included in the kit. Instructions may include variations that can be implemented.

It is contemplated that such reagents are embodiments of kits of the invention. Such kits, however, are not limited to the particular items identified above and may include any reagent used for the manipulation or characterization of miRNA.

25

D. Vectors for Cloning, Gene Transfer and Expression

30 Within certain embodiments expression vectors are employed to express a nucleic acid of interest, such as a miRNA that inhibits the expression of CHXP1. Expression requires that appropriate signals be provided in the vectors, and which include various regulatory elements, such as enhancers/promoters from both viral and mammalian sources that drive expression of the genes of interest in host cells. Elements designed to optimize messenger RNA stability and translatability in host cells also are defined. The conditions for the use of a number of dominant drug selection markers for establishing permanent, stable

cell clones expressing the products are also provided, as is an element that links expression of the drug selection markers to expression of the polypeptide.

1. Regulatory Elements

Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. In certain embodiments, expression includes both transcription of a gene and translation of mRNA into a gene product. In other embodiments, expression only includes transcription of the nucleic acid encoding a gene of interest.

In certain embodiments, the nucleic acid encoding a gene product is under transcriptional control of a promoter. 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. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (*tk*) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another.

In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

In other embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, rat insulin promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose.

By employing a promoter with well-known properties, the level and pattern of expression of the protein of interest following transfection or transformation can be optimized. Further, selection of a promoter that is regulated in response to specific physiologic signals can permit inducible expression of the gene product. Tables 1 and 2 list several regulatory elements that may be employed, in the context of the present invention, to regulate the expression of the gene of interest. This list is not intended to be exhaustive of all the possible elements involved in the promotion of gene expression but, merely, to be exemplary thereof.

Enhancers are genetic elements that increase transcription from a promoter located at a distant position on the same molecule of DNA. Enhancers are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Below is a list of viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the nucleic acid encoding a gene of interest in an expression construct (Table 3 and Table 4). Additionally, any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of the gene. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is

provided, either as part of the delivery complex or as an additional genetic expression construct.

TABLE 3 Promoter and/or Enhancer	
Promoter/Enhancer	References
Immunoglobulin Heavy Chain	Banerji <i>et al.</i> , 1983; Gilles <i>et al.</i> , 1983; Grosschedl <i>et al.</i> , 1985; Atchinson <i>et al.</i> , 1986, 1987; Imler <i>et al.</i> , 1987; Weinberger <i>et al.</i> , 1984; Kiledjian <i>et al.</i> , 1988; Porton <i>et al.</i> ; 1990
Immunoglobulin Light Chain	Queen <i>et al.</i> , 1983; Picard <i>et al.</i> , 1984
T-Cell Receptor	Luria <i>et al.</i> , 1987; Winoto <i>et al.</i> , 1989; Redondo <i>et al.</i> ; 1990
HLA DQ α and/or DQ β	Sullivan <i>et al.</i> , 1987
β -Interferon	Goodbourn <i>et al.</i> , 1986; Fujita <i>et al.</i> , 1987; Goodbourn <i>et al.</i> , 1988
Interleukin-2	Greene <i>et al.</i> , 1989
Interleukin-2 Receptor	Greene <i>et al.</i> , 1989; Lin <i>et al.</i> , 1990
MHC Class II 5	Koch <i>et al.</i> , 1989
MHC Class II HLA-DRA	Sherman <i>et al.</i> , 1989
β -Actin	Kawamoto <i>et al.</i> , 1988; Ng <i>et al.</i> ; 1989
Muscle Creatine Kinase (MCK)	Jaynes <i>et al.</i> , 1988; Horlick <i>et al.</i> , 1989; Johnson <i>et al.</i> , 1989
Prealbumin (Transthyretin)	Costa <i>et al.</i> , 1988
Elastase I	Ornitz <i>et al.</i> , 1987
Metallothionein (MTII)	Karin <i>et al.</i> , 1987; Culotta <i>et al.</i> , 1989
Collagenase	Pinkert <i>et al.</i> , 1987; Angel <i>et al.</i> , 1987a
Albumin	Pinkert <i>et al.</i> , 1987; Tronche <i>et al.</i> , 1989, 1990
α -Fetoprotein	Godbout <i>et al.</i> , 1988; Campere <i>et al.</i> , 1989
t-Globin	Bodine <i>et al.</i> , 1987; Perez-Stable <i>et al.</i> , 1990
β -Globin	Trudel <i>et al.</i> , 1987
c-fos	Cohen <i>et al.</i> , 1987
c-HA-ras	Triesman, 1986; Deschamps <i>et al.</i> , 1985
Insulin	Edlund <i>et al.</i> , 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsh <i>et al.</i> , 1990

TABLE 3
Promoter and/or Enhancer

Promoter/Enhancer	References
α_1 -Antitrypsin	Latimer <i>et al.</i> , 1990
H2B (TH2B) Histone	Hwang <i>et al.</i> , 1990
Mouse and/or Type I Collagen	Ripe <i>et al.</i> , 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang <i>et al.</i> , 1989
Rat Growth Hormone	Larsen <i>et al.</i> , 1986
Human Serum Amyloid A (SAA)	Edbrooke <i>et al.</i> , 1989
Troponin I (TN I)	Yutzey <i>et al.</i> , 1989
Platelet-Derived Growth Factor (PDGF)	Pech <i>et al.</i> , 1989
Duchenne Muscular Dystrophy	Klamut <i>et al.</i> , 1990
SV40	Banerji <i>et al.</i> , 1981; Moreau <i>et al.</i> , 1981; Sleight <i>et al.</i> , 1985; Firak <i>et al.</i> , 1986; Herr <i>et al.</i> , 1986; Imbra <i>et al.</i> , 1986; Kadesch <i>et al.</i> , 1986; Wang <i>et al.</i> , 1986; Ondek <i>et al.</i> , 1987; Kuhl <i>et al.</i> , 1987; Schaffner <i>et al.</i> , 1988
Polyoma	Swartzendruber <i>et al.</i> , 1975; Vasseur <i>et al.</i> , 1980; Katinka <i>et al.</i> , 1980, 1981; Tyndell <i>et al.</i> , 1981; Dandolo <i>et al.</i> , 1983; de Villiers <i>et al.</i> , 1984; Hen <i>et al.</i> , 1986; Satake <i>et al.</i> , 1988; Campbell and/or Villarreal, 1988
Retroviruses	Kriegler <i>et al.</i> , 1982, 1983; Levinson <i>et al.</i> , 1982; Kriegler <i>et al.</i> , 1983, 1984a, b, 1988; Bosze <i>et al.</i> , 1986; Miksicek <i>et al.</i> , 1986; Celander <i>et al.</i> , 1987; Thiesen <i>et al.</i> , 1988; Celander <i>et al.</i> , 1988; Choi <i>et al.</i> , 1988; Reisman <i>et al.</i> , 1989
Papilloma Virus	Campo <i>et al.</i> , 1983; Lusky <i>et al.</i> , 1983; Spandidos and/or Wilkie, 1983; Spalholz <i>et al.</i> , 1985; Lusky <i>et al.</i> , 1986; Cripe <i>et al.</i> , 1987; Gloss <i>et al.</i> , 1987; Hirochika <i>et al.</i> , 1987; Stephens <i>et al.</i> , 1987
Hepatitis B Virus	Bulla <i>et al.</i> , 1986; Jameel <i>et al.</i> , 1986; Shaul <i>et al.</i> , 1987; Spandau <i>et al.</i> , 1988; Vannice <i>et al.</i> , 1988
Human Immunodeficiency Virus	Muesing <i>et al.</i> , 1987; Hauber <i>et al.</i> , 1988; Jakobovits <i>et al.</i> , 1988; Feng <i>et al.</i> , 1988; Takebe <i>et al.</i> , 1988; Rosen <i>et al.</i> , 1988; Berkhout <i>et al.</i> , 1989; Laspias <i>et al.</i> , 1989; Sharp <i>et al.</i> , 1989; Braddock <i>et al.</i> , 1989
Cytomegalovirus (CMV)	Weber <i>et al.</i> , 1984; Boshart <i>et al.</i> , 1985; Foecking <i>et al.</i> , 1986
Gibbon Ape Leukemia Virus	Holbrook <i>et al.</i> , 1987; Quinn <i>et al.</i> , 1989

TABLE 4 Inducible Elements		
Element	Inducer	References
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter <i>et al.</i> , 1982; Haslinger <i>et al.</i> , 1985; Searle <i>et al.</i> , 1985; Stuart <i>et al.</i> , 1985; Imagawa <i>et al.</i> , 1987, Karin <i>et al.</i> , 1987; Angel <i>et al.</i> , 1987b; McNeill <i>et al.</i> , 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang <i>et al.</i> , 1981; Lee <i>et al.</i> , 1981; Majors <i>et al.</i> , 1983; Chandler <i>et al.</i> , 1983; Ponta <i>et al.</i> , 1985; Sakai <i>et al.</i> , 1988
β -Interferon	poly(rI)x poly(rc)	Tavernier <i>et al.</i> , 1983
Adenovirus 5 <u>E2</u>	E1A	Imperiale <i>et al.</i> , 1984
Collagenase	Phorbol Ester (TPA)	Angel <i>et al.</i> , 1987a
Stromelysin	Phorbol Ester (TPA)	Angel <i>et al.</i> , 1987b
SV40	Phorbol Ester (TPA)	Angel <i>et al.</i> , 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	Hug <i>et al.</i> , 1988
GRP78 Gene	A23187	Resendez <i>et al.</i> , 1988
α -2-Macroglobulin	IL-6	Kunz <i>et al.</i> , 1989
Vimentin	Serum	Rittling <i>et al.</i> , 1989
MHC Class I Gene H-2 κ b	Interferon	Blonar <i>et al.</i> , 1989
HSP70	E1A, SV40 Large T Antigen	Taylor <i>et al.</i> , 1989, 1990a, 1990b
Proliferin	Phorbol Ester-TPA	Mordacq <i>et al.</i> , 1989
Tumor Necrosis Factor	PMA	Hensel <i>et al.</i> , 1989
Thyroid Stimulating Hormone α Gene	Thyroid Hormone	Chatterjee <i>et al.</i> , 1989

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; Kelly *et al.*, 1995), the alpha actin promoter (Moss *et al.*, 1996), the troponin 1 promoter (Bhavsar *et al.*, 1996); the Na⁺/Ca²⁺ exchanger promoter (Barnes *et al.*, 1997), the dystrophin promoter (Kimura *et al.*, 1997), the alpha7 integrin promoter (Ziober and Kramer, 1996), the

brain natriuretic peptide promoter (LaPointe *et al.*, 1996) and the alpha B-crystallin/small heat shock protein promoter (Gopal-Srivastava, 1995), alpha myosin heavy chain promoter (Yamauchi-Takahara *et al.*, 1989) and the ANF promoter (LaPointe *et al.*, 1988).

Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

2. Selectable Markers

In certain embodiments of the invention, the cells contain nucleic acid constructs of the present invention, a cell may be identified *in vitro* or *in vivo* by including a marker in the expression construct. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression construct. Usually the inclusion of a drug selection marker aids in cloning and in the selection of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. Alternatively, enzymes such as herpes simplex virus thymidine kinase (*tk*) or chloramphenicol acetyltransferase (CAT) may be employed. Immunologic markers also can be employed. The selectable marker employed is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable markers are well known to one of skill in the art.

3. Multigene Constructs and IRES

In certain embodiments of the invention, the use of internal ribosome binding sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5' methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is

accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

Any heterologous open reading frame can be linked to IRES elements. This includes genes for secreted proteins, multi-subunit proteins, encoded by independent genes, intracellular or membrane-bound proteins and selectable markers. In this way, expression of several proteins can be simultaneously engineered into a cell with a single construct and a single selectable marker.

4. Delivery of Expression Vectors

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

One of the preferred methods for *in vivo* delivery involves the use of an adenovirus expression vector. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to express an antisense polynucleotide that has been cloned therein. In this context, expression does not require that the gene product be synthesized.

The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kB, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kB (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus

can infect virtually all epithelial cells regardless of their cell cycle stage. So far, adenoviral infection appears to be linked only to mild disease such as acute respiratory disease in humans.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are *cis* elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP, (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNA's issued from this promoter possess a 5'-tripartite leader (TPL) sequence which makes them preferred mRNA's for translation.

In a current system, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector. Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated from this process. Therefore, it is critical to isolate a single clone of virus from an individual plaque and examine its genomic structure.

Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham *et al.*, 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the D3 or both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury *et al.*, 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone

and is the source of vector-borne cytotoxicity. Also, the replication deficiency of the E1-deleted virus is incomplete.

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, *e.g.*, Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Racher *et al.* (1995) disclosed improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1 to 4 h. The medium is then replaced with 50 ml of fresh medium and shaking initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking commenced for another 72 h.

Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the polynucleotide encoding the gene of interest at the position from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical to the invention. The polynucleotide encoding the gene of interest may also be inserted in lieu of the deleted E3 region in E3 replacement

vectors, as described by Karlsson *et al.* (1986), or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Adenovirus is easy to grow and manipulate and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, *e.g.*, 10^9 - 10^{12} plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch *et al.*, 1963; Top *et al.*, 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression (Levrero *et al.*, 1991; Gomez-Foix *et al.*, 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1991). Recently, animal studies suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.*, 1990; Rich *et al.*, 1993). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et al.*, 1991; Rosenfeld *et al.*, 1992), muscle injection (Ragot *et al.*, 1993), peripheral intravenous injections (Herz and Gerard, 1993) and stereotactic inoculation into the brain (Le Gal La Salle *et al.*, 1993).

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, *gag*, *pol*, and *env* that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the *gag* gene contains a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988) adeno-associated virus (AAV) (Ridgeway, 1988; Baichwal and Sugden, 1986; Hermonat and Muzycska, 1984) and herpesviruses may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990).

With the recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. *In vitro* studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich *et al.*, 1990). This suggested that large portions of the genome could be replaced with foreign genetic material. The hepatotropism and persistence (integration) were particularly attractive properties for liver-directed gene transfer. Chang *et al.*, introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding sequences. It was co-transfected with wild-type virus into an avian hepatoma cell line. Culture media containing high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang *et al.*, 1991).

In order to effect expression of sense or antisense gene constructs, the expression construct must be delivered into a cell. This delivery may be accomplished *in vitro*, as in laboratory procedures for transforming cells lines, or *in vivo* or *ex vivo*, as in the treatment of certain disease states. One mechanism for delivery is via viral infection where the expression construct is encapsidated in an infectious viral particle.

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979) and lipofectamine-DNA complexes, cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

Once the expression construct has been delivered into the cell the nucleic acid encoding the gene of interest may be positioned and expressed at different sites. In certain embodiments, the nucleic acid encoding the gene may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation via homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of

or in synchronization with the host cell cycle. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed.

In yet another embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is particularly applicable for transfer *in vitro* but it may be applied to *in vivo* use as well. Dubensky *et al.* (1984) successfully injected polyomavirus DNA in the form of calcium phosphate precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of calcium phosphate-precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding a gene of interest may also be transferred in a similar manner *in vivo* and express the gene product.

In still another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

Selected organs including the liver, skin, and muscle tissue of rats and mice have been bombarded *in vivo* (Yang *et al.*, 1990; Zelenin *et al.*, 1991). This may require surgical exposure of the tissue or cells, to eliminate any intervening tissue between the gun and the target organ, *i.e.*, *ex vivo* treatment. Again, DNA encoding a particular gene may be delivered via this method and still be incorporated by the present invention.

In a further embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated are lipofectamine-DNA complexes.

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful. Wong *et al.*, (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells. Nicolau *et al.*, (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection.

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression constructs have been successfully employed in transfer and expression of nucleic acid *in vitro* and *in vivo*, then they are applicable for the present invention. Where a bacterial promoter is employed in the DNA construct, it also will be desirable to include within the liposome an appropriate bacterial polymerase.

Other expression constructs which can be employed to deliver a nucleic acid encoding a particular gene into cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993).

Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and transferrin (Wagner *et al.*, 1990). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol *et al.*, 1993; Perales *et al.*, 1994) and epidermal growth factor (EGF) has also been used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau *et al.* (1987) employed lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. Thus, it is feasible that a nucleic acid encoding a particular gene also may be specifically delivered into a cell type by any number of receptor-ligand systems with or without liposomes. For example, epidermal growth factor (EGF) may be used as the

receptor for mediated delivery of a nucleic acid into cells that exhibit upregulation of EGF receptor. Mannose can be used to target the mannose receptor on liver cells. Also, antibodies to CD5 (CLL), CD22 (lymphoma), CD25 (T-cell leukemia) and MAA (melanoma) can similarly be used as targeting moieties.

5 In a particular example, the oligonucleotide may be administered in combination with a cationic lipid. Examples of cationic lipids include, but are not limited to, lipofectin, DOTMA, DOPE, and DOTAP. The publication of WO/0071096, which is specifically incorporated by reference, describes different formulations, such as a DOTAP:cholesterol or cholesterol derivative formulation that can effectively be used for gene therapy. Other
10 disclosures also discuss different lipid or liposomal formulations including nanoparticles and methods of administration; these include, but are not limited to, U.S. Patent Publication 20030203865, 20020150626, 20030032615, and 20040048787, which are specifically incorporated by reference to the extent they disclose formulations and other related aspects of administration and delivery of nucleic acids. Methods used for forming particles are also
15 disclosed in U.S. Patents 5,844,107, 5,877,302, 6,008,336, 6,077,835, 5,972,901, 6,200,801, and 5,972,900, which are incorporated by reference for those aspects.

In certain embodiments, gene transfer may more easily be performed under *ex vivo* conditions. *Ex vivo* gene therapy refers to the isolation of cells from an animal, the delivery of a nucleic acid into the cells *in vitro*, and then the return of the modified cells back into an
20 animal. This may involve the surgical removal of tissue/organs from an animal or the primary culture of cells and tissues.

E. Clinical Information

1. Definitions

“Treatment” and “treating” as used herein refer to administration or application of a
25 therapeutic agent to a subject or performance of a procedure or modality on a subject for the purpose of obtaining a therapeutic benefit of a disease or health-related condition. For example, an inhibitor of CHXP1 may be administered to reduce the symptoms of congestive heart failure.

The term “therapeutic benefit” or “therapeutically effective” as used throughout this
30 application refers to anything that promotes or enhances the well-being of the subject with respect to the medical treatment of this condition. This includes, but is not limited to, a reduction in the frequency or severity of the signs or symptoms of a disease. For example,

administration of an inhibitor of CHXP1 may result in improvement in symptoms of congestive heart failure in a patient that has been diagnosed with congestive heart failure.

“Prevention” and “preventing” are used according to their ordinary and plain meaning to mean “acting before” or such an act. In the context of a particular disease or health-related condition, those terms refer to administration or application of an agent, drug, or remedy to a subject or performance of a procedure or modality on a subject for the purpose of blocking the onset of a disease or health-related condition. For example, an inhibitor of CHXP1 may be administered to a patient with a history of left ventricular dysfunction to prevent onset of signs or symptoms of congestive heart failure.

As used herein, “left ventricular dysfunction” refers to decreased contractility of the left ventricle of any cause.

