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(54) Title: INHIBITION OF EXTRACELLULAR SIGNAL-REGULATED KINASE 1/2 AS A TREATMENT FOR CARDIAC HYPERTROPHY AND HEART FAILURE

(57) Abstract: The present invention provides for methods of treating and preventing cardiac hypertrophy and heart failure. The present invention provides the link between ERK1/2 and calcineurin and CamKII. The present invention further demonstrates that inhibitors of ERK1/2 inhibit cardiac hypertrophy and heart disease by inhibiting, in part, the fetal cardiac gene expression that occurs when Ca²⁺-dependent signalling occurs in the heart.



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**INHIBITION OF EXTRACELLULAR SIGNAL-REGULATED KINASE 1/2 AS A
TREATMENT FOR CARDIAC HYPERTROPHY AND HEART FAILURE**

BACKGROUND OF THE INVENTION

5 The present invention claims priority to U.S. Provisional Application Serial Nos. 60/701,600 and 60/701,768, filed July 22, 2005, and the entire contents of which are hereby incorporated by reference. The United States government owns rights in the application by virtue of funding under Grant Nos. 2R01HL48013 and R01 HL48013-10S1 from the National Institutes of Health.

10 **1. Field of the Invention**

 The present invention relates generally to the fields of developmental biology and molecular biology. More particularly, it concerns gene regulation and cellular physiology in cardiomyocytes. Specifically, the invention relates to the use inhibitors of Extracellular Signal-Related Kinase (ERK) 1/2 to block fetal gene expression in myocytes and to treat
15 cardiac hypertrophy and heart failure.

2. Description of Related Art

 Cardiac hypertrophy in response to an increased workload imposed on the heart is a fundamental adaptive mechanism. It is a specialized process reflecting a quantitative increase in cell size and mass (rather than cell number) as the result of any, or a combination of,
20 neural, endocrine or mechanical stimuli. When heart failure occurs, the left ventricle usually is hypertrophied and dilated and indices of systolic function, such as ejection fraction, are reduced. Clearly, the cardiac hypertrophic response is a complex syndrome and the elucidation of the pathways leading to cardiac hypertrophy will be beneficial in the treatment of heart disease resulting from various stimuli.

25 A family of transcription factors, the myocyte enhancer factor-2 family (MEF2), is involved in cardiac hypertrophy. For example, a variety of stimuli can elevate intracellular calcium, resulting in a cascade of intracellular signaling systems or pathways, including calcineurin, CAM kinases, PKC and MAP kinases. All of these signals activate MEF2 and result in cardiac hypertrophy. Further, it is known that certain histone deacetylase proteins

(HDAC's) are involved in modulating MEF2 activity. However, it is still not completely understood how the various signal systems exert their effects on MEF2 and modulate its hypertrophic signaling.

SUMMARY OF THE INVENTION

5 Thus, in accordance with the present invention, there is provided a method of treating pathologic cardiac hypertrophy or heart failure comprising identifying a patient having cardiac hypertrophy or heart failure and administering to said patient an inhibitor of ERK1/2. The inhibitor of ERK1/2 may be selected from the group consisting of a ERK1/2 RNAi molecule, a ERK1/2 antisense molecule, a ERK1/2 ribozyme molecule or a ERK1/2-binding
10 single-chain antibody, an expression construct that encodes a ERK1/2-binding single-chain antibody, an inhibitor of ERK1/2 phosphorylation, YY1 and an expression construct encoding YY1. Administering may be performed intravenously, by direct injection into cardiac tissue, by oral, transdermal, or sublingual routes, or by sustained release, controlled release, delayed release, or suppository vehicles.

15 The method may further comprise administering to said patient a second cardiac hypertrophic therapy, such as a beta blocker, an ionotrope, a diuretic, ACE-I, AII antagonist, BNP, a Ca^{++} -blocker, an inhibitor of calcineurin, an inhibitor of CamKII or an HDAC inhibitor. The second therapy may be administered at the same time as said inhibitor of ERK1/2, or either before or after said inhibitor of ERK1/2. Treating may comprise improving
20 one or more symptoms of pathologic cardiac hypertrophy or one or more symptoms of heart failure. The one or more improved symptom may comprise increased exercise capacity, increased cardiac ejection volume, decreased left ventricular end diastolic pressure, decreased pulmonary capillary wedge pressure, increased cardiac output, or cardiac index, lowered pulmonary artery pressures, decreased left ventricular end systolic and diastolic dimensions,
25 decreased left and right ventricular wall stress, decreased wall tension, increased quality of life, and decreased disease related morbidity or mortality.

In another embodiment, there is provided a method of preventing pathologic hypertrophy or heart failure comprising identifying a patient at risk of developing pathologic cardiac hypertrophy or heart failure and administering to said patient an inhibitor of ERK1/2.
30 The inhibitor of ERK1/2 may be selected from the group consisting of a ERK1/2 RNAi molecule, a ERK1/2 antisense molecule, a ERK1/2 ribozyme molecule or a ERK1/2-binding single-chain antibody, or expression construct that encodes a ERK1/2-binding single-chain antibody, an inhibitor of ERK1/2 phosphorylation, YY1 and an expression construct encoding

YY1. Administering may be performed intravenously or by direct injection into cardiac tissue, by oral, transdermal, or sublingual routes, or with sustained release, controlled release, delayed release, or suppository vehicles.

The patient at risk may exhibit one or more of a list of risk factors comprising long standing uncontrolled hypertension, uncorrected valvular disease, chronic angina, recent myocardial infarction, congenital predisposition to heart disease or pathological hypertrophy, may be diagnosed as having a genetic predisposition to cardiac hypertrophy, or may have a familial history of cardiac hypertrophy.

In yet another embodiment, there is provided a method of assessing an inhibitor of ERK1/2 for efficacy in treatment of cardiac hypertrophy or heart failure comprising (a) providing an inhibitor of ERK1/2; (b) treating a cell with said inhibitor of ERK1/2; and (c) measuring the expression of one or more cardiac hypertrophy parameters, wherein a change in said one or more cardiac hypertrophy parameters, as compared to one or more cardiac hypertrophy parameters in a cell not treated with said inhibitor of ERK1/2, identifies said inhibitor of ERK1/2 as an inhibitor of cardiac hypertrophy or heart failure. Treating may be performed *in vitro* or *in vivo*.

The cell may be a myocyte, such as an isolated myocyte or a cardiomyocyte. The myocyte maybe a neonatal rat ventricular myocyte or an H9C2 cell. The cardiomyocyte or myocyte may be comprised in isolated intact tissue or *in vivo* in a functioning intact heart muscle, which may be a transgenic tissue or animal and may be subjected to a stimulus that triggers a hypertrophic response in one or more cardiac hypertrophy parameters. The stimulus may be aortic banding, rapid cardiac pacing, induced myocardial infarction, transgene expression, such as ANP, β -MyHC, BNP, and α -skeletal actin, or a chemical or pharmaceutical agent, such as angiotensin II, isoproterenol, phenylephrine, endothelin-I, vasoconstrictors, or antidiuretics.

The one or more cardiac hypertrophy parameters comprises right ventricular ejection fraction, left ventricular ejection fraction, ventricular wall thickness, heart weight/body weight ratio, right or left ventricular weight/body weight ratio, cardiac weight normalization measurement, total protein synthesis, ERK1/2 phosphorylation, one or mor aspects of cellular morphology, or the expression level of one or more target genes in said myocyte, wherein expression level of said one or more target genes is indicative of cardiac hypertrophy. Transgene expression levels may measured using a reporter protein coding region operably linked to a target gene promoter, such as luciferase, β -gal, or green fluorescent protein. Alternatively, the expression level may be measured using hybridization of a nucleic acid

probe to a target mRNA or amplified nucleic acid product. The one or more aspects of cellular morphology may comprise sarcomere assembly, cell size, or cell contractility. The method may further comprise measuring cell toxicity.

In still yet another embodiment, there is provided a method of identifying an inhibitor of cardiac hypertrophy or heart failure comprising (a) providing a ERK1/2; (b) contacting the ERK1/2 with a candidate inhibitor substance; and (c) assessing the phosphorylation of said ERK1/2, wherein a decrease in the phosphorylation of the ERK1/2 identifies said candidate inhibitor substance as an inhibitor of cardiac hypertrophy or heart failure. The ERK1/2 may be purified away from whole cells, such as heart cells, or located in an intact cell, such as a myocyte or a cardiomyocyte. The candidate inhibitor substance may be an interfering RNA, an antibody preparation, a single chain antibodies, an antisense construct, or an enzyme, chemical, pharmaceutical, or small compound. More specifically, the candidate substance may be selected from the group consisting of a ERK1/2 RNAi molecule, a ERK1/2 antisense molecule, a ERK1/2 ribozyme molecule or a ERK1/2-binding single-chain antibody, or an expression construct that encodes a ERK1/2-binding single-chain antibody, YY1 and an expression construct encoding YY1.

The invention may further comprise:

- a method of preventing cardiac hypertrophy and dilated cardiomyopathy comprising decreasing ERK1/2 activity in heart cells of a subject;
- a method of inhibiting progression of cardiac hypertrophy comprising decreasing ERK1/2 activity in heart cells of a subject;
- a method of treating heart failure comprising decreasing ERK1/2 activity in heart cells of a subject;
- a method of inhibiting progression of heart failure comprising decreasing ERK1/2 activity in heart cells of a subject;
- a method of increasing exercise tolerance in a subject with heart failure or cardiac hypertrophy comprising decreasing ERK1/2 activity in heart cells of a subject;
- a method of reducing hospitalization in a subject with heart failure or cardiac hypertrophy comprising decreasing ERK1/2 activity in heart cells of a subject;
- a method of improving quality of life in a subject with heart failure or cardiac hypertrophy comprising decreasing ERK1/2 activity in heart cells of a subject;
- a method of decreasing morbidity in a subject with heart failure or cardiac hypertrophy comprising decreasing ERK1/2 activity in heart cells of a subject; or

a method of decreasing mortality in a subject with heart failure or cardiac hypertrophy comprising decreasing ERK1/2 activity in heart cells of a subject.

It is contemplated that any method or composition described herein can be implemented with respect to any other method or composition described herein.

5 The use of the word “a” or “an” when used in conjunction with the term “comprising” in the claims and/or the specification may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.”

 These, and other, embodiments of the invention will be better appreciated and understood when considered in conjunction with the following description and the
10 accompanying drawings. It should be understood, however, that the following description, while indicating various embodiments of the invention and numerous specific details thereof, is given by way of illustration and not of limitation. Many substitutions, modifications, additions and/or rearrangements may be made within the scope of the invention without departing from the spirit thereof, and the invention includes all such substitutions,
15 modifications, additions and/or rearrangements.

BRIEF DESCRIPTION OF THE DRAWINGS

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

FIG. 1 – Activation of β_1 -AR-mediated fetal gene expression is dependent on CaMKII. NRVMs were pretreated with KN-93 for 1 hour followed by isoproterenol
25 treatment as described in the text. RNA was extracted and submitted to RPA. Quantification of RPA – the graph bars represent a total of three individual experiments. Each mRNA was normalized to GAPDH.

FIG. 2 – Calcineurin is involved in β_1 -AR-mediated fetal gene expression. NRVMs were pretreated with cyclosporine for 1 hour followed by isoproterenol
30 treatment. RNA was extracted and submitted to RPA. Quantification of RPA – the graph bars represent a total of three individual experiments. Each mRNA was normalized to GAPDH.

FIG. 3 – Activation of β_1 -AR-mediated fetal gene expression is dependent on ERK1/2. NRVMs were pretreated with UO126 for 1 hour followed by isoproterenol treatment. RNA was extracted and submitted to RPA. Quantification of RPA – the graph bars represent a total of three individual experiments. Each mRNA was normalized to GAPDH.

FIGS. 4A-C – ERK1/2 is activated at five minutes and 33 hours post-isoproterenol treatment – role of CaMKII and calcineurin. (FIG. 4A) Western blot of phosphorylated ERK1/2. ERK1/2 phosphorylation in response to isoproterenol has a dual activation phase. Lane 1: untreated cells; Lane 2: cells treated with isoproterenol for 5 min.; Lane 3: 30 min treatment with isoproterenol; Lane 4: 1 hr treatment with isoproterenol; Lane 5: 6 hr treatment with isoproterenol; Lane 6: 24 hr treatment with isoproterenol; Lane 7: 33 hr isoproterenol treatment; Lane 8: 48 hr isoproterenol treatment. (FIG. 4B) Phosphorylation of ERK1/2 after 5 min of isoproterenol stimulation is inhibited by calcineurin inhibition but not by CaMKII or PKA inhibition. Lane 1: no treatment; Lane 2: 5 min isoproterenol treatment; Lane 3: pretreatment with cyclosporine followed by isoproterenol treatment; Lane 4: pretreatment with PKA inhibitor followed by isoproterenol treatment; Lane 5: pretreatment with CaMKII inhibitor followed by isoproterenol treatment. (FIG. 4C) CaMKII is involved in the phosphorylation of ERK1/2 at 33 and 48 hours. Lane 1: untreated cells; Lane 2: 33 hr isoproterenol treatment; Lane 3: 48 hr isoproterenol treatment. Pretreatment with CaMKII inhibitor followed by isoproterenol treatment for 33 hrs (Lane 4) or 48 hrs (Lane 5).

FIG. 5 – LTCCs are involved in β_1 -AR-mediated fetal gene expression. NRVMs were pretreated with nifedipine for 1 hour followed by isoproterenol treatment. RNA was extracted and submitted to RPA. Quantification of RPA – the graph bars represent a total of three individual experiments. Each mRNA was normalized to GAPDH.

FIG. 6 – Activation of β_1 -AR-mediated fetal gene expression is dependent on TRPC channels. NRVMs were pretreated with 2-APB for 1 hour followed by isoproterenol treatment. RNA was extracted and submitted to RPA. Quantification of RPA – the graph bars represent a total of three individual experiments. Each mRNA was normalized to GAPDH.

FIG. 7 - β_1 -AR activation of the fetal gene program is dependent on Ca^{2+} influx.

Stimulation of β_1 -AR with isoproterenol results in an upload of Ca^{2+} into the cells through LTCCs and TRPC channels. This, in turn, activates CaMKII, calcineurin and ERK1/2. Activation of ERK1/2 occurs through CaMKII- and calcineurin-dependent mechanisms.

FIGS. 8A-B - MEK1 inhibition prevents ISO-mediated changes in gene expression. Cells were treated with the MEK1 inhibitor U0126 1 hour prior to ISO treatment (FIG. 8A) or 1 hour after ISO treatment (FIG. 8B). mRNA was extracted 48 hours later and analyzed by RPAs. Results were compared to vehicle-treated cells, defined as 100%.

FIG. 9 - YY1 prevents ERK1/2 phosphorylation in response to ISO treatment.

Cells were infected with CMV (Lanes 1-3) or YY1 (Lanes 4-6) adenovirus constructs. Lanes 1 and 4: No treatment; Lanes 2 and 5: PE treatment; Lanes 3 and 6: ISO treatment. Western blot was done with an anti-phospho ERK1/2 antibody.

FIGS. 10A-B - Phosphorylated ERK1/2 prevents YY1 and GATA interaction.

(FIG. 10A) ERK1/2 phosphorylation in COS7 cells. Lane 1: infection with YY1 and GATA4 adenovirus constructs; Lane 2: YY1 and GATA4 infection and MEK1 transfection; Lane 3: YY1 and GATA4 infection, MEK1 transfection and treatment with MEK1 inhibitor U0126. (FIG. 10B) YY1 and GATA4 interaction in COS7 cells.

Cells were immunoprecipitated with anti-YY1 antibody and GATA4 was detected by Western Blot. Lane 1: Cells infected with YY1 and GATA4 adenovirus construct and transfected with a control plasmid; Lane 2: cells infected with YY1 and GATA4 adenovirus construct and transfected with MEK1 cDNA.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Heart failure is one of the leading causes of morbidity and mortality in the world. In the U.S. alone, estimates indicate that 3 million people are currently living with cardiomyopathy and another 400,000 are diagnosed on a yearly basis. Dilated cardiomyopathy (DCM), also referred to as "congestive cardiomyopathy," is the most common form of the cardiomyopathies and has an estimated prevalence of nearly 40 per 100,000 individuals (Durand *et al.*, 1995). Although there are other causes of DCM, familial dilated cardiomyopathy has been indicated as representing approximately 20% of "idiopathic" DCM. Approximately half of the DCM cases are idiopathic, with the remainder being

associated with known disease processes. For example, serious myocardial damage can result from certain drugs used in cancer chemotherapy (*e.g.*, doxorubicin and daunoribucin). In addition, many DCM patients are chronic alcoholics. Fortunately, for these patients, the progression of myocardial dysfunction may be stopped or reversed if alcohol consumption is reduced or stopped early in the course of disease. Peripartum cardiomyopathy is another idiopathic form of DCM, as is disease associated with infectious sequelae. In sum, cardiomyopathies, including DCM, are significant public health problems.

Heart disease and its manifestations, including coronary artery disease, myocardial infarction, congestive heart failure and cardiac hypertrophy, clearly presents a major health risk in the United States today. The cost to diagnose, treat and support patients suffering from these diseases is well into the billions of dollars. Two particularly severe manifestations of heart disease are myocardial infarction and cardiac hypertrophy. With respect to myocardial infarction, typically an acute thrombotic coronary occlusion occurs in a coronary artery as a result of atherosclerosis and causes myocardial cell death. Because cardiomyocytes, the heart muscle cells, are terminally differentiated and generally incapable of cell division, they are generally replaced by scar tissue when they die during the course of an acute myocardial infarction. Scar tissue is not contractile, fails to contribute to cardiac function, and often plays a detrimental role in heart function by expanding during cardiac contraction, or by increasing the size and effective radius of the ventricle, for example, becoming hypertrophic. With respect to cardiac hypertrophy, one theory regards this as a disease that resembles aberrant development and, as such, raises the question of whether developmental signals in the heart can contribute to hypertrophic disease. Cardiac hypertrophy is an adaptive response of the heart to virtually all forms of cardiac disease, including those arising from hypertension, mechanical load, myocardial infarction, cardiac arrhythmias, endocrine disorders, and genetic mutations in cardiac contractile protein genes. While the hypertrophic response is initially a compensatory mechanism that augments cardiac output, sustained hypertrophy can lead to DCM, heart failure, and sudden death. In the United States, approximately half a million individuals are diagnosed with heart failure each year, with a mortality rate approaching 50%.

The causes and effects of cardiac hypertrophy have been extensively documented, but the underlying molecular mechanisms have not been elucidated. Understanding these mechanisms is a major concern in the prevention and treatment of cardiac disease and will be crucial as a therapeutic modality in designing new drugs that specifically target cardiac hypertrophy and cardiac heart failure. As pathologic cardiac hypertrophy typically does not produce any symptoms until the cardiac damage is severe enough to produce heart failure, the

symptoms of cardiomyopathy are those associated with heart failure. These symptoms include shortness of breath, fatigue with exertion, the inability to lie flat without becoming short of breath (orthopnea), paroxysmal nocturnal dyspnea, enlarged cardiac dimensions, and/or swelling in the lower legs. Patients also often present with increased blood pressure, extra heart sounds, cardiac murmurs, pulmonary and systemic emboli, chest pain, pulmonary congestion, and palpitations. In addition, DCM causes decreased ejection fractions (*i.e.*, a measure of both intrinsic systolic function and remodeling). The disease is further characterized by ventricular dilation and grossly impaired systolic function due to diminished myocardial contractility, which results in dilated heart failure in many patients. Affected hearts also undergo cell/chamber remodeling as a result of the myocyte/myocardial dysfunction, which contributes to the “DCM phenotype.” As the disease progresses so do the symptoms. Patients with DCM also have a greatly increased incidence of life-threatening arrhythmias, including ventricular tachycardia and ventricular fibrillation. In these patients, an episode of syncope (dizziness) is regarded as a harbinger of sudden death.

Diagnosis of dilated cardiomyopathy typically depends upon the demonstration of enlarged heart chambers, particularly enlarged ventricles. Enlargement is commonly observable on chest X-rays, but is more accurately assessed using echocardiograms. DCM is often difficult to distinguish from acute myocarditis, valvular heart disease, coronary artery disease, and hypertensive heart disease. Once the diagnosis of dilated cardiomyopathy is made, every effort is made to identify and treat potentially reversible causes and prevent further heart damage. For example, coronary artery disease and valvular heart disease must be ruled out. Anemia, abnormal tachycardias, nutritional deficiencies, alcoholism, thyroid disease and/or other problems need to be addressed and controlled.

As mentioned above, treatment with pharmacological agents still represents the primary mechanism for reducing or eliminating the manifestations of heart failure. Diuretics constitute the first line of treatment for mild-to-moderate heart failure. Unfortunately, many of the commonly used diuretics (*e.g.*, the thiazides) have numerous adverse effects. For example, certain diuretics may increase serum cholesterol and triglycerides. Moreover, diuretics are generally ineffective for patients suffering from severe heart failure.