As used herein, the term “heart failure” is broadly used to mean any condition that reduces the ability of the heart to pump blood. As a result, congestion and edema develop in the tissues. Most frequently, heart failure is caused by decreased contractility of the myocardium, resulting from reduced coronary blood flow; however, many other factors may result in heart failure, including damage to the heart valves, vitamin deficiency, and primary cardiac muscle disease. Though the precise physiological mechanisms of heart failure are not entirely understood, heart failure is generally believed to involve disorders in several cardiac autonomic properties, including sympathetic, parasympathetic, and baroreceptor responses.

The phrase “manifestations (or signs and symptoms) of heart failure” is used broadly to encompass all of the sequelae associated with heart failure, such as shortness of breath, pitting edema, an enlarged tender liver, engorged neck veins, pulmonary rales and the like including laboratory findings associated with heart failure.

“Improvement in the physiologic function” of the heart may be assessed using any method known to those of ordinary skill in the art, such as by measurement of ejection fraction, fractional shortening, left ventricular internal dimension, heart rate, *etc.* as well as any effect upon survival.

The term “compound” refers to any chemical entity, pharmaceutical, drug, and the like that can be used to treat or prevent a disease, illness, sickness, or disorder of bodily function. Compounds comprise both known and potential therapeutic compounds. A compound can be determined to be therapeutic by screening using the screening methods of the present invention. A “known therapeutic compound” refers to a therapeutic compound that has been shown (*e.g.*, through animal trials or prior experience with administration to

humans) to be effective in such treatment. In other words, a known therapeutic compound is not limited to a compound efficacious in the treatment of heart failure.

As used herein, the terms “antagonist” and “inhibitor” refer to molecules, compounds, or nucleic acids that inhibit the action of a cellular factor that may be involved in left ventricular dysfunction. Antagonists may or may not be homologous to these natural compounds in respect to conformation, charge or other characteristics. Thus, antagonists may be recognized by the same or different receptors that are recognized by an agonist. Antagonists may have allosteric effects that prevent the action of an agonist. Alternatively, antagonists may prevent the function of the agonist. In contrast to the agonists, antagonistic compounds do not result in pathologic and/or biochemical changes within the cell such that the cell reacts to the presence of the antagonist in the same manner as if the cellular factor was present. Antagonists and inhibitors may include proteins, nucleic acids, carbohydrates, or any other molecules that bind or interact with a receptor, molecule, and/or pathway of interest. “Antagonist” and “inhibitor” also refer to agents that decrease the expression of a particular gene of interest.

As used herein, the term “modulate” refers to a change or an alteration in a biological activity. Modulation may be an increase or a decrease in protein activity, a change in kinase activity, a change in binding characteristics, or any other change in the biological, functional, or immunological properties associated with the activity of a protein or other structure of interest. The term “modulator” refers to any molecule or compound which is capable of changing or altering biological activity as described above.

A “sample” is any biological material obtained from an individual. For example, a “sample” may be a blood sample or an endomyocardial tissue sample.

“Gene” refers to a polynucleotide sequence that comprises sequences that are expressed in a cell as RNA and control sequences necessary for the production of a transcript or precursor.

“Polynucleotide” refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. Thus, the term includes, but is not limited to, single-, double-, or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases, as well as polynucleotides that have been modified in order to introduce a means for attachment (*e.g.*, to a support for use as a microarray).

2. Dosage

A pharmaceutically effective amount of a therapeutic agent as set forth herein is determined based on the intended goal, for example inhibition of cell death. The quantity to be administered, both according to number of treatments and dose, depends on the subject to be treated, the state of the subject, the protection desired, and the route of administration. Precise amounts of the therapeutic agent also depend on the judgment of the practitioner and are peculiar to each individual.

For example, a dose of the therapeutic agent may be about 0.0001 milligrams to about 1.0 milligrams, or about 0.001 milligrams to about 0.1 milligrams, or about 0.1 milligrams to about 1.0 milligrams, or even about 10 milligrams per dose or so. Multiple doses can also be administered. In some embodiments, a dose is at least about 0.0001 milligrams. In further embodiments, a dose is at least about 0.001 milligrams. In still further embodiments, a dose is at least 0.01 milligrams. In still further embodiments, a dose is at least about 0.1 milligrams. In more particular embodiments, a dose may be at least 1.0 milligrams. In even more particular embodiments, a dose may be at least 10 milligrams. In further embodiments, a dose is at least 100 milligrams or higher.

In other non-limiting examples, a dose may also comprise from about 1 microgram/kg/body weight, about 5 microgram/kg/body weight, about 10 microgram/kg/body weight, about 50 microgram/kg/body weight, about 100 microgram/kg/body weight, about 200 microgram/kg/body weight, about 350 microgram/kg/body weight, about 500 microgram/kg/body weight, about 1 milligram/kg/body weight, about 5 milligram/kg/body weight, about 10 milligram/kg/body weight, about 50 milligram/kg/body weight, about 100 milligram/kg/body weight, about 200 milligram/kg/body weight, about 350 milligram/kg/body weight, about 500 milligram/kg/body weight, to about 1000 mg/kg/body weight or more per administration, and any range derivable therein. In non-limiting examples of a derivable range from the numbers listed herein, a range of about 5 mg/kg/body weight to about 100 mg/kg/body weight, about 5 microgram/kg/body weight to about 500 milligram/kg/body weight, *etc.*, can be administered, based on the numbers described above.

The dose can be repeated as needed as determined by those of ordinary skill in the art. Thus, in some embodiments of the methods set forth herein, a single dose is contemplated. In other embodiments, two or more doses are contemplated. Where more than one dose is administered to a subject, the time interval between doses can be any time interval as

determined by those of ordinary skill in the art. For example, the time interval between doses may be about 1 hour to about 2 hours, about 2 hours to about 6 hours, about 6 hours to about 10 hours, about 10 hours to about 24 hours, about 1 day to about 2 days, about 1 week to about 2 weeks, or longer, or any time interval derivable within any of these recited ranges.

5 In certain embodiments, it may be desirable to provide a continuous supply of a pharmaceutical composition to the patient. This could be accomplished by catheterization, followed by continuous administration of the therapeutic agent. The administration could be intra-operative or post-operative.

F. Drug Formulations and Routes for Administration to Patients

10 Some embodiments of the present invention include compositions that include one or more inhibitors of CMPX1. 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.

15 One will generally desire to employ appropriate salts and buffers in preparing compositions of therapeutic agents. Buffers also will be employed when recombinant cells are introduced into a patient. Aqueous compositions of the present invention comprise an effective amount of the therapeutic agent, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. The phrases “pharmaceutically acceptable” or
20 “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,
25 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
30 the therapeutic agents of the compositions.

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.

Administration may be by any method known to those of ordinary skill in the art, such as intravenous, intradermal, subcutaneous, intramuscular, intraperitoneal or intrathecal injection, or by direct injection into cardiac tissue. Other modes of administration include oral, buccal, and nasogastric administration. The active compounds may also be administered parenterally or intraperitoneally. Such compositions would normally be administered as pharmaceutically acceptable compositions, as described *supra*. In particular embodiments, the composition is administered to a subject using a drug delivery device. For example, the drug delivery device may be a catheter or syringe. In some embodiments, the composition is applied as a coating to a medical device, such as a stent.

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 may contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use 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.

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
5 yield a powder of the active ingredient(s) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

For oral administration the therapeutic agents of the present invention generally may be incorporated with excipients. Any excipient known to those of ordinary skill in the art is contemplated.

10 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
15 bases (*e.g.*, sodium, potassium, ammonium, calcium, or ferric hydroxides) or from organic bases (*e.g.*, isopropylamine, trimethylamine, histidine, procaine and the like.

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
20 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,
25 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
30 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.

G. Combined Therapy

In another embodiment, it is envisioned to use an inhibitor of CMPX1 in combination with other therapeutic modalities. Thus, in addition to the therapies described above, one may also provide to the patient more "standard" pharmaceutical cardiac therapies. Examples of other therapies include, without limitation, so-called " β blockers," anti-hypertensives, cardiotonics, anti-thrombotics, vasodilators, hormone antagonists, ionotropes, diuretics, endothelin receptor antagonists, calcium channel blockers, phosphodiesterase inhibitors, ACE inhibitors, angiotensin type 2 antagonists and cytokine blockers/inhibitors, and HDAC inhibitors.

The other therapeutic modality may be administered before, concurrently with, or following administration of the inhibitor of CMPX1. The therapy using an inhibitor of CMPX1 may precede or follow administration of the other agent(s) by intervals ranging from minutes to weeks. In embodiments where the other agent and the CMPX1 inhibitor are administered separately, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that each agent would still be able to exert an advantageously combined effect. In such instances, it is contemplated that one would typically administer the inhibitor of CMPX1 and the other therapeutic agent within about 12-24 hours of each other and, more preferably, within about 6-12 hours of each other, with a delay time of only about 12 hours being most preferred. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

It also is conceivable that more than one administration of either an inhibitor of CMPX1, or the other agent will be desired. In this regard, various combinations may be employed. By way of illustration, where the inhibitor of CMPX1 is "A" and the other agent is "B", the following permutations based on 3 and 4 total administrations are exemplary:

A/B/A B/A/B B/B/A A/A/B B/A/A A/B/B B/B/B/A B/B/A/B

A/A/B/B A/B/A/B A/B/B/A B/B/A/A B/A/B/A B/A/A/B B/B/B/A

A/A/A/B B/A/A/A A/B/A/A A/A/B/A A/B/B/B B/A/B/B B/B/A/B

Other combinations are likewise contemplated.

Non-limiting examples of pharmacological agents that may be used in the present invention include any pharmacological agent known to those of ordinary skill in the art. For

example, the pharmacological agent may be an agent that can be applied in the treatment of left ventricular dysfunction. Other examples of pharmacological agents include an antihyperlipoproteinemic agent, an antiarteriosclerotic agent, an antithrombotic/fibrinolytic agent, a blood coagulant, an antiarrhythmic agent, an antihypertensive agent, a vasopressor, an antianginal agent, an antibacterial agent or a combination thereof. Non-limiting examples of pharmacological agents that may be used in the present invention include the following.