If diuretics are ineffective, vasodilatory agents may be used; the angiotensin converting (ACE) inhibitors (*e.g.*, enalapril and lisinopril) not only provide symptomatic relief, they also have been reported to decrease mortality (Young *et al.*, 1989). Again, however, the ACE inhibitors are associated with adverse effects that result in their being contraindicated in patients with certain disease states (*e.g.*, renal artery stenosis). Similarly,

inotropic agent therapy (*i.e.*, a drug that improves cardiac output by increasing the force of myocardial muscle contraction) is associated with a panoply of adverse reactions, including gastrointestinal problems and central nervous system dysfunction.

Thus, the currently used pharmacological agents have severe shortcomings in particular patient populations. The availability of new, safe and effective agents would undoubtedly benefit patients who either cannot use the pharmacological modalities presently available, or who do not receive adequate relief from those modalities. The prognosis for patients with DCM is variable, and depends upon the degree of ventricular dysfunction, with the majority of deaths occurring within five years of diagnosis.

I. The Present Invention

β -adrenergic signaling plays an important role in the natural history of dilated cardiac myopathy (DCM), exerting both compensatory effects on cardiac function and promoting the development and progression of the DCM phenotype. Activation of β -adrenergic receptors (β_1 -AR and β_2 -AR) during periods of cardiac stress initially results in increases in heart rate and contractility, effectively improving cardiac output, but then ultimately harms the failing heart (Bristow, 1997; Esler *et al.*, 1997) by mechanisms that include alterations in gene expression (Lowes *et al.*, 2002). At the cellular level, myocardial failure is characterized by changes in the gene expression of many components of the heart, including the contractile apparatus. These molecular changes have been described as a recapitulation of a "fetal" gene program (FGP) because many embryonically-expressed genes that are down-regulated postnatally are reactivated, while several "adult" genes are repressed (Lompre *et al.*, 1979). Of the changes that are observed in failing hearts, increases in β myosin heavy chain (β -MyHC), skeletal α -actin, and atrial natriuretic peptide (ANP), with coordinate decreases in α myosin heavy chain (α -MyHC) and sarcoplasmic reticulum ATPase 2a (SRCA2a), are perhaps the most widely recognized.

Chronic β -adrenergic stimulation of the myocardium has been shown to change gene expression patterns in a manner that also mimics the "fetal" gene program observed in failing hearts (Lowes *et al.*, 2002; Boluyt *et al.*, 1995; Rothermel *et al.*, 2001). Several β -adrenergic receptor blocking agents have been identified that successfully treat heart failure patients with DCMs, generally by acting to favorably alter the biology of the failing heart (Eichhorn and Bristow, 1996). It was recently shown that patients with DCMs who respond to β_1 -adrenergic receptor blockade by an improvement in systolic function and a reversal of remodeling also

have a partial reversal of the fetal gene program (Lowes *et al.*, 2002).. In these patients' hearts, α -MyHC and SRCA2a are up-regulated and β -MyHC and ANP expression are reduced (Lowes *et al.*, 2002). Unfortunately, not all patients respond to β -blocker therapy and the need to find additional targets for the treatment of heart failure is imperative.

5 The involvement of ERK1/2 in the development of cardiac hypertrophy has been well documented, and over expression of MEK1 (upstream activator of ERK1/2) in a transgenic animal model results in profound cardiac hypertrophy accompanied by repression of α Myosin Heavy Chain (α MyHC) and activation of β Myosin Heavy Chain (β MyHC), ANP, BNP and skeletal α -actin, considered markers of pathologic hypertrophy (the so-called fetal
10 gene induction). It has recently been shown that CaMK is involved in ERK1/2 activation in non-myocytes and that calcineurin is involved in ERK activation in cardiac myocytes, making ERK1/2 a potential candidate for the involvement in the β_1 -adrenergic-mediated activation of the fetal program.

15 The inventors have recently shown that β_1 -adrenergic stimulation in neonatal rat cardiac myocytes results in changes in the fetal gene program consistent with pathologic hypertrophic response. Here, they now show that blockade of calcineurin or CaMKII previous to β_1 -adrenergic stimulation with isoproterenol blocks the induction of the fetal gene program and the inventors show that blockade of ERK1/2 activation with U0126 prevents β_1 -
20 adrenergic mediated activation of β MyHC, ANP, BNP and skeletal α -actin and repression of α MyHC but does not suppress repression of SRCA2a. The inventors also show that ERK1/2 activation in response to β_1 -adrenergic stimulation in neonate rat cardiac myocytes (NRCMs) occurs at an early time point (5 minutes) and a later time point (33 and 48 hours). Interestingly, activation at the first time point is dependent on calcineurin since treatment with cyclosporine decreases isoproterenol-mediated ERK1/2 activation, and activation at the later
25 time point is dependent on CaMKII since treatment of the cells with KN-93 prior to isoproterenol treatment results in complete blockade of ERK1/2 activation. These results suggest that ERK1/2 is a central integrator of signal pathways involved in the β_1 -adrenergic mediated induction of the fetal gene program.

30 II. β -AR and ERK1/2

Activation of β -adrenergic (β -AR) signaling is involved in a variety of cellular processes, including cell growth, muscle contraction, cell survival and gene expression (Zhu *et al.*, 2003). Stimulation of β -ARs during periods of cardiac stress initially results in

increased myocardial performance by a classical mechanism that involves the coupling protein G α s, cAMP and protein kinase A (PKA) (Rockman *et al.*, 1996). However, sustained activation of the β -AR signaling ultimately harms the failing heart (Bristow, 1997; Esler *et al.*, 1997).

5 β -AR stimulation in adult rodent models results in changes in gene expression that includes repression of genes that are expressed during adult development (α -Myosin Heavy Chain (α -MyHC) and sarcoplasmatic reticulum ATPase 2a (SRCA2a)), and re-expression of genes that are present during the fetal development (β -Myosin Heavy Chain (β -MyHC), atrial and brain natriuretic peptide (ANP and BNP), and skeletal α -actin) (Boluyt *et al.*, 1995; 10 Rothermel *et al.*, 2001). The repression of adult genes and activation of fetal genes is referred to as induction of a “fetal” gene program. In humans, multiple studies have demonstrated that β -adrenergic stimulation plays an important role in the natural history of dilated cardiomyopathy (DCM), the myocardial phenotype that is the most common cause of chronic heart failure (Devereux and Roman, 1999; Iwanaga *et al.*, 2001; Kono *et al.*, 1992; Bonow, 15 2002). The inventors have recently shown that DCM patients have changes in gene expression that recapitulate the fetal gene program, and that these changes are partially reversed in patients that favorably respond to β -blocker therapy (Lowes *et al.*, 2002).

The inventors have recently shown that β -AR receptor stimulation in neonatal cardiac myocytes results in changes in the contractile gene program with a decrease in 20 α MyHC: β MyHC and SRCA2a gene expression and an increase in the expression of the adult genes, ANP, BNP and skeletal α -actin. The inventors also showed that these changes are specific for the β_1 -AR and not the β_2 -AR, and are independent of α_1 -AR signaling. Furthermore, β_1 -AR mediated fetal gene induction is independent of the classical cAMP/PKA pathway.

25 The Ca²⁺/calmodulin kinase (CaMK) has been shown to be an important component of β_1 -AR signal transduction pathway for a variety of effector responses, including, apoptosis and cell contractility (Wang *et al.*, 2004). CaMK has also been implicated in the induction of the hypertrophic marker genes skeletal α -actin, BNP, ANF and β -MyHC (reviewed in Zhang *et al.*, 2004), making it a potential candidate for involvement in β_1 -AR mediated induction of 30 the fetal gene program expression. Other candidates for a role in cAMP independent intracellular signaling of β -adrenergic fetal gene induction include MAP kinases (Bogoyevitch *et al.*, 1996) and calcineurin (Zou *et al.*, 2001).

A. ERK Function

The ERKs (MAPK p42/p44) are the prototypic MAPKs and are activated by a variety of mitogenic stimuli as well as differentiation signals. MAPK activation largely requires Ras activation. In *Drosophila*, the ERK homologue is encoded by the Rolled locus (an activated allele) which has been placed downstream of Ras in the sevenless receptor kinase pathway. ERKs are inactivated by dephosphorylation by specific protein phosphatases such as MKP1 (CL100) and PAC1. Downstream substrates include Elk1, phospholipase A2 and p90Rsk1. ERK2 is the only enzyme in the family to have its x-ray structure solved.

Targets for ERK phosphorylation include alphaB-crystallin-Ser45(activation), C/EBPbeta-Thr235(activation), c-Jun-Ser243(inhibition), Elk1-Ser383(activation), Estrogen receptor alpha-Ser118(activation), GAIIP-Ser151(activation), HSF-1-Ser-307(activation), MAPKAPK-2-Thr222, Ser272 and Thr334(activation), MBP-Thr98(activation), MEK1-Thr292 and Thr386(feedback), PHAS-I- Ser64(activation), p90 RSK-Ser364 and Thr574(activation), STAT5a-Ser780(activation) and Tyrosine hydroxylase-Ser31(activation).

B. ERK Protein and Nucleic Acid Structure

The accession nos. for the ERK1/2 protein and mRNA are found at CAA42744.1 and X60188, respectively, and are hereby incorporated by reference. Cloning and expression vectors for use with and production of ERK1/2 nucleic acids and proteins are discussed below.

C. ERK1/2 Inhibitors

As shown below in the Examples, the protein YY1 and its corresponding gene, when provided in an expression cassette, both constitute inhibitors of ERK1/2. In addition to the aforementioned, there are also generic, non-pharmacological methods of inhibiting genes, which are discussed below.

Because ERK1/2 appears to act at several time points in the hypertrophic signaling pathway, it may be desirable to avoid global inhibition of ERK1/2, but instead, to selectively inhibit ERK1/2 at particular time points, such as the 33 hour time point which appears most related to the pathologic function of ERK1/2. It may also be desirable to titrate inhibition in terms of total dose, or time of doses, following a particular event.

i. Nucleic Acids**a. Antisense Constructs**

Antisense methodology takes advantage of the fact that nucleic acids tend to pair with "complementary" sequences. By complementary, it is meant that polynucleotides are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs will include regions complementary to intron/exon splice junctions. Thus, it is proposed that a preferred embodiment includes an antisense construct with complementarity to regions within 50-200 bases of an intron-exon splice junction. It has been observed that some exon sequences can be included in the construct without seriously affecting the target selectivity thereof. The amount of exonic material included will vary depending on the particular exon and intron sequences used. One can readily test whether too much exon DNA is included simply by testing the constructs *in vitro* to determine whether normal cellular function is affected or whether the expression of related genes having complementary sequences is affected.