3. Treatment Agents for Congestive Heart Failure

Non-limiting examples of agents for the treatment of congestive heart failure include anti-angiotension II agents, afterload-preload reduction treatment, diuretics and inotropic agents.

a. Afterload-Preload Reduction

In certain embodiments, an animal patient that can not tolerate an angiotension antagonist may be treated with a combination therapy. Such therapy may combine administration of hydralazine (apresoline) and isosorbide dinitrate (isordil, sorbitrate).

b. Diuretics

Non-limiting examples of a diuretic include a thiazide or benzothiadiazine derivative (*e.g.*, althiazide, bendroflumethazide, benzthiazide, benzyhydrochlorothiazide, buthiazide, chlorothiazide, chlorothiazide, chlorthalidone, cyclopenthiazide, epithiazide, ethiazide, ethiazide, fenquizone, hydrochlorothiazide, hydroflumethiazide, methyclothiazide, meticrane, metolazone, paraflutizide, polythizide, tetrachloromethiazide, trichlormethiazide), an organomercurial (*e.g.*, chlormerodrin, meralluride, mercamphamide, mercaptomerin sodium, mercuriallylic acid, mercuratiline, mercurous chloride, mersalyl), a pteridine (*e.g.*, furterene, triamterene), purines (*e.g.*, acefylline, 7-morpholinomethyltheophylline, pamobrom, protheobromine, theobromine), steroids including aldosterone antagonists (*e.g.*, canrenone, oleandrin, spironolactone), a sulfonamide derivative (*e.g.*, acetazolamide, ambuside, azosemide, bumetanide, butazolamide, chloraminophenamide, clofenamide, clopamide, clorexolone, diphenylmethane-4,4'-disulfonamide, disulfamide, ethoxzolamide, furosemide, indapamide, mefruside, methazolamide, piretanide, quinethazone, torasemide, tripamide, xipamide), a uracil (*e.g.*, aminometradine, amisometradine), a potassium sparing antagonist (*e.g.*, amiloride, triamterene) or a miscellaneous diuretic such as aminozine, arbutin, chlorazasil, ethacrynic acid, etozolin, hydracarbazine, isosorbide, mannitol, metochalcone, muzolimine, perhexiline, ticnafene and urea.

c. Inotropic Agents

Non-limiting examples of a positive inotropic agent, also known as a cardiotonic, include acefylline, an acetyldigitoxin, 2-amino-4-picoline, amrinone, benfurodil hemisuccinate, bucladesine, cerberosine, camphotamide, convallatoxin, cymarin, denopamine, deslanoside, digitalin, digitalis, digitoxin, digoxin, dobutamine, dopamine, dopexamine, enoximone, erythrophleine, fenalcomine, gitalin, gitoxin, glycoamine, heptaminol, hydrastinine, ibopamine, a lanatoside, metamivam, milrinone, nerifolin, oleandrin, ouabain, oxyfedrine, prenalterol, proscillaridine, resibufogenin, scillaren, scillarenin, strphanthin, sulmazole, theobromine and xamoterol.

In particular aspects, an inotropic agent is a cardiac glycoside, a β -adrenergic agonist or a phosphodiesterase inhibitor. Non-limiting examples of a cardiac glycoside includes digoxin (lanoxin) and digitoxin (crystodigin). Non-limiting examples of a β -adrenergic agonist include albuterol, bambuterol, bitolterol, carbuterol, clenbuterol, clorprenaline, denopamine, dioxethedrine, dobutamine (dobutrex), dopamine (intropin), dopexamine, ephedrine, etafedrine, ethylnorepinephrine, fenoterol, formoterol, hexoprenaline, ibopamine, isoetharine, isoproterenol, mabuterol, metaproterenol, methoxyphenamine, oxyfedrine, pirbuterol, procaterol, protokylol, reproterol, rimiterol, ritodrine, soterenol, terbutaline, tretoquinol, tulobuterol and xamoterol. Non-limiting examples of a phosphodiesterase inhibitor include amrinone (inocor).

d. Antianginal Agents

Antianginal agents may comprise organonitrates, calcium channel blockers, β blockers and combinations thereof.

Non-limiting examples of organonitrates, also known as nitrovasodilators, include nitroglycerin (nitro-bid, nitrostat), isosorbide dinitrate (isordil, sorbitrate) and amyl nitrate (aspirol, vaporole).

e. Endothelin Receptor Antagonists

Endothelin (ET) is a 21-amino acid peptide that has potent physiologic and pathophysiologic effects that appear to be involved in the development of heart failure. The effects of ET are mediated through interaction with two classes of cell surface receptors. The type A receptor (ET-A) is associated with vasoconstriction and cell growth while the type B receptor (ET-B) is associated with endothelial-cell mediated vasodilation and with the release of other neurohormones, such as aldosterone. Pharmacologic agents that can inhibit either the production of ET or its ability to stimulate relevant cells are known in the art. Inhibiting the production of ET involves the use of agents that block an enzyme termed endothelin-

converting enzyme that is involved in the processing of the active peptide from its precursor. Inhibiting the ability of ET to stimulate cells involves the use of agents that block the interaction of ET with its receptors. Non-limiting examples of endothelin receptor antagonists (ERA) include Bosentan, Enrasentan, Ambrisentan, Darusentan, Tezosentan, 5 Atrasentan, Avosentan, Clazosentan, Edonentan, sitaxsentan, TBC 3711, BQ 123, and BQ 788.

4. Antihyperlipoproteinemics

In certain embodiments, administration of an agent that lowers the concentration of one of more blood lipids and/or lipoproteins, known herein as an “antihyperlipoproteinemic,” 10 may be combined with a cardiovascular therapy according to the present invention, particularly in treatment of atherosclerosis and thickenings or blockages of vascular tissues. In certain aspects, an antihyperlipoproteinemic agent may comprise an aryloxyalkanoic/fibric acid derivative, a resin/bile acid sequesterant, a HMG CoA reductase inhibitor, a nicotinic acid derivative, a thyroid hormone or thyroid hormone analog, a miscellaneous agent or a 15 combination thereof.

a. Aryloxyalkanoic Acid/Fibric Acid Derivatives

Non-limiting examples of aryloxyalkanoic/fibric acid derivatives include beclobrate, enzaifibrate, binifibrate, ciprofibrate, clinofibrate, clofibrate (atromide-S), clofibric acid, 20 etofibrate, fenofibrate, gemfibrozil (lobid), nicofibrate, pirifibrate, ronifibrate, simfibrate and theofibrate.

b. Resins/Bile Acid Sequesterants

Non-limiting examples of resins/bile acid sequesterants include cholestyramine (cholybar, questran), colestipol (colestid) and polidexide.

c. HMG CoA Reductase Inhibitors

Non-limiting examples of HMG CoA reductase inhibitors include lovastatin (mevacor), pravastatin (pravochol) or simvastatin (zocor).

d. Nicotinic Acid Derivatives

Non-limiting examples of nicotinic acid derivatives include nicotinate, acepimox, 30 niceritrol, nicoclonate, nicomol and oxiniacic acid.

e. Thyroid Hormones and Analogs

Non-limiting examples of thyroid hormones and analogs thereof include etoroxate, thyropropic acid and thyroxine.

f. Miscellaneous Antihyperlipoproteinemics

Non-limiting examples of miscellaneous antihyperlipoproteinemics include acifran, azacosterol, benfluorex, β -benzalbutyramide, carnitine, chondroitin sulfate, clomestron, detaxtran, dextran sulfate sodium, 5,8, 11, 14, 17-eicosapentaenoic acid, eritadenine, furazabol, meglutol, melinamide, mytatrienediol, ornithine, γ -oryzanol, pantethine, pentaerythritol tetraacetate, α -phenylbutyramide, pirozadil, probucol (lorelco), β -sitosterol, sultosilic acid-piperazine salt, tiadenol, triparanol and xenbucin.

5. Antiartherosclerotics

Non-limiting examples of an antiatherosclerotic include pyridinol carbamate.

6. Antithrombotic/Fibrinolytic Agents

In certain embodiments, administration of an agent that aids in the removal or prevention of blood clots may be combined with administration of a modulator, particularly in treatment of atherosclerosis and vasculature (e.g., arterial) blockages. Non-limiting examples of antithrombotic and/or fibrinolytic agents include anticoagulants, anticoagulant antagonists, antiplatelet agents, thrombolytic agents, thrombolytic agent antagonists or combinations thereof.

In certain aspects, antithrombotic agents that can be administered orally, such as, for example, aspirin and warfarin (coumadin), are preferred.

a. Anticoagulants

A non-limiting example of an anticoagulant include acenocoumarol, ancrod, anisindione, bromindione, chlorindione, coumatrol, cyclocoumarol, dextran sulfate sodium, dicoumarol, diphenadione, ethyl biscoumacetate, ethylidene dicoumarol, fluindione, heparin, hirudin, lyapolate sodium, oxazindione, pentosan polysulfate, phenindione, phenprocoumon, phosvitin, picotamide, tiocoumarol and warfarin.

b. Antiplatelet Agents

Non-limiting examples of antiplatelet agents include aspirin, a dextran, dipyridamole (persantin), heparin, sulfinpyranone (anturane) and ticlopidine (ticlid).

c. Thrombolytic Agents

Non-limiting examples of thrombolytic agents include tissue plasminogen activator (activase), plasmin, pro-urokinase, urokinase (abbokinase) streptokinase (streptase), anistreplase/APSAC (eminase).

7. Blood Coagulants

In certain embodiments wherein a patient is suffering from a hemorrhage or an increased likelihood of hemorrhaging, an agent that may enhance blood coagulation may be used. Non-limiting examples of a blood coagulation promoting agent include thrombolytic agent antagonists and anticoagulant antagonists.

a. Anticoagulant Antagonists

Non-limiting examples of anticoagulant antagonists include protamine and vitamin K1.

b. Thrombolytic Agent Antagonists and Antithrombotics

Non-limiting examples of thrombolytic agent antagonists include aminocaproic acid (amicar) and tranexamic acid (amstat). Non-limiting examples of antithrombotics include anagrelide, argatroban, cilostazol, daltroban, defibrotide, enoxaparin, fraxiparine, indobufen, lamoparan, ozagrel, picotamide, plafibride, tedelparin, ticlopidine and triflusal.

8. Antiarrhythmic Agents

Non-limiting examples of antiarrhythmic agents include Class I antiarrhythmic agents (sodium channel blockers), Class II antiarrhythmic agents (β -adrenergic blockers), Class III antiarrhythmic agents (repolarization prolonging drugs), Class IV antiarrhythmic agents (calcium channel blockers) and miscellaneous antiarrhythmic agents.

a. Sodium Channel Blockers

Non-limiting examples of sodium channel blockers include Class IA, Class IB and Class IC antiarrhythmic agents. Non-limiting examples of Class IA antiarrhythmic agents include disopyramide (norpace), procainamide (pronestyl) and quinidine (quinidex). Non-limiting examples of Class IB antiarrhythmic agents include lidocaine (xylocaine), tocainide (tonocard) and mexiletine (mexitil). Non-limiting examples of Class IC antiarrhythmic agents include encainide (enkaide) and flecainide (tambocor).

b. Beta Blockers

Non-limiting examples of a β blocker, otherwise known as a β -adrenergic blocker, a β -adrenergic antagonist or a Class II antiarrhythmic agent, include acebutolol (sectral), alprenolol, amosulalol, arotinolol, atenolol, befunolol, betaxolol, bevantolol, bisoprolol, bopindolol, bucumolol, bufetolol, bufuralol, bunitrolol, bupranolol, butidine hydrochloride, butofilolol, carazolol, carteolol, carvedilol, celiprolol, cetamolol, cloranolol, dilevalol, epanolol, esmolol (brevibloc), indenolol, labetalol, levobunolol, mepindolol, metipranolol,

metoprolol, moprolol, nadolol, nadoxolol, nifenalol, nipradilol, oxprenolol, penbutolol, pindolol, practolol, pronethalol, propanolol (inderal), sotalol (betapace), sulfinalol, talinolol, tertatolol, timolol, toliprolol and xibinolol. In certain aspects, the β blocker comprises an aryloxypropanolamine derivative. Non-limiting examples of aryloxypropanolamine derivatives include acebutolol, alprenolol, arotinolol, atenolol, betaxolol, bevantolol, bisoprolol, bopindolol, bunitrolol, butofilolol, carazolol, carteolol, carvedilol, celiprolol, cetamolol, epanolol, indenolol, mepindolol, metipranolol, metoprolol, moprolol, nadolol, nipradilol, oxprenolol, penbutolol, pindolol, propanolol, talinolol, tertatolol, timolol and toliprolol.

c. Repolarization Prolonging Agents

Non-limiting examples of an agent that prolong repolarization, also known as a Class III antiarrhythmic agent, include amiodarone (cordarone) and sotalol (betapace).

d. Calcium Channel Blockers/Antagonist

Non-limiting examples of a calcium channel blocker, otherwise known as a Class IV antiarrhythmic agent, include an arylalkylamine (*e.g.*, bepridile, diltiazem, fendiline, gallopamil, prenylamine, terodiline, verapamil), a dihydropyridine derivative (felodipine, isradipine, nicardipine, nifedipine, nimodipine, nisoldipine, nitrendipine) a piperazine derivative (*e.g.*, cinnarizine, flunarizine, lidoflazine) or a miscellaneous calcium channel blocker such as bencyclane, etafenone, magnesium, mibefradil or perhexiline. In certain embodiments a calcium channel blocker comprises a long-acting dihydropyridine (nifedipine-type) calcium antagonist.

e. Miscellaneous Antiarrhythmic Agents

Non-limiting examples of miscellaneous antiarrhythmic agents include adenosine (adenocard), digoxin (lanoxin), acecainide, ajmaline, amoproxan, aprindine, bretylium tosylate, bunaftine, butobendine, capobenic acid, cifenline, disopyranide, hydroquinidine, indecainide, ipatropium bromide, lidocaine, lorajmine, lorcaïnide, meobentine, moricizine, pirmenol, prajmaline, propafenone, pyrinoline, quinidine polygalacturonate, quinidine sulfate and viquidil.

9. Antihypertensive Agents

Non-limiting examples of antihypertensive agents include sympatholytic, α/β blockers, α blockers, anti-angiotensin II agents, β blockers, calcium channel blockers, vasodilators and miscellaneous antihypertensives.

a. Alpha Blockers

Non-limiting examples of an α blocker, also known as an α -adrenergic blocker or an α -adrenergic antagonist, include amosulalol, arotinolol, dapiprazole, doxazosin, ergoloid mesylates, fenspiride, indoramin, labetalol, nicergoline, prazosin, terazosin, tolazoline, trimazosin and yohimbine. In certain embodiments, an α blocker may comprise a quinazoline derivative. Non-limiting examples of quinazoline derivatives include alfuzosin, bunazosin, doxazosin, prazosin, terazosin and trimazosin.

b. Alpha/Beta Blockers

In certain embodiments, an antihypertensive agent is both an α and β adrenergic antagonist. Non-limiting examples of an α/β blocker comprise labetalol (normodyne, trandate).

c. Anti-Angiotension II Agents

Non-limiting examples of anti-angiotension II agents include include angiotensin converting enzyme inhibitors and angiotension II receptor antagonists. Non-limiting examples of angiotension converting enzyme inhibitors (ACE inhibitors) include alacepril, enalapril (vasotec), captopril, cilazapril, delapril, enalaprilat, fosinopril, lisinopril, moveltopril, perindopril, quinapril and ramipril.. Non-limiting examples of an angiotensin II receptor blocker, also known as an angiotension II receptor antagonist, an ANG receptor blocker or an ANG-II type-1 receptor blocker (ARBS), include angiocandesartan, eprosartan, irbesartan, losartan and valsartan.

d. Sympatholytics

Non-limiting examples of a sympatholytic include a centrally acting sympatholytic or a peripherially acting sympatholytic. Non-limiting examples of a centrally acting sympatholytic, also known as an central nervous system (CNS) sympatholytic, include clonidine (catapres), guanabenz (wytensin) guanfacine (tenex) and methyldopa (aldomet). Non-limiting examples of a peripherally acting sympatholytic include a ganglion blocking agent, an adrenergic neuron blocking agent, a β -adrenergic blocking agent or a $\alpha 1$ -adrenergic blocking agent. Non-limiting examples of a ganglion blocking agent include mecamylamine (inversine) and trimethaphan (arfonad). Non-limiting of an adrenergic neuron blocking agent

include guanethidine (ismelin) and reserpine (serpasil). Non-limiting examples of a β -adrenergic blocker include acenitlolol (sectral), atenolol (tenormin), betaxolol (kerlone), carteolol (cartrol), labetalol (normodyne, trandate), metoprolol (lopressor), nadanol (corgard), penbutolol (levatol), pindolol (visken), propranolol (inalderal) and timolol (blocadren). Non-limiting examples of α 1-adrenergic blocker include prazosin (minipress), doxazosin (cardura) and terazosin (hytrin).

e. Vasodilators

In certain embodiments a cardiovascular therapeutic agent may comprise a vasodilator (*e.g.*, a cerebral vasodilator, a coronary vasodilator or a peripheral vasodilator).

In certain preferred embodiments, a vasodilator comprises a coronary vasodilator. Non-limiting examples of a coronary vasodilator include amotriphene, bendazol, benfurodil hemisuccinate, benziodarone, chloracizine, chromonar, clobenfurol, clonitrate, dilazep, dipyridamole, droprenilamine, efloxate, erythrityl tetranitrate, etafenone, fendiline, floredil, ganglefene, herestrol bis(β -diethylaminoethyl ether), hexobendine, itramin tosylate, khellin, lidoflanine, mannitol hexanitrate, medibazine, nicorglycerin, pentaerythritol tetranitrate, pentritinol, perhexiline, pimefylline, trapidil, tricromyl, trimetazidine, trolnitrate phosphate and visnadine.

In certain aspects, a vasodilator may comprise a chronic therapy vasodilator or a hypertensive emergency vasodilator. Non-limiting examples of a chronic therapy vasodilator include hydralazine (apresoline) and minoxidil (loniten). Non-limiting examples of a hypertensive emergency vasodilator include nitroprusside (nipride), diazoxide (hyperstat IV), hydralazine (apresoline), minoxidil (loniten) and verapamil.

f. Miscellaneous Antihypertensives

Non-limiting examples of miscellaneous antihypertensives include ajmaline, γ -aminobutyric acid, bufeniodide, cicletamine, ciclosidomine, a cryptenamine tannate, fenoldopam, flosequinan, ketanserine, mebutamate, mecamlamine, methyl dopa, methyl 4-pyridyl ketone thiosemicarbazone, muzolimine, pargyline, pempidine, pinacidil, piperoxan, primaperone, a protoveratrine, raubasine, rescimetol, rilmenidine, saralasin, sodium nitroprusside, ticrynafene, trimethaphan camsylate, tyrosinase and urapidil.