As stated above, "complementary" or "antisense" means polynucleotide sequences that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have complementary nucleotides at thirteen or fourteen positions. Naturally, sequences which are completely complementary will be sequences which are entirely complementary throughout their entire length and have no base mismatches. Other sequences with lower

degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (*e.g.*, ribozyme; see below) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

It may be advantageous to combine portions of genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

ii. Ribozymes

Although proteins traditionally have been used for catalysis of nucleic acids, another class of macromolecules has emerged as useful in this endeavor. Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cook, 1987; Gerlach *et al.*, 1987; Forster and Symons, 1987). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cook *et al.*, 1981; Michel and Westhof, 1990; Reinhold-Hurek and Shub, 1992). This specificity has been attributed to the requirement that the substrate bind *via* specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Ribozyme catalysis has primarily been observed as part of sequence-specific cleavage/ligation reactions involving nucleic acids (Joyce, 1989; Cook *et al.*, 1981). For example, U.S. Patent 5,354,855 reports that certain ribozymes can act as endonucleases with a sequence specificity greater than that of known ribonucleases and approaching that of the DNA restriction enzymes. Thus, sequence-specific ribozyme-mediated inhibition of gene expression may be particularly suited to therapeutic applications (Scanlon *et al.*, 1991; Sarver *et al.*, 1990). Recently, it was reported that ribozymes elicited genetic changes in some cells lines to which they were applied; the altered genes included the oncogenes H-ras, c-fos and genes of HIV. Most of this work involved the modification of a target mRNA, based on a specific mutant codon that is cleaved by a specific ribozyme.

iii. RNAi

RNA interference (also referred to as "RNA-mediated interference" or RNAi) is a mechanism by which gene expression can be reduced or eliminated. Double-stranded RNA

(dsRNA) has been observed to mediate the reduction, which is a multi-step process. dsRNA activates post-transcriptional gene expression surveillance mechanisms that appear to function to defend cells from virus infection and transposon activity (Fire *et al.*, 1998; Grishok *et al.*, 2000; Ketting *et al.*, 1999; Lin and Avery *et al.*, 1999; Montgomery *et al.*, 1998; Sharp and Zamore, 2000; Tabara *et al.*, 1999). Activation of these mechanisms targets mature, dsRNA-complementary mRNA for destruction. RNAi offers major experimental advantages for study of gene function. These advantages include a very high specificity, ease of movement across cell membranes, and prolonged down-regulation of the targeted gene (Fire *et al.*, 1998; Grishok *et al.*, 2000; Ketting *et al.*, 1999; Lin and Avery *et al.*, 1999; Montgomery *et al.*, 1998; Sharp *et al.*, 1999; Sharp and Zamore, 2000; Tabara *et al.*, 1999). Moreover, dsRNA has been shown to silence genes in a wide range of systems, including plants, protozoans, fungi, *C. elegans*, *Trypanosoma*, *Drosophila*, and mammals (Grishok *et al.*, 2000; Sharp *et al.*, 1999; Sharp and Zamore, 2000; Elbashir *et al.*, 2001). It is generally accepted that RNAi acts post-transcriptionally, targeting RNA transcripts for degradation. It appears that both nuclear and cytoplasmic RNA can be targeted (Bosher and Labouesse, 2000).

siRNAs must be designed so that they are specific and effective in suppressing the expression of the genes of interest. Methods of selecting the target sequences, *i.e.*, those sequences present in the gene or genes of interest to which the siRNAs will guide the degradative machinery, are directed to avoiding sequences that may interfere with the siRNA's guide function while including sequences that are specific to the gene or genes. Typically, siRNA target sequences of about 21 to 23 nucleotides in length are most effective. This length reflects the lengths of digestion products resulting from the processing of much longer RNAs as described above (Montgomery *et al.*, 1998).

The making of siRNAs has been mainly through direct chemical synthesis; through processing of longer, double-stranded RNAs through exposure to *Drosophila* embryo lysates; or through an *in vitro* system derived from S2 cells. Use of cell lysates or *in vitro* processing may further involve the subsequent isolation of the short, 21-23 nucleotide siRNAs from the lysate, *etc.*, making the process somewhat cumbersome and expensive. Chemical synthesis proceeds by making two single-stranded RNA-oligomers followed by the annealing of the two single-stranded oligomers into a double stranded RNA. Methods of chemical synthesis are diverse. Non-limiting examples are provided in U.S. Patents 5,889,136, 4,415,723, and 4,458,066, expressly incorporated herein by reference, and in Wincott *et al.* (1995).

Several further modifications to siRNA sequences have been suggested in order to alter their stability or improve their effectiveness. It is suggested that synthetic

complementary 21-mer RNAs having di-nucleotide overhangs (*i.e.*, 19 complementary nucleotides + 3' non-complementary dimers) may provide the greatest level of suppression. These protocols primarily use a sequence of two (2'-deoxy) thymidine nucleotides as the di-nucleotide overhangs. These dinucleotide overhangs are often written as dTdT to distinguish
5 them from the typical nucleotides incorporated into RNA. The literature has indicated that the use of dT overhangs is primarily motivated by the need to reduce the cost of the chemically synthesized RNAs. It is also suggested that the dTdT overhangs might be more stable than UU overhangs, though the data available shows only a slight (< 20%) improvement of the dTdT overhang compared to an siRNA with a UU overhang.

10 Chemically synthesized siRNAs are found to work optimally when they are in cell culture at concentrations of 25-100 nM, but concentrations of about 100 nM have achieved effective suppression of expression in mammalian cells. siRNAs have been most effective in mammalian cell culture at about 100 nM. In several instances, however, lower concentrations of chemically synthesized siRNA have been used (Caplen, *et al.*, 2000; Elbashir *et al.*, 2001).

15 WO 99/32619 and WO 01/68836 suggest that RNA for use in siRNA may be chemically or enzymatically synthesized. Both of these texts are incorporated herein in their entirety by reference. The enzymatic synthesis contemplated in these references is by a cellular RNA polymerase or a bacteriophage RNA polymerase (*e.g.*, T3, T7, SP6) via the use and production of an expression construct as is known in the art. For example, see U.S.
20 Patent 5,795,715. The contemplated constructs provide templates that produce RNAs that contain nucleotide sequences identical to a portion of the target gene. The length of identical sequences provided by these references is at least 25 bases, and may be as many as 400 or more bases in length. An important aspect of this reference is that the authors contemplate digesting longer dsRNAs to 21-25mer lengths with the endogenous nuclease complex that
25 converts long dsRNAs to siRNAs *in vivo*. They do not describe or present data for synthesizing and using *in vitro* transcribed 21-25mer dsRNAs. No distinction is made between the expected properties of chemical or enzymatically synthesized dsRNA in its use in RNA interference.

30 Similarly, WO 00/44914, incorporated herein by reference, suggests that single strands of RNA can be produced enzymatically or by partial/total organic synthesis. Preferably, single-stranded RNA is enzymatically synthesized from the PCR products of a DNA template, preferably a cloned cDNA template and the RNA product is a complete transcript of the cDNA, which may comprise hundreds of nucleotides. WO 01/36646, incorporated herein by reference, places no limitation upon the manner in which the siRNA is

synthesized, providing that the RNA may be synthesized *in vitro* or *in vivo*, using manual and/or automated procedures. This reference also provides that *in vitro* synthesis may be chemical or enzymatic, for example using cloned RNA polymerase (*e.g.*, T3, T7, SP6) for transcription of the endogenous DNA (or cDNA) template, or a mixture of both. Again, no distinction in the desirable properties for use in RNA interference is made between chemically or enzymatically synthesized siRNA.

U.S. Patent 5,795,715 reports the simultaneous transcription of two complementary DNA sequence strands in a single reaction mixture, wherein the two transcripts are immediately hybridized. The templates used are preferably of between 40 and 100 base pairs, and which is equipped at each end with a promoter sequence. The templates are preferably attached to a solid surface. After transcription with RNA polymerase, the resulting dsRNA fragments may be used for detecting and/or assaying nucleic acid target sequences.

III. Genes Involved in Modulation of of ERK1/2 Expression and Function

A. Calcineurin

It is known that Ca^{2+} activation is involved in a variety of forms of heart failure and heart disease. Ca^{2+} store depletion, or a raise in the cytoplasmic Ca^{2+} levels in the cell, has been show to stimulate a calcineurin dependent pathway for cardiac hypertrophy. Calcineurin is a ubiquitously expressed serine/threonine phosphatase that exists as a heterodimer, comprised of a 59 kD calmodulin-binding catalytic A subunit and a 19 kD Ca^{2+} -binding regulatory B subunit (Stemmer and Klee, 1994; Su *et al.*, 1995). Calcineurin is uniquely suited to mediate the prolonged hypertrophic response of a cardiomyocyte to Ca^{2+} signaling because the enzyme is activated by a sustained Ca^{2+} plateau and is insensitive to transient Ca^{2+} fluxes as occur in response to cardiomyocyte contraction (Dolmetsch *et al.*, 1997).

Activation of calcineurin is mediated by binding of Ca^{2+} and calmodulin to the regulatory and catalytic subunits, respectively. Previous studies showed that over-expression of calmodulin in the heart also results in hypertrophy, but the mechanism involved was not determined (Gruver *et al.*, 1993). It is now clear that calmodulin acts through the calcineurin pathway to induce the hypertrophic response. Calcineurin has been shown previously by the inventors to phosphorylate NF-AT3, which subsequently acts on the transcription factor MEF-2 (Olson *et al.*, 2000). Once this event occurs, MEF-2 activates a variety of genes known as fetal genes, the activation of which inevitably results in hypertrophy and heart failure.

CsA and FK-506 bind the immunophilins cyclophilin and FK-506-binding protein (FKBP12), respectively, forming complexes that bind the calcineurin catalytic subunit and inhibit its activity. CsA and FK-506 block the ability of cultured cardiomyocytes to undergo hypertrophy in response to AngII and PE (both strong agonists of hypertrophy *in vitro* and *in vivo*). Both of these hypertrophic agonists have been shown to act by elevating intracellular Ca^{2+} , which results in activation of the PKC and MAP kinase signaling pathways (Sadoshima *et al.*, 1993; Sadoshima and Izumo, 1993; Kudoh *et al.*, 1997; Yamazaki *et al.*, 1997; Zou *et al.*, 1996). CsA does not interfere with early signaling events at the cell membrane, such as PI turnover, Ca^{2+} mobilization, or PKC activation (Emmel *et al.*, 1989). Thus, its ability to abrogate the hypertrophic responses of AngII and PE suggests that calcineurin activation is an essential step in the AngII and PE signal transduction pathways.