In certain aspects, an antihypertensive may comprise an aryethanolamine derivative, a benzothiadiazine derivative, a *N*-carboxyalkyl(peptide/lactam) derivative, a dihydropyridine derivative, a guanidine derivative, a hydrazines/phthalazine, an imidazole derivative, a quaternary ammonium compound, a reserpine derivative or a suflonamide derivative. Non-

limiting examples of aryethanolamine derivatives include amosulalol, bufuralol, dilevalol, labetalol, pronethalol, sotalol and sulfinalol. Non-limiting examples of benzothiadiazine derivatives include althizide, bendroflumethiazide, benzthiazide, benzyhydrochlorothiazide, buthiazide, chlorothiazide, chlorthalidone, cyclopenthiiazide, cyclothiazide, diazoxide, 5 epithiazide, ethiazide, fenquizone, hydrochlorothizide, hydroflumethizide, methyclothiazide, meticrane, metolazone, paraflutizide, polythizide, tetrachlormethiazide and trichlormethiazide. Non-limiting examples of *N*-carboxyalkyl(peptide/lactam) derivatives include alacepril, captopril, cilazapril, delapril, enalapril, enalaprilat, fosinopril, lisinopril, moveltipril, perindopril, quinapril and ramipril. Non-limiting examples of dihydropyridine 10 derivatives include amlodipine, felodipine, isradipine, nicardipine, nifedipine, nilvadipine, nisoldipine and nitrendipine. Non-limiting examples of guanidine derivatives include bethanidine, debrisoquin, guanabenz, guanacine, guanadrel, guanazodine, guanethidine, guanfacine, guanochlor, guanoxabenz and guanoxan. Non-limiting examples of hydrazines/phthalazines include budralazine, cadralazine, dihydralazine, endralazine, 15 hydracarbazine, hydralazine, pheniprazine, pildralazine and todralazine. Non-limiting examples of imidazole derivatives include clonidine, lofexidine, phentolamine, tiamenidine and tolondine. Non-limiting examples of quaternary ammonium compounds include azamethonium bromide, chlorisondamine chloride, hexamethonium, pentacynium bis(methylsulfate), pentamethonium bromide, pentolinium tartrate, phenactropinium chloride 20 and trimethidinium methosulfate. Non-limiting examples of reserpine derivatives include bictaserpine, deserpidine, rescinnamine, reserpine and syrosingopine. Non-limiting examples of sulfonamide derivatives include ambuside, clopamide, furosemide, indapamide, quinethazone, tripamide and xipamide.

Vasopressors generally are used to increase blood pressure during shock, which may 25 occur during a surgical procedure. Non-limiting examples of a vasopressor, also known as an antihypotensive, include amezinium methyl sulfate, angiotensin amide, dimetofrine, dopamine, etifelmin, etilefrin, gepefrine, metaraminol, midodrine, norepinephrine, pholedrine and synephrine.

10. Surgical Therapeutic Agents

30 In certain aspects, the secondary therapeutic agent may comprise a surgery of some type, which includes, for example, preventative, diagnostic or staging, curative and palliative surgery. Surgery, and in particular a curative surgery, may be used in conjunction with other therapies, such as the present invention and one or more other agents.

Such surgical therapeutic agents for vascular and cardiovascular diseases and disorders are well known to those of skill in the art, and may comprise, but are not limited to, performing surgery on an organism, providing a cardiovascular mechanical prostheses, angioplasty, coronary artery reperfusion, catheter ablation, providing an implantable cardioverter defibrillator to the subject, mechanical circulatory support or a combination thereof. Non-limiting examples of a mechanical circulatory support that may be used in the present invention comprise an intra-aortic balloon counterpulsation, left ventricular assist device or combination thereof.

H. Methods of Making Transgenic Mice

A particular embodiment of the present invention provides transgenic animals that lack one or both functional alleles of an miRNA of interest, such as any of these set forth in the foregoing sections. Also, transgenic animals that express an miRNA of interest under the control of an inducible, tissue selective or a constitutive promoter, recombinant cell lines derived from such animals, and transgenic embryos may be useful in determining the exact role that a particular miRNA plays in left ventricular dysfunction. Furthermore, these transgenic animals may provide an insight into heart development. The use of constitutively expressed miRNA encoding nucleic acid provides a model for over- or unregulated expression. Also, transgenic animals that are “knocked out” for a particular miRNA, in one or both alleles, are contemplated.

In a general aspect, a transgenic animal is produced by the integration of a given transgene into the genome in a manner that permits the expression of the transgene. Methods for producing transgenic animals are generally described by Wagner and Hoppe (U.S. Patent 4,873,191; incorporated herein by reference), and Brinster *et al.* (1985; incorporated herein by reference).

Typically, a gene flanked by genomic sequences is transferred by microinjection into a fertilized egg. The microinjected eggs are implanted into a host female, and the progeny are screened for the expression of the transgene. Transgenic animals may be produced from the fertilized eggs from a number of animals including, but not limited to reptiles, amphibians, birds, mammals, and fish.

I. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for

its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5

EXAMPLE 1

A Micro RNA Signature to Determine Prognosis in Patients with Left Ventricular Dysfunction

10 **Methods**

Patient Samples and Selection. Patients with new onset left ventricular dysfunction presenting to the Johns Hopkins Hospital between 1998 and 2008 underwent a detailed clinical assessment, cardiac catheterization, and endomyocardial biopsy as part of a clinical research protocol approved by the Institutional Review Board at the Johns Hopkins
15 University School of Medicine. Patients were followed clinically until they reached a defined endpoint of need for left ventricular assist device, cardiac transplantation, or death. In the current study, patients were included if they had no evidence of significant coronary artery disease that would account for their cardiac dysfunction (non-ischemic cardiomyopathy). Patients with established causes of non-ischemic cardiomyopathy (*e.g.*, HIV, infiltrative heart
20 disease, arrhythmogenic right ventricular dysplasia) were not selected for analysis. Using these criteria, 10 patients were selected for detailed analysis in the current study.

Clinical Data. Detailed clinical data from the time of initial presentation and endomyocardial biopsy was obtained for each patient. This includes patient demographics, hemodynamic data, echocardiographic measurement of ventricular size and function, blood
25 pressures, serum chemistries, medication usage, and assessment of NYHA heart failure classification. Follow up data obtained included number of hospitalizations for heart failure within one year of index presentation, time until left ventricular assist device (LVAD) implantation, time until death, and time until cardiac transplantation. A composite end point of time until death, need for LVAD implantation, and need for heart transplantation was used
30 as the primary clinical endpoint. Patients were divided into two groups; Group 1, consisting of time until the primary endpoint >250 days, and Group 2, time until primary endpoint < 250 days. Tests of comparison for clinical parameters, hemodynamic parameters, serum chemistries, numbers of hospitalization for heart failure, and time until the primary endpoint were performed using an unpaired, two-sided Student's t-test.

RNA extraction. Endomyocardial biopsy samples that had been flash frozen in liquid nitrogen and stored at -190 degrees C were used to extract RNA. Tissue was disrupted using mortar and pestle that were continuously cooled with liquid nitrogen. Tissue was homogenized using a PowerGen Tissue Homogenizer (Fisher Scientific, Pittsburgh, PA).
5 RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) as previously described (Di Leva *et al.*, 2006).

RNA Quality Control. To determine preservation of RNA integrity and retention of small RNA species, RNA samples were loaded onto a 2% formaldehyde-agarose gel stained with ethidium bromide, allowing for visualization of the 28S, 18S, and 5S ribosomal RNA
10 bands. In addition, RNA integrity and preservation of small RNA species was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and run against an RNA ladder of specified molecular weights (Eukaryote Total RNA Nano Series II, Agilent Technologies).

MicroRNA RT-PCR. Quantification of microRNA species from cardiac tissue RNA
15 samples was performed using the Taqman Human MicroRNA Array v1.0 (Applied Biosciences Inc, Foster City, CA). This RT-PCR based array contains primer-probe pairs for 365 independent human microRNAs. Individual microRNA that were assessed using this technology can be found at
[http://www.appliedbiosystems.com/support/TaqMan_Array_Human_microRNA_panel_AIF.](http://www.appliedbiosystems.com/support/TaqMan_Array_Human_microRNA_panel_AIF.xls)
20 xls. Samples were reverse transcribed using a multiplex RT strategy with 8 independent RT primer pools, and were then hybridized on the microRNA array cards. Endogenous controls on the array for small nucleolar RNA species (snoRNAs) were used for data normalization.

MicroRNA data analysis. For determination of microRNAs that were differentially expressed in patients with divergent outcomes (Group 1 vs. Group 2), a class comparison
25 significance analysis of microarray (SAM) was performed. Hierarchical clustering analysis was performed to determine higher order relationships between groups. A class prediction analysis of microarray (PAM) was performed to identify and validate a microRNA classifier to be used to predict group allocation. All of the above were performed using BRB-Array
Tools Version 3.7.0-Beta_2 Release (NCI Biometric Research Branch, Bethesda, MD) using
30 previously described methods (Schetter *et al.*, 2008). Determination of overall group differences in microRNA expression profiles were determined using principal component analysis (PCA) mapping using the Partek Genomics Suite (Partek, Inc., St. Louis, MO) as previously described (Downey, 2006).

mRNA Gene Expression Arrays. Gene expression data was obtained by hybridizing mRNA obtained from patient samples using Affymetrix Human Genome U133A 2.0 arrays (Affymetrix, Inc., Santa Clara, CA). SAM, PAM, and Class Prediction Analysis were performed using BRB-ArrayTools software as described above.

Results

Baseline Clinical Parameters in Studied Patients. The clinical data and endomyocardial biopsy samples from 10 patients with non-ischemic cardiomyopathy were analyzed. Patients were separated into two groups based upon time to the hard endpoint of death, need for LVAD, or need for cardiac transplantation. A summary of the baseline demographic data of the two groups of patients is detailed in Table 5. Data shown in Table 5 was obtained at time of initial cardiac catheterization, during which time endomyocardial biopsy samples were obtained. All patients had documentation of non-ischemic cardiomyopathy by coronary angiography.

Table 5. Demographic data of patients with heart failure analyzed in the current study.

	Group 1	Group 2	p-value
Age (years)	56.4 ± 7.5	58.6 ± 8.0	N.S.
Ethnicity	2 C, 3 AA	3C, 2 AA	
Gender	3F, 2M	2F, 3M	
Weight (kg)	71.6 ± 21	67 ± 13	N.S.
Height (cm)	166 ± 13	166 ± 7	N.S.
BSA	1.78 ± 0.27	1.75 ± 0.20	N.S.

Analysis of the clinical data at index presentation (time of endomyocardial biopsy sample) is shown in Table 6.

Table 6. Baseline clinical, echocardiographic and hemodynamic measurements of studied patients.

	Group 1	Group 2	p-value
Ejection Fraction (%)	20 ± 7.9	17 ± 5.7	N.S.
LVEDD (mm)	5.69 ± 0.39	5.90 ± 0.35	N.S.
SBP (mm Hg)	108.6 ± 27.7	100.8 ± 17.4	N.S.
DBP (mm Hg)	66.4 ± 10.9	68.0 ± 9.3	N.S.
Mean RA (mm Hg)	17 ± 7.7	8.6 ± 5.9	N.S.
RVSP (mm Hg)	53.4 ± 9.5	41.0 ± 12.6	N.S.
Mean PAP (mm Hg)	38.2 ± 8.5	27.2 ± 9.7	N.S.
PCWP (mm Hg)	28.8 ± 9.1	17.6 ± 6.8	N.S.
Cardiac Output (L/min)	3.56 ± 1.17	2.83 ± 0.72	N.S.
Arterial O ₂ Sat (%)	97 ± 2	97.6 ± 1.9	N.S.
PA O ₂ Sat (%)	55 ± 15	58 ± 11	N.S.
Blood Urea Nitrogen (mg/dl)	42.2 ± 10.8	60.4 ± 14.0	N.S.
Creatinine (mg/dl)	1.54 ± 0.36	2.12 ± 0.60	N.S.

For tests of significance of the data shown in Table 6, unpaired Student's t-test was used, with a p-value of 0.05 to determine significance. LVEDD = left ventricular end diastolic diameter; SBP= systolic blood pressure; DBP = diastolic blood pressure; RVSP = right ventricular systolic pressure; PCWP = pulmonary capillary wedge pressure.

Patients were divided into two groups base upon time to primary endpoint. As shown in Table 7, Group 1 patients had a significantly longer time to the primary endpoint compared with Group 2 patients. Notably, both groups of patients had severe cardiac dysfunction (20% vs. 17%, p = N.S.) and marked left ventricular dilatation (LVEDD), but neither of these parameters was significantly different between the two groups.

Table 7. Clinical endpoints for the two groups of study patients. LVAD= left ventricular assist device

	Group 1	Group 2	p-value
Hospitalizations for CHF in one year	1.0 ± 1	4.4 ± 2.7	0.03
Time until Transplant/LVAD/Death (days)	396.4 ± 79.8	147.6 ± 85.7	0.001

Furthermore, there were no significant differences in any measurable clinical parameters between the two patient groups (see Table 6). As shown in Table 8, medication usage was similar between the two groups.

Table 8. Heart failure medication usage in studied patients.

	Group 1	Group 2
Beta blocker	4/5	2/5
ACE/ARB	5/5	3/5
ISDN/hydralazine	0/5	2/5
Aldosterone antagonist	4/5	3/5
Diuretic	5/5	5/5
Inotropes	0/5	0/5

Analysis of clinical outcome data (Table 7) reveals a significant difference between the numbers of hospitalizations for heart failure within one year after index presentation between the two groups, consistent with multiple prior studies correlating hospitalizations for heart failure and increased mortality (Shahar and Lee, 2007). In addition, there was a significant difference in the time to the primary endpoint between the two groups (Table 7), which is by design based upon the strategy used to create the two patient groups for further analysis.

MicroRNA integrity in studied patient samples. Since the endomyocardial biopsy samples from the patients studied represented relatively small pieces of tissue that had been obtained up to 10 years ago, extensive quality control analysis was performed to determine that the samples were suitable for mRNA and microRNA profiling. The integrity of the samples was found to be excellent, as assessed by the presence of crisp 28S, 18S, and 5S rRNA bands for all 10 patient samples.

In addition, all 10 samples passed initial quality control analysis as assessed using Agilent Bioanalyzer RNA integrity determination. Specifically, all samples had RNA integrity numbers (RINs) of 8.0 or greater, indicative of high quality, intact RNA samples (Schroeder *et al.*, 2006).

MicroRNA signatures in heart failure patients with divergent outcomes. To determine levels of microRNA expression in endomyocardial biopsy samples from patients with

advanced heart failure, the human TaqMan microRNA array (Applied Biosystems, Foster City, CA) was used, which allows for quantification of 365 independent microRNA species from a single input sample using stem-loop RT-PCR. This assay contains the vast majority of microRNAs with known biological function, including those whose function in cardiac disease has been reported, as well as many micro RNA species whose biological function is not well known. Bioinformatics predictions suggest that there may be as many as 1000 micro RNAs in the human genome, of which approximately 650 have been identified to date.

To determine if a micro RNA signature that could distinguish patients with heart failure and poor outcomes (Group 2) from those with better outcomes (Group 1) could be identified, a Principal Component Analysis was performed. This analysis is a mathematical transformation which accounts for the highest degrees of variance within sample sets, and has been widely used as an unbiased clustering tool for microarray data analysis, and has recently been successfully applied in the analysis of microRNA profiles (Eisenberg *et al.*, 2007; Feber *et al.*, 2008). Strikingly, PCA analysis found that the two sample groups formed two distinct sample clusters (FIG. 1), suggesting meaningful biological differences exist between microRNA profiles of the two groups. Furthermore, dendogram clustering analysis, an independent, unbiased clustering technique, reveals that the microRNA data sets from the two groups of samples cluster independently into two distinct families, again revealing important differences in microRNA profiles between the two groups (FIG. 2).

Differential expression of individual microRNA species between heart failure patients with divergent outcomes. Analysis of differential expression of individual microRNA species revealed a total of 9 micro RNAs that were significantly differentially expressed ($p < 0.01$, Table 9).

Table 9. Identities of differentially expressed microRNAs from hearts of heart failure patients with divergent outcomes.

Number	microRNA species ID	Fold Difference	p-value
1	hsa-miR-367	4.58	0.0004
2	hsa-miR-10a	-5.58	0.001
3	hsa-miR-187	-4.62	0.002
4	hsa-miR-452	-2.27	0.002
5	hsa-miR-218	-2.25	0.002
6	hsa-miR-10b	2.81	0.005
7	hsa-miR-214	1.61	0.007
8	hsa-miR-193a	1.83	0.007
9	hsa-miR-565	2.10	0.008

As shown in Table 9, nine micro RNA species were identified which were significantly differentially expressed ($p < 0.01$) between patients with non-ischemic cardiomyopathy with divergent outcomes. FDR = false discovery rate. Fold difference numbers are positive when the selected microRNAs species is increased in patients with better outcomes (Group 1) and are negative when the selected microRNAs species is decreased in patients with better outcomes.

Among these micro RNA species, the three most significantly differentially expressed microRNAs, hsa-miR-367, 10a, and 187 were each expressed at 4 fold or greater higher levels in samples from patients with good outcomes. Using these 3 microRNAs as a classifier, 90% of the patients could be classified into their appropriate outcome groups. Furthermore, the one “mis-classified” patient, was the patient in the poor outcome group who had the longest event-free survival.

Gene expression analysis from heart failure patients with divergent outcomes: biological validation of microRNA profiles. Microarray gene expression analysis was performed on the same 10 test patient samples. Using a high stringency test of significance, a single gene that was differentially expressed at a very high level between the two sample groups was identified. This gene, CMPX1, a zinc finger transcription factor of unknown function, was expressed 7 fold higher in the hearts of patients with poor outcomes ($p < 1 \times 10^{-6}$). Importantly, there is a highly conserved putative binding site for hsa-miR-367 in the 3'

untranslated region of CMPX1. Since miR-367 is overexpressed in hearts of patients with good outcomes, the finding of overexpression of CMPX1 in patients with poor outcomes provides strong evidence that CMPX1 is regulated by miR-367, and furthermore, demonstrates the biological relevance underlying of the differentially expressed micro RNA species that was observed.

* * * * *

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

REFERENCES

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

- 5 U.S. Patent 4,873,191
 - U.S. Patent 5,510,270
 - U.S. Patent 5,556,752
 - U.S. Patent 5,578,832
 - 10 U.S. Patent 5,844,107
 - U.S. Patent 5,877,302
 - U.S. Patent 5,972,900
 - U.S. Patent 5,972,901
 - U.S. Patent 6,008,336
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CLAIMS

1. A method for determining prognosis in a subject with left ventricular dysfunction, comprising:

5 (a) determining expression level of one or more miRNA species selected from the group consisting of hsa-miR-367, hsa-miR-10a, hsa-miR-187, hsa-miR-452, hsa-miR-218, hsa-miR-10b, hsa-miR-214, hsa-miR-193a, and hsa-miR-565 in a heart tissue sample from a subject with left ventricular dysfunction; and

(b) comparing the expression level of the one or more miRNA species to a reference
10 level to determine prognosis, wherein:

(i) reduced expression level of hsa-miR-10a, hsa-miR-187, hsa-miR-452, or hsa-miR-218 relative to the reference level is indicative of left ventricular dysfunction that is not associated with a high mortality rate;

(ii) reduced expression level of hsa-miR-10a, hsa-miR-187, hsa-miR-452, or hsa-miR-218 in the sample from the subject relative to the expression level of hsa-miR-10a, hsa-miR-187, hsa-miR-452, or hsa-miR-218 in a reference set of samples obtained from subjects with severe left ventricular dysfunction known to be associated with a poor prognosis, indicates a reduced likelihood that the subject has severe left ventricular dysfunction associated with a poor
20 prognosis;

(iii) increased expression level of hsa-miR-367, hsa-miR-10b, hsa-miR-214, hsa-miR-193a, or hsa-miR-565 relative to a reference level is indicative of left ventricular dysfunction that is not associated with a high mortality rate; or

(iv) increased expression level of hsa-miR-367, hsa-miR-10b, hsa-miR-214, hsa-miR-193a, or hsa-miR-565 in the sample from the subject relative to the expression level of hsa-miR-367, hsa-miR-10b, hsa-miR-214, hsa-miR-193a, or hsa-miR-565 in a reference set of samples obtained from subjects with severe left ventricular dysfunction known to be associated with a poor prognosis, indicates a reduced likelihood that the subject has severe left
25 ventricular dysfunction associated with a poor prognosis.
30

2. The method of claim 1, wherein the reference level is the expression level of the one or more miRNA species in one or more subjects with severe left ventricular dysfunction (positive control).