B. CamKII

Signaling by CaMK has been implicated in a broad range of cellular, developmental, and physiological processes, including learning and memory, neurotransmission, maturation of oocytes, myocyte contractility, and muscle growth and gene expression, each of which requires phosphorylation of specific substrates within different subcellular compartments. The complexity of CaMK signaling is underscored by the existence of 6 different *CaMK* genes (*CaMKI*, *Ila*, *Ilb*, *Ilg*, *IId*, and *IV*), which give rise to multiple protein variants through alternative splicing.

Specificity of signaling events can be achieved by scaffold proteins, which mediate compartmentalization of kinases and phosphatases, as described, in general, for A-kinase anchoring proteins and, in the case of CaMKII, for A-kinase anchoring proteins. A distinct but related mechanism involves the tethering of the kinase to its substrate at a site that is distinct from the phosphorylation site. Backs *et al.* (2006) identified a unique CaMKII docking site on HDAC4 that is not present on other HDACs. The docking site (amino acids 585–608) is located in relatively close proximity to the sites of HDAC4, which we show to be phosphorylated by CaMKII (S467 and S632). Interestingly, only the active form of CaMKII, mimicked by the T287D point mutation, interacts with HDAC4, suggesting that autophosphorylation induces a conformational change in CaMKII that enables it to bind HDAC4.

There is increasing evidence for the involvement of CaMKII in pathological cardiac hypertrophy. Inhibition of CaMKII in mice by overexpression of a CaMKII inhibitory peptide in the heart blocks adverse myocardial remodeling in response to β -adrenergic stimulation and

myocardial infarction, providing strong evidence that CaMKII is required for a pathological hypertrophic response. CaMKII has also been implicated in endothelin-1-induced cardiomyocyte hypertrophy and in ANP gene expression during hypertrophy. Moreover, forced overexpression of calmodulin or CaMKII in the heart is sufficient to induce cardiac hypertrophy. However, the downstream mechanisms and targets of CaMKII signaling that lead to changes in cardiac gene regulation have remained elusive. CaMKII exists as a multimer consisting of 6–12 α , β , δ , or γ subunits, each encoded by a different gene. Whereas CaMKII α and β are mainly expressed in neuronal tissues, CaMKII δ and γ are abundant in the heart and upregulated in clinical and experimental heart failure.

C. YY1

Yin Yang 1 (YY1) is a ubiquitously distributed transcription factor belonging to the GLI-Kruppel class of zinc finger proteins. The protein is involved in repressing and activating a diverse number of promoters. The inventors previously reported that YY1 was a negative regulator of the α -myosin heavy chain (α MyHC) gene, which, with β MyHC are the molecular motors of the heart. α MyHC mRNA and protein levels were down-regulated in hypertrophy and heart failure, and this is thought to be detrimental for cardiac contractility. YY1 was shown to specifically interact with the α MyHC promoter and that overexpression of YY1 in cardiac cells represses the activity of the α MyHC promoter. It also was shown that the 170-200-amino acid region of YY1, important for its interaction with histone acetyl transferases and histone deacetylases, was important for its repressive activity and that YY1 deleted in this region was an activator of the α MyHC promoter. They also showed that YY1 levels and DNA binding activity were increased in failing human left ventricles and in a mouse model of hypertrophic cardiomyopathy, where α MyHC levels are decreased. These results suggest that YY1 is a negative regulator of α MyHC gene expression, and thus pro-hypertrophic (Sucharov *et al.*, 2003).

More recently, the inventors have discovered that overexpression of YY1 actually prevents induction of the fetal gene program in isoproterenol-mediated hypertrophy suggesting that YY1 could have a protective function in β_1 -mediated hypertrophy. They also showed that knockdown of YY1 expression by siRNA in neonate rat cardiac myocytes (NRCMs) induces a hypertrophic response that is measure by an increase in cell size consistent with eccentric hypertrophy and by the induction of the fetal gene program. These results also suggest that inhibition of CaMKII in isoproterenol treated NRCMs increases YY1

repression of the FGP in these cells. Finally, the results show that YY1 interacts with HDAC5 in differentiated cells and that HDAC5 increases YY1 repression of the α MyHC promoter activity suggesting that YY1 and HDAC5 are important components of the transcription machinery necessary to maintain the repression of the fetal components of the FGP. U.S. Provisional Application Serial No. 60/701,788.

IV. Methods of Treating Cardiac Hypertrophy

A. Therapeutic Regimens

Current medical management of cardiac hypertrophy in the setting of a cardiovascular disorder includes the use of at least two types of drugs: inhibitors of the rennin-angiotensin system, and β -adrenergic blocking agents (Bristow, 1999). Therapeutic agents to treat pathologic hypertrophy in the setting of heart failure include angiotensin II converting enzyme (ACE) inhibitors and β -adrenergic receptor blocking agents (Eichhorn and Bristow, 1996). Other pharmaceutical agents that have been disclosed for treatment of cardiac hypertrophy include angiotensin II receptor antagonists (U.S. Patent 5,604,251) and neuropeptide Y antagonists (WO 98/33791). Despite currently available pharmaceutical compounds, prevention and treatment of cardiac hypertrophy, and subsequent heart failure, continue to present a therapeutic challenge.

Non-pharmacological treatment is primarily used as an adjunct to pharmacological treatment. One means of non-pharmacological treatment involves reducing the sodium in the diet. In addition, non-pharmacological treatment also entails the elimination of certain precipitating drugs, including negative inotropic agents (*e.g.*, certain calcium channel blockers and antiarrhythmic drugs like disopyramide), cardiotoxins (*e.g.*, amphetamines), and plasma volume expanders (*e.g.*, nonsteroidal anti-inflammatory agents and glucocorticoids).

In one embodiment of the present invention, methods for the treatment of cardiac hypertrophy or heart failure utilizing inhibitors of ERK1/2 are provided. For the purposes of the present application, treatment comprises reducing one or more of the symptoms of cardiac hypertrophy, such as reduced exercise capacity, reduced blood ejection volume, increased left ventricular end diastolic pressure, increased pulmonary capillary wedge pressure, reduced cardiac output, cardiac index, increased pulmonary artery pressures, increased left ventricular end systolic and diastolic dimensions, and increased left ventricular wall stress, wall tension and wall thickness-same for right ventricle. In addition, use of inhibitors of ERK1/2 may prevent cardiac hypertrophy and its associated symptoms from arising.

Treatment regimens would vary depending on the clinical situation. However, long term maintenance would appear to be appropriate in most circumstances. It also may be desirable treat hypertrophy with inhibitors of ERK1/2 intermittently, such as within brief window during disease progression.

5 **B. Combined Therapy**

In another embodiment, it is envisioned to use an inhibitor of ERK1/2 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 "beta blockers," anti-hypertensives, 10 cardiotonics, anti-thrombotics, vasodilators, hormone antagonists, ionotropes, diuretics, endothelin antagonists, calcium channel blockers, phosphodiesterase inhibitors, ACE inhibitors, angiotensin type 2 antagonists and cytokine blockers/inhibitors, and HDAC inhibitors.

Combinations may be achieved by contacting cardiac cells with a single composition 15 or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the expression construct and the other includes the agent. Alternatively, the therapy using an inhibitor of ERK1/2 may precede or follow administration of the other agent(s) by intervals ranging from minutes to weeks. In embodiments where the other agent and expression 20 construct are applied separately to the cell, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the agent and expression construct would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one would typically contact the cell with both modalities within about 12-24 hours of each other and, more preferably, within about 6-12 25 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 30 ERK1/2, or the other agent will be desired. In this regard, various combinations may be employed. By way of illustration, where the inhibitor of ERK1/2 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.

5 C. Pharmacological Therapeutic Agents

Pharmacological therapeutic agents and methods of administration, dosages, *etc.*, are well known to those of skill in the art (see for example, the “Physicians Desk Reference,” Klaassen’s “The Pharmacological Basis of Therapeutics,” “Remington’s Pharmaceutical Sciences,” and “The Merck Index, Eleventh Edition,” incorporated herein by reference in
 10 relevant parts), and may be combined with the invention in light of the disclosures herein. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject, and such individual determinations are within the skill of those of ordinary skill in the art.

15 Non-limiting examples of a pharmacological therapeutic agent that may be used in the present invention include an antihyperlipoproteinemic agent, an antiarteriosclerotic agent, an antithrombotic/fibrinolytic agent, a blood coagulant, an antiarrhythmic agent, an antihypertensive agent, a vasopressor, a treatment agent for congestive heart failure, an antianginal agent, an antibacterial agent or a combination thereof.

20 In addition, it should be noted that any of the following may be used to develop new sets of cardiac therapy target genes as β -blockers were used in the present examples (see below). While it is expected that many of these genes may overlap, new gene targets likely can be developed.

i. Antihyperlipoproteinemics

25 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,” 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
 30 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 combination thereof.

a. Aryloxyalkanoic Acid/Fibric Acid Derivatives

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

10 **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

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

d. Nicotinic Acid Derivatives

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

e. Thyroid Hormones and Analogs

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

25

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, 30 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.

ii. Antiarteriosclerotics

Non-limiting examples of an antiarteriosclerotic include pyridinol carbamate.

iii. Antithrombotic/Fibrinolytic Agents

5 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
10 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, anisindione, anisindione, bromindione, chlorindione, coumatrol, cyclocoumarol, dextran sulfate sodium,
15 dicoumarol, diphenadione, ethyl biscoumatate, ethylidene dicoumarol, fluindione, heparin, hirudin, lyapolate sodium, oxazindione, pentosan polysulfate, phenindione, phenprocoumon, phosvitin, picotamide, ticlopidine and warfarin.

b. Antiplatelet Agents

20 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
25 (activase), plasmin, pro-urokinase, urokinase (abbokinase) streptokinase (streptase), anistreplase/APSAC (eminase).

iv. Blood Coagulants

30 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 vitamine K1.

b. Thrombolytic Agent Antagonists and Antithrombotics

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

v. Antiarrhythmic Agents

Non-limiting examples of antiarrhythmic agents include Class I antiarrhythmic agents (sodium channel blockers), Class II antiarrhythmic agents (beta-adrenergic blockers), Class II 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 dispyramide (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 (enkaid) and flecainide (tambocor).

b. Beta Blockers

Non-limiting examples of a beta 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 (inalderal), sotalol (betapace), sulfinalol, talinolol, tertatolol, timolol, toliprolol and xibinolol. In certain aspects, the beta 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
5 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
15 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, lorcainide, meobentine, moricizine,
25 pirmenol, prajmaline, propafenone, pyrinoline, quinidine polygalacturonate, quinidine sulfate and viquidil.

vi. Antihypertensive Agents

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

a. Alpha Blockers

Non-limiting examples of an alpha 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 alpha 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 alpha and beta adrenergic antagonist. Non-limiting examples of an alpha/beta blocker comprise labetalol (normodyne, trandate).

c. Anti-Angiotension II Agents

Non-limiting examples of anti-angiotension II agents 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 peripherally 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 α 1-adrenergic blocking agent. Non-limiting examples of a ganglion blocking agent include mecamlamine (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 acenitolol (sectral), atenolol (tenormin), betaxolol

(kerlone), carteolol (cartrol), labetalol (normodyne, trandate), metoprolol (lopressor), nadanol (corgard), penbutolol (levatol), pindolol (visken), propranolol (inderal) and timolol (blocadren). Non-limiting examples of alpha1-adrenergic blocker include prazosin (minipress), doxazocin (cardura) and terazosin (hytrin).

5

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
10 examples of a coronary vasodilator include amotriphene, bendazol, benfurodil hemisuccinate, benziadarone, 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,
15 perhexiline, pimefylline, trapidil, tricromyl, trimetazidine, toltrinate 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),
20 hydralazine (apresoline), minoxidil (loniten) and verapamil.

f. Miscellaneous Antihypertensives

Non-limiting examples of miscellaneous antihypertensives include ajmaline, γ -aminobutyric acid, bufeniodol, cicletanine, ciclosporin, a cryptenamine tannate, fenoldopam, flosequin, ketanserin, mebutamate, mecamlamine, methyl dopa, methyl 4-
25 pyridyl ketone thiosemicarbazone, muzolimine, pargyline, pempidine, pinacidil, piperoxan, primaperone, a protoveratrine, raubasine, rescimetol, rilmenidine, saralasin, sodium nitroprusside, ticrynafen, trimethaphan camsylate, tyrosinase and urapidil.

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

Arylethanolamine Derivatives. Non-limiting examples of arylethanolamine derivatives include amosulalol, bufuralol, dilevalol, labetalol, pronethalol, sotalol and sulfinalol.

Benzothiadiazine Derivatives. Non-limiting examples of benzothiadiazine derivatives include althizide, bendroflumethiazide, benzthiazide, benzylhydrochlorothiazide, buthiazide, chlorothiazide, chlorthalidone, cyclopentthiazide, cyclothiazide, diazoxide, epithiazide, ethiazide, fenquizone, hydrochlorothizide, hydroflumethizide, methyclothiazide, meticrane, metolazone, paraflutizide, polythizide, tetrachlormethiazide and trichlormethiazide.

***N*-carboxyalkyl(peptide/lactam) Derivatives.** Non-limiting examples of *N*-carboxyalkyl(peptide/lactam) derivatives include alacepril, captopril, cilazapril, delapril, enalapril, enalaprilat, fosinopril, lisinopril, moveltipril, perindopril, quinapril and ramipril.

Dihydropyridine Derivatives. Non-limiting examples of dihydropyridine derivatives include amlodipine, felodipine, isradipine, nicardipine, nifedipine, nilvadipine, nisoldipine and nitrendipine.

Guanidine Derivatives. Non-limiting examples of guanidine derivatives include bethanidine, debrisoquin, guanabenz, guanacine, guanadrel, guanazodine, guanethidine, guanfacine, guanochlor, guanoxabenz and guanozan.

Hydrazines/Phthalazines. Non-limiting examples of hydrazines/phthalazines include budralazine, cadralazine, dihydralazine, endralazine, hydracarbazine, hydralazine, pheniprazine, pildralazine and todralazine.

Imidazole Derivatives. Non-limiting examples of imidazole derivatives include clonidine, lofexidine, phentolamine, tiamenidine and tolonidine.

Quaternary Ammonium Compounds. Non-limiting examples of quaternary ammonium compounds include azamethonium bromide, chlorisondamine chloride, hexamethonium, pentacynium bis(methylsulfate), pentamethonium bromide, pentolinium tartrate, phenactropinium chloride and trimethidinium methosulfate.

Reserpine Derivatives. Non-limiting examples of reserpine derivatives include bietaserpine, deserpidine, rescinnamine, reserpine and syrosingopine.

Sulfonamide Derivatives. Non-limiting examples of sulfonamide derivatives include ambuside, clopamide, furosemide, indapamide, quinethazone, tripamide and xipamide.

g. Vasopressors

Vasopressors generally are used to increase blood pressure during shock, which may 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.

vii. 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, mercumallylic acid, mercumatilin dodium, 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,

chlorazanyl, ethacrynic acid, etozolin, hydracarbazine, isosorbide, mannitol, metochalcone, muzolimine, perhexiline, ticrfafen and urea.

c. Inotropic Agents

Non-limiting examples of a positive inotropic agent, also known as a cardiotonic, include acefylline, an acetyldigoxin, 2-amino-4-picoline, amrinone, benfurodil hemisuccinate, bucladesine, cerberosine, camphotamide, convallatoxin, cymarln, denopamine, deslanoside, digitalin, digitalis, digitoxin, digoxin, dobutamine, dopamine, dopexamine, enoximone, erythrophleine, fenalcomine, gitalin, gitoxin, glycoeyamine, 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 beta-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, beta 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).

30 D. Surgical Therapeutic Agents

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.

E. Drug Formulations and Routes for Administration to Patients

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.

One will generally desire to employ appropriate salts and buffers to render delivery vectors stable and allow for uptake by target cells. Buffers also will be employed when recombinant cells are introduced into a patient. Aqueous compositions of the present invention comprise an effective amount of the vector or cells, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes solvents, buffers, solutions, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like acceptable for use in formulating pharmaceuticals, such as pharmaceuticals suitable for administration to humans. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredients of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions, provided they do not inactivate the vectors or cells 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. This includes oral, nasal, or buccal. Alternatively, administration may be by intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection, or by direct injection into cardiac tissue. Such compositions would normally be administered as pharmaceutically acceptable compositions, as described *supra*.

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

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

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

For oral administration the polypeptides of the present invention generally may be incorporated with excipients and used in the form of non-ingestible mouthwashes and dentifrices. A mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active ingredient may be incorporated into an antiseptic wash containing sodium borate, glycerin and potassium bicarbonate. The active ingredient may also be dispersed in dentifrices, including: gels, pastes, powders and slurries. The active ingredient may be added in a therapeutically effective amount to a paste dentifrice that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants.

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

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

sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

V. Screening Methods

The present invention further comprises methods for identifying inhibitors of ERK1/2 that are useful in the prevention or treatment or reversal of cardiac hypertrophy or heart failure. These assays may comprise random screening of large libraries of candidate substances; alternatively, the assays may be used to focus on particular classes of compounds selected with an eye towards structural attributes that are believed to make them more likely to inhibit the function of ERK1/2.

To identify an inhibitor of ERK1/2, one generally will determine the function of a ERK1/2 in the presence and absence of the candidate substance. For example, a method generally comprises:

- (a) providing a candidate modulator;
- (b) admixing the candidate modulator with a ERK1/2;
- (c) measuring ERK1/2 expression or activity; and
- (d) comparing the activity in step (c) with the activity in the absence of the candidate modulator,

wherein a difference between the measured activities indicates that the candidate modulator is, indeed, a modulator of the compound, cell or animal.

Assays also may be conducted in isolated cells, organs, or in living organisms. Typically, the activity of ERK1/2 is measured by assessing fetal gene expression.

It will, of course, be understood that all the screening methods of the present invention are useful in themselves notwithstanding the fact that effective candidates may not be found. The invention provides methods for screening for such candidates, not solely methods of finding them.

A. Modulators

As used herein the term "candidate substance" refers to any molecule that may potentially inhibit the kinase activity or cellular functions of ERK1/2. The candidate substance may be a protein or fragment thereof, a small molecule, or even a nucleic acid. It may prove to be the case that the most useful pharmacological compounds will be compounds that are structurally related to known ERK1/2 inhibitors, listed elsewhere in this document. Using lead compounds to help develop improved compounds is known as "rational drug

design” and includes not only comparisons with known inhibitors and activators, but predictions relating to the structure of target molecules.

The goal of rational drug design is to produce structural analogs of biologically active polypeptides or target compounds. By creating such analogs, it is possible to fashion drugs which are more active or stable than the natural molecules, which have different susceptibility to alteration, or which may affect the function of various other molecules. In one approach, one would generate a three-dimensional structure for a target molecule, or a fragment thereof. This could be accomplished by x-ray crystallography, computer modeling, or by a combination of both approaches.

It also is possible to use antibodies to ascertain the structure of a target compound, activator, or inhibitor. In principle, this approach yields a pharmacore upon which subsequent drug design can be based. It is possible to bypass protein crystallography altogether by generating anti-idiotypic antibodies to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of anti-idiotypic would be expected to be an analog of the original antigen. The anti-idiotypic could then be used to identify and isolate peptides from banks of chemically- or biologically-produced peptides. Selected peptides would then serve as the pharmacore. Anti-idiotypes may be generated using the methods described herein for producing antibodies, using an antibody as the antigen.

On the other hand, one may simply acquire, from various commercial sources, small molecular libraries that are believed to meet the basic criteria for useful drugs in an effort to “brute force” the identification of useful compounds. Screening of such libraries, including combinatorially-generated libraries (e.g., peptide libraries), is a rapid and efficient way to screen large number of related (and unrelated) compounds for activity. Combinatorial approaches also lend themselves to rapid evolution of potential drugs by the creation of second, third, and fourth generation compounds modeled on active, but otherwise undesirable compounds.

Candidate compounds may include fragments or parts of naturally-occurring compounds, or may be found as active combinations of known compounds, which are otherwise inactive. It is proposed that compounds isolated from natural sources, such as animals, bacteria, fungi, plant sources, including leaves and bark, and marine samples may be assayed as candidates for the presence of potentially useful pharmaceutical agents. It will be understood that the pharmaceutical agents to be screened could also be derived or synthesized from chemical compositions or man-made compounds. Thus, it is understood that the candidate substance identified by the present invention may be peptide, polypeptide,

polynucleotide, small molecule inhibitors or any other compounds that may be designed through rational drug design starting from known inhibitors or stimulators.

Other suitable modulators include antisense molecules, ribozymes, and antibodies (including single chain antibodies), each of which would be specific for the target molecule.