3. The method of claim 1, wherein the reference level is the expression level of the one or more miRNA species in one or more subjects without left ventricular dysfunction (negative control).

5

4. The method of claim 1, further comprising determining the level of expression of CMPX1 in a sample from the subject and comparing the expression level of CMPX1 to a reference level.

10 5. A method for determining prognosis in a subject with left ventricular dysfunction, comprising:

(a) determining the expression level of CMPX1 in a heart tissue sample from the subject; and

(b) comparing the expression level of CMPX1 in the sample from the subject to a
15 reference level to determine prognosis,

wherein an increased expression level of CMPX1 relative to the reference level is indicative of left ventricular dysfunction that is associated with a high mortality rate.

20 6. The method of claim 5, wherein the reference level is the level of CMPX1 in a subject without left ventricular dysfunction that is associated with a high mortality rate.

7. A composition comprising an inhibitor of CMPX1 for treating left ventricular dysfunction in a subject, wherein the inhibitor of CMPX1 is a polynucleotide, a protein, a polypeptide, a peptide, an antibody, an antibody fragment, or a small molecule.

25

8. The composition of claim 7, wherein the inhibitor of CMPX1 is a polynucleotide.

9. The composition of claim 8, wherein the polynucleotide is a miRNA.

30 10. The composition of claim 9, wherein the miRNA is hsa-miR-367.

11. The composition of claim 7, further comprising administering to the subject a secondary form of therapy selected from the group consisting of a pharmaceutical agent, a left ventricular assist device, or cardiac transplantation.

12. The composition of claim 11, wherein the secondary form of therapy is a pharmaceutical agent selected from the group consisting of a beta-blocker, an angiotensin converting enzyme inhibitor, hydralazine, an aldosterone antagonist, a diuretic, or an inotrope.

13. A kit for determining prognosis of a subject with left ventricular dysfunction, comprising polynucleotides for analysis of at least one miRNA species selected from the group consisting of hsa-miR-367, hsa-miR-10a, hsa-miR-187, hsa-miR-452, hsa-miR-218, hsa-miR-10b, hsa-miR-214, hsa-miR-193a, and hsa-miR-565, wherein each polynucleotide specifically hybridizes to at least one miRNA species selected from the group consisting of hsa-miR-367, hsa-miR-10a, hsa-miR-187, hsa-miR-452, hsa-miR-218, hsa-miR-10b, hsa-miR-214, hsa-miR-193a, and hsa-miR-565.

14. The kit of claim 13, further comprising a set of primers specific for transcription or reverse transcription of one or more miRNA species selected from the group consisting of hsa-miR-367, hsa-miR-10a, hsa-miR-187, hsa-miR-452, hsa-miR-218, hsa-miR-10b, hsa-miR-214, hsa-miR-193a, and hsa-miR-565.

15. The kit of claim 13, further comprising a miRNA array card, wherein the one or more polynucleotides are arrayed on said card.

16. A pharmaceutical composition comprising an inhibitor of CMPX1 and a secondary compound that can be applied in the treatment of left ventricular dysfunction in a subject.

17. The pharmaceutical composition of claim 24, wherein the inhibitor of CMPX1 is hsa-miR-367, a polynucleotide that encodes hsa-miR-267, or a polynucleotide that hybridizes to a polynucleotide that encodes CMPX1.

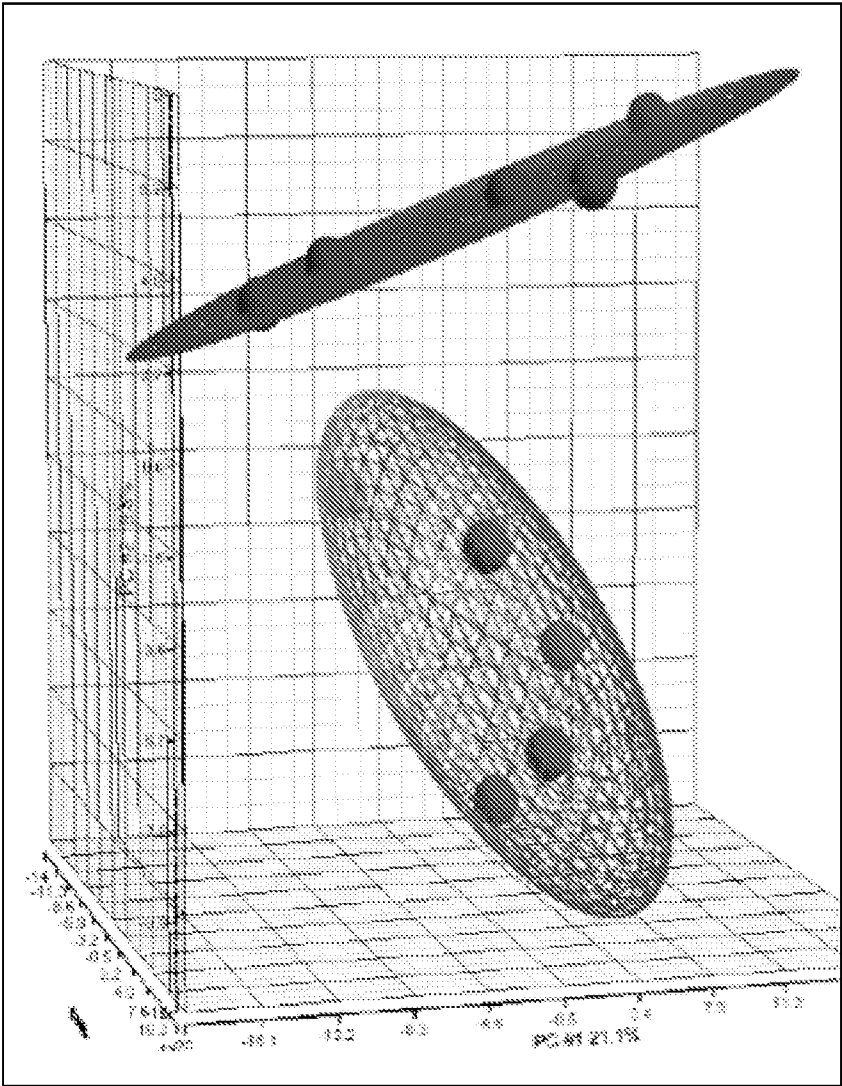


FIG. 1

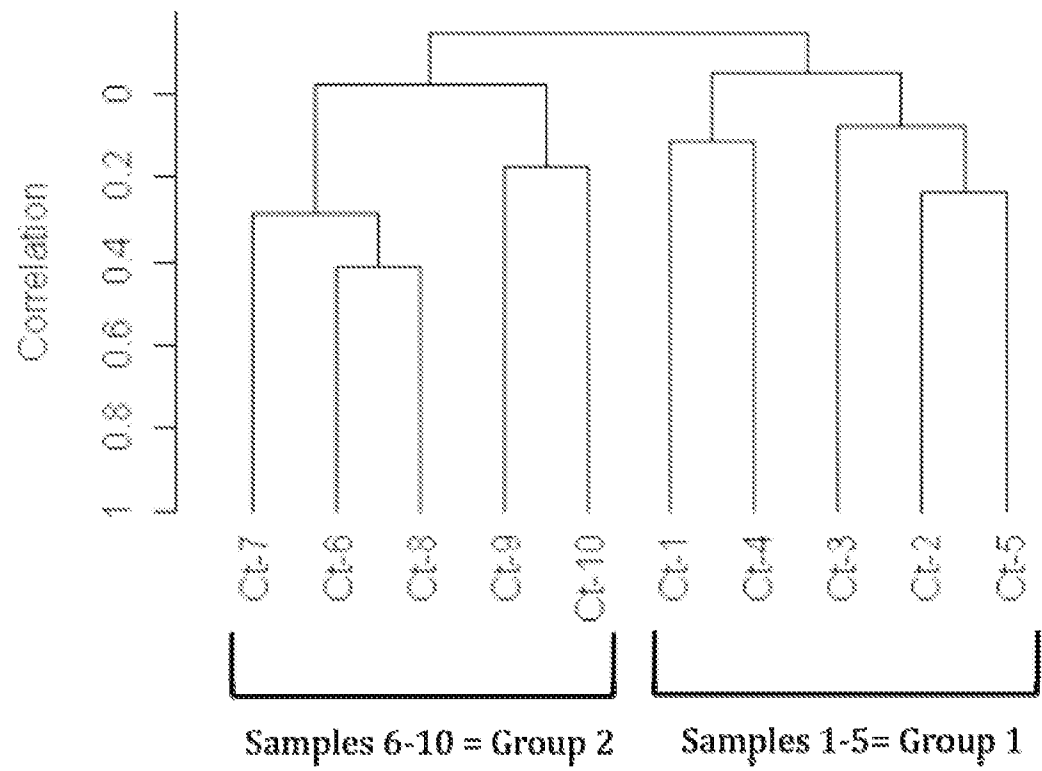


FIG. 2