- 5 Such compounds are described in greater detail elsewhere in this document. For example, an antisense molecule that bound to a translational or transcriptional start site, or splice junctions, would be ideal candidate inhibitors.

10 In addition to the modulating compounds initially identified, the inventors also contemplate that other sterically similar compounds may be formulated to mimic the key portions of the structure of the modulators. Such compounds, which may include peptidomimetics of peptide modulators, may be used in the same manner as the initial modulators.

B. *In vitro* Assays

15 A quick, inexpensive and easy assay to run is an *in vitro* assay. Such assays generally use isolated molecules, can be run quickly and in large numbers, thereby increasing the amount of information obtainable in a short period of time. A variety of vessels may be used to run the assays, including test tubes, plates, dishes and other surfaces such as dipsticks or beads.

20 A technique for high throughput screening of compounds is described in WO 84/03564. Large numbers of small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. Such peptides could be rapidly screening for their ability to bind and inhibit ERK1/2.

C. *In cyto* Assays

25 The present invention also contemplates the screening of compounds for their ability to modulate ERK1/2 function and/or expression in cells. Various cell lines can be utilized for such screening assays, including cells specifically engineered for this purpose.

D. *In vivo* Assays

30 *In vivo* assays involve the use of various animal models of heart disease, including transgenic animals, that have been engineered to have specific defects, or carry markers that can be used to measure the ability of a candidate substance to reach and effect different cells within the organism. Due to their size, ease of handling, and information on their physiology

and genetic make-up, mice are a preferred embodiment, especially for transgenics. However, other animals are suitable as well, including rats, rabbits, hamsters, guinea pigs, gerbils, woodchucks, cats, dogs, sheep, goats, pigs, cows, horses and monkeys (including chimps, gibbons and baboons). Assays for inhibitors may be conducted using an animal model
5 derived from any of these species.

Treatment of animals with test compounds will involve the administration of the compound, in an appropriate form, to the animal. Administration will be by any route that could be utilized for clinical purposes. Determining the effectiveness of a compound *in vivo* may involve a variety of different criteria, including but not limited to . Also, measuring
10 toxicity and dose response can be performed in animals in a more meaningful fashion than in *in vitro* or *in cyto* assays.

VI. Purification of Proteins

In certain embodiments, it will be desirable to purify ERK1/2. Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level,
15 the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. Having separated the polypeptide from other proteins, the polypeptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, exclusion
20 chromatography; polyacrylamide gel electrophoresis; isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or even HPLC.

Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of an encoded protein or peptide. The term "purified protein or peptide" as used herein, is intended to refer to a composition, isolatable
25 from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state. A purified protein or peptide therefore also refers to a protein or peptide, free from the environment in which it may naturally occur.

Generally, "purified" will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition
30 substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95% or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the
5 purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a “-fold purification number.” The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

10 Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other
15 techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will
20 have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater “-fold” purification than the same technique utilizing a low pressure chromatography system.
25 Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi *et al.*, 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or
30 partially purified expression products may vary.

High Performance Liquid Chromatography (HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain an adequate flow rate. Separation can be accomplished in a matter of minutes, or at most an hour. Moreover, only a very small volume

of the sample is needed because the particles are so small and close-packed that the void volume is a very small fraction of the bed volume. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

5 Gel chromatography, or molecular sieve chromatography, is a special type of partition chromatography that is based on molecular size. The theory behind gel chromatography is that the column, which is prepared with tiny particles of an inert substance that contain small pores, separates larger molecules from smaller molecules as they pass through or around the pores, depending on their size. As long as the material of which the particles are made does
10 not adsorb the molecules, the sole factor determining rate of flow is the size. Hence, molecules are eluted from the column in decreasing size, so long as the shape is relatively constant. Gel chromatography is unsurpassed for separating molecules of different size because separation is independent of all other factors such as pH, ionic strength, temperature, *etc.* There also is virtually no adsorption, less zone spreading and the elution volume is
15 related in a simple matter to molecular weight.

 Affinity Chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule that it can specifically bind to. This is a receptor-ligand type interaction. The column material is synthesized by covalently coupling one of the binding partners to an insoluble matrix. The column material is then able
20 to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (alter pH, ionic strength, temperature, *etc.*).

 A particular type of affinity chromatography useful in the purification of carbohydrate containing compounds is lectin affinity chromatography. Lectins are a class of substances
25 that bind to a variety of polysaccharides and glycoproteins. Lectins are usually coupled to agarose by cyanogen bromide. Concanavalin A coupled to Sepharose was the first material of this sort to be used and has been widely used in the isolation of polysaccharides and glycoproteins other lectins that have been include lentil lectin, wheat germ agglutinin which has been useful in the purification of N-acetyl glucosaminyl residues and *Helix pomatia*
30 lectin. Lectins themselves are purified using affinity chromatography with carbohydrate ligands. Lactose has been used to purify lectins from castor bean and peanuts; maltose has been useful in extracting lectins from lentils and jack bean; N-acetyl-D galactosamine is used for purifying lectins from soybean; N-acetyl glucosaminyl binds to lectins from wheat germ;

D-galactosamine has been used in obtaining lectins from clams and L-fucose will bind to lectins from lotus.

The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide relatively tight binding. And it should be possible to elute the substance without destroying the sample or the ligand. One of the most common forms of affinity chromatography is immunoaffinity chromatography. The generation of antibodies that would be suitable for use in accord with the present invention is discussed below.

VII. Vectors for Cloning, Gene Transfer and Expression

Within certain embodiments expression vectors are employed to express a ERK1/2 polypeptide product, which can then be purified. In other embodiments, the expression vectors may be used in gene therapy. 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.

A. 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 certain embodiments, the native ERK1/2 promoter will be employed to drive expression of either the corresponding ERK1/2 gene, a heterologous ERK1/2 gene, a screenable or selectable marker gene, or any other gene of interest.

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 1 and Table 2). 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 1	
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

TABLE 1	
Promoter and/or Enhancer	
Promoter/Enhancer	References
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
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

TABLE 1	
Promoter and/or Enhancer	
Promoter/Enhancer	References
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, 1983a; Levinson <i>et al.</i> , 1982; Kriegler <i>et al.</i> , 1983b, 1984, 1988; Bosze <i>et al.</i> , 1986; Miksicsek <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; Laspia <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 2		
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>	ElA	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-2kb	Interferon	Blonar <i>et al.</i> , 1989
HSP70	ElA, 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
5 (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
10 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.

B. Selectable Markers

In certain embodiments of the invention, the cells contain nucleic acid constructs of
15 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
20 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
25 of skill in the art.

C. 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
30 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.

D. 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
5 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.
10 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
15 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
20 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.
25 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
30 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
5 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
10 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
15 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,
20 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
25 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
30 contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a gene of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag,

pol, and env genes but without the LTR and packaging components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

A novel approach designed to allow specific targeting of retrovirus vectors was recently developed based on the chemical modification of a retrovirus by the chemical addition of lactose residues to the viral envelope. This modification could permit the specific infection of hepatocytes via sialoglycoprotein receptors.

A different approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled via the biotin components by using streptavidin (Roux *et al.*, 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus in vitro (Roux *et al.*, 1989).

There are certain limitations to the use of retrovirus vectors in all aspects of the present invention. For example, retrovirus vectors usually integrate into random sites in the cell genome. This can lead to insertional mutagenesis through the interruption of host genes or through the insertion of viral regulatory sequences that can interfere with the function of flanking genes (Varmus *et al.*, 1981). Another concern with the use of defective retrovirus vectors is the potential appearance of wild-type replication-competent virus in the packaging cells. This can result from recombination events in which the intact- sequence from the recombinant virus inserts upstream from the gag, pol, env sequence integrated in the host cell genome. However, new packaging cell lines are now available that should greatly decrease the likelihood of recombination (Markowitz *et al.*, 1988; Hersdorffer *et al.*, 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
5 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*
10 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
15 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
20 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
25 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
30 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
5 to CD5 (CLL), CD22 (lymphoma), CD25 (T-cell leukemia) and MAA (melanoma) can similarly be used as targeting moieties.

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
10 animal. This may involve the surgical removal of tissue/organs from an animal or the primary culture of cells and tissues.

VIII. Methods of Making Transgenic Mice

A particular embodiment of the present invention provides transgenic animals that
15 express a heterologous ERK1/2 gene under the control of a promoter. Transgenic animals expressing a ERK1/2 encoding nucleic acid under the control of an inducible or a constitutive promoter, recombinant cell lines derived from such animals, and transgenic embryos may be useful in determining the exact role that ERK1/2 plays in the development and differentiation of cardiomyocytes and in the development of pathologic cardiac hypertrophy and heart
20 failure. Furthermore, these transgenic animals may provide an insight into heart development. The use of constitutively expressed ERK1/2 encoding nucleic acid provides a model for over- or unregulated expression. Also, transgenic animals which are “knocked out” for ERK1/2, in one or both alleles are contemplated.

In a general aspect, a transgenic animal is produced by the integration of a given
25 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; which is incorporated herein by reference), and Brinster *et al.*, 1985; which is incorporated herein by reference in its entirety).

Typically, a gene flanked by genomic sequences is transferred by microinjection into a
30 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.

DNA clones for microinjection can be prepared by any means known in the art. For example, DNA clones for microinjection can be cleaved with enzymes appropriate for removing the bacterial plasmid sequences, and the DNA fragments electrophoresed on 1% agarose gels in TBE buffer, using standard techniques. The DNA bands are visualized by staining with ethidium bromide, and the band containing the expression sequences is excised. The excised band is then placed in dialysis bags containing 0.3 M sodium acetate, pH 7.0. DNA is electroeluted into the dialysis bags, extracted with a 1:1 phenol:chloroform solution and precipitated by two volumes of ethanol. The DNA is redissolved in 1 ml of low salt buffer (0.2 M NaCl, 20 mM Tris, pH 7.4, and 1 mM EDTA) and purified on an Elutip-D™ column. The column is first primed with 3 ml of high salt buffer (1 M NaCl, 20 mM Tris, pH 7.4, and 1 mM EDTA) followed by washing with 5 ml of low salt buffer. The DNA solutions are passed through the column three times to bind DNA to the column matrix. After one wash with 3 ml of low salt buffer, the DNA is eluted with 0.4 ml high salt buffer and precipitated by two volumes of ethanol. DNA concentrations are measured by absorption at 260 nm in a UV spectrophotometer. For microinjection, DNA concentrations are adjusted to 3 µg/ml in 5 mM Tris, pH 7.4 and 0.1 mM EDTA. Other methods for purification of DNA for microinjection are described in in Palmiter *et al.* (1982); and in Sambrook *et al.* (2001).

In an exemplary microinjection procedure, female mice six weeks of age are induced to superovulate with a 5 IU injection (0.1 cc, ip) of pregnant mare serum gonadotropin (PMSG; Sigma) followed 48 hours later by a 5 IU injection (0.1 cc, ip) of human chorionic gonadotropin (hCG; Sigma). Females are placed with males immediately after hCG injection. Twenty-one hours after hCG injection, the mated females are sacrificed by CO₂ asphyxiation or cervical dislocation and embryos are recovered from excised oviducts and placed in Dulbecco's phosphate buffered saline with 0.5% bovine serum albumin (BSA; Sigma). Surrounding cumulus cells are removed with hyaluronidase (1 mg/ml). Pronuclear embryos are then washed and placed in Earle's balanced salt solution containing 0.5 % BSA (EBSS) in a 37.5°C incubator with a humidified atmosphere at 5% CO₂, 95% air until the time of injection. Embryos can be implanted at the two-cell stage.

Randomly cycling adult female mice are paired with vasectomized males. C57BL/6 or Swiss mice or other comparable strains can be used for this purpose. Recipient females are mated at the same time as donor females. At the time of embryo transfer, the recipient females are anesthetized with an intraperitoneal injection of 0.015 ml of 2.5 % avertin per gram of body weight. The oviducts are exposed by a single midline dorsal incision. An incision is then made through the body wall directly over the oviduct. The ovarian bursa is

then torn with watchmakers forceps. Embryos to be transferred are placed in DPBS (Dulbecco's phosphate buffered saline) and in the tip of a transfer pipet (about 10 to 12 embryos). The pipet tip is inserted into the infundibulum and the embryos transferred. After the transfer, the incision is closed by two sutures.

5

IX. Antibodies Reactive With ERK1/2

In another aspect, the present invention contemplates an antibody that is immunoreactive with a ERK1/2 molecule of the present invention, or any portion thereof. An antibody can be a polyclonal or a monoclonal antibody. In a preferred embodiment, an antibody is a monoclonal antibody. Means for preparing and characterizing antibodies are well known in the art (see, *e.g.*, Harlow and Lane, 1988).

Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide of the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically an animal used for production of anti-antisera is a non-human animal including rabbits, mice, rats, hamsters, pigs or horses. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

Antibodies, both polyclonal and monoclonal, specific for isoforms of antigen may be prepared using conventional immunization techniques, as will be generally known to those of skill in the art. A composition containing antigenic epitopes of the compounds of the present invention can be used to immunize one or more experimental animals, such as a rabbit or mouse, which will then proceed to produce specific antibodies against the compounds of the present invention. Polyclonal antisera may be obtained, after allowing time for antibody generation, simply by bleeding the animal and preparing serum samples from the whole blood.

It is proposed that the monoclonal antibodies of the present invention will find useful application in standard immunochemical procedures, such as ELISA and Western blot methods and in immunohistochemical procedures such as tissue staining, as well as in other procedures which may utilize antibodies specific to ERK1/2-related antigen epitopes.

In general, both polyclonal, monoclonal, and single-chain antibodies against ERK1/2 may be used in a variety of embodiments. A particularly useful application of such antibodies is in purifying native or recombinant ERK1/2, for example, using an antibody affinity column. The operation of all accepted immunological techniques will be known to those of skill in the art in light of the present disclosure.

Means for preparing and characterizing antibodies are well known in the art (see, e.g., Harlow and Lane, 1988; incorporated herein by reference). More specific examples of monoclonal antibody preparation are given in the examples below.

As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known in the art and include glutaraldehyde, *m*-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

As also is well known in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*), incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster, injection may also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate mAbs.

MAbs may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable animal with a selected immunogen composition, e.g., a purified or partially purified ERK1/2 protein, polypeptide or peptide or cell expressing high levels of ERK1/2. The immunizing composition is administered in a manner effective to stimulate antibody producing cells. Rodents such as mice and rats are preferred animals, however, the use of rabbit, sheep frog cells is also possible. The use of rats may provide certain advantages (Goding, 1986), but mice are preferred, with the BALB/c mouse being

most preferred as this is most routinely used and generally gives a higher percentage of stable fusions.

Following immunization, somatic cells with the potential for producing antibodies, specifically B-lymphocytes (B-cells), are selected for use in the mAb generating protocol.

5 These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the spleen of animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. 10 Typically, a spleen from an immunized mouse contains approximately 5×10^7 to 2×10^8 lymphocytes.

The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that 15 was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render them incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in 20 the art (Goding, 1986; Campbell, 1984). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, P3-X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with cell fusions.

25 Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 ratio, though the ratio may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described (Kohler and Milstein, 1975; 1976), and those 30 using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Geftter *et al.*, (1977). The use of electrically induced fusion methods is also appropriate (Goding, 1986).

Fusion procedures usually produce viable hybrids at low frequencies, around 1×10^{-6} to 1×10^{-8} . However, this does not pose a problem, as the viable, fused hybrids are

differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, *e.g.*, hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B-cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide mAbs. The cell lines may be exploited for mAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide mAbs in high concentration. The individual cell lines could also be cultured *in vitro*, where the mAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations. mAbs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography.

X. Definitions

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 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.

The term “treatment” or grammatical equivalents encompasses the improvement and/or reversal of the symptoms of heart failure (*i.e.*, the ability of the heart to pump blood). “Improvement in the physiologic function” of the heart may be assessed using any of the measurements described herein (*e.g.*, measurement of ejection fraction, fractional shortening, left ventricular internal dimension, heart rate, *etc.*), as well as any effect upon the animal’s survival. In use of animal models, the response of treated transgenic animals and untreated transgenic animals is compared using any of the assays described herein (in addition, treated and untreated non-transgenic animals may be included as controls). A compound which causes an improvement in any parameter associated with heart failure used in the screening methods of the instant invention may thereby be identified as a therapeutic compound.

The term “dilated cardiomyopathy” refers to a type of heart failure characterized by the presence of a symmetrically dilated left ventricle with poor systolic contractile function and, in addition, frequently involves the right ventricle.

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 term “agonist” refers to molecules or compounds which mimic the action of a “native” or “natural” compound. Agonists may be homologous to these natural compounds in respect to conformation, charge or other characteristics. Thus, agonists may be recognized by receptors expressed on cell surfaces. This recognition may result in physiologic and/or biochemical changes within the cell, such that the cell reacts to the presence of the agonist in the same manner as if the natural compound was present. Agonists may include proteins, nucleic acids, carbohydrates, or any other molecules that interact with a molecule, receptor, and/or pathway of interest.

As used herein, the term “cardiac hypertrophy” refers to the process in which adult cardiac myocytes respond to stress through hypertrophic growth. Such growth is characterized by cell size increases without cell division, assembling of additional sarcomeres within the cell to maximize force generation, and an activation of a fetal cardiac gene program. Cardiac hypertrophy is often associated with increased risk of morbidity and mortality, and thus studies aimed at understanding the molecular mechanisms of cardiac hypertrophy could have a significant impact on human health.

As used herein, the terms “antagonist” and “inhibitor” refer to molecules, compounds, or nucleic acids which inhibit the action of a cellular factor that may be involved in cardiac hypertrophy. 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 which 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 which bind or interact with a receptor, molecule, and/or pathway 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.

The term “ β -adrenergic receptor antagonist” refers to a chemical compound or entity that is capable of blocking, either partially or completely, the beta (β) type of adrenoreceptors (*i.e.*, receptors of the adrenergic system that respond to catecholamines, especially norepinephrine). Some β -adrenergic receptor antagonists exhibit a degree of specificity for one receptor subtype (generally β_1); such antagonists are termed “ β_1 -specific adrenergic receptor antagonists” and “ β_2 -specific adrenergic receptor antagonists.” The term “ β -adrenergic receptor antagonist” refers to chemical compounds that are selective and non-selective antagonists. Examples of β -adrenergic receptor antagonists include, but are not limited to, acebutolol, atenolol, butoxamine, carteolol, esmolol, labetalol, metoprolol, nadolol, penbutolol, propranolol, and timolol. The use of derivatives of known β -adrenergic receptor antagonists is encompassed by the methods of the present invention. Indeed any compound, which functionally behaves as a β -adrenergic receptor antagonist is encompassed by the methods of the present invention.

The terms “angiotensin-converting enzyme inhibitor” or “ACE inhibitor” refer to a chemical compound or entity that is capable of inhibiting, either partially or completely, the enzyme involved in the conversion of the relatively inactive angiotensin I to the active angiotensin II in the rennin-angiotensin system. In addition, the ACE inhibitors concomitantly inhibit the degradation of bradykinin, which likely significantly enhances the antihypertensive effect of the ACE inhibitors. Examples of ACE inhibitors include, but are not limited to, benazepril, captopril, enalapril, fosinopril, lisinopril, quinopril and ramipril. The use of derivatives of known ACE inhibitors is encompassed by the methods of the present invention. Indeed any compound, which functionally behaves as an ACE inhibitor, is encompassed by the methods of the present invention.

As used herein, the term “genotypes” refers to the actual genetic make-up of an organism, while “phenotype” refers to physical traits displayed by an individual. In addition, the “phenotype” is the result of selective expression of the genome (*i.e.*, it is an expression of the cell history and its response to the extracellular environment). Indeed, the human genome contains an estimated 30,000-35,000 genes. In each cell type, only a small (*i.e.*, 10-15%) fraction of these genes are expressed.

XI. Examples

The following examples are included to further illustrate various aspects 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 and/or compositions 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
5 embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1: Materials and Methods

Materials. Isoproterenol (Sigma) was dissolved in sterile H₂O containing 1 mmol/L Ascorbic Acid, and the concentrations used are described in the results section. Additional
10 reagents were added to the cells with the following final concentrations: 2 µmol/L KN-93 (Sigma), 0.8 µmol/L 14-22 Amide myristoylated (Calbiochem), 1 µmol/L Nicarpidine HCl (Sigma), 30 µmol/L 2-APB (Cayman Chemicals), 1 µmol/L cyclosporine A (Sigma), 10 µmol/L U0126 (Promega).

Cell Culture. Neonatal rat cardiac ventricular myocytes (NRVMs) were cultured as
15 previously described (Simpson *et al.*, 1982; Simpson and Savion, 1982; Long *et al.*, 1992; Waspe *et al.*, 1990). Briefly, cells were isolated by trypsin digestion from the ventricles of 1-3 day old rats. Isolation procedures were done in a very gentle manner and cells were plated at a concentration of 1.5×10^5 cells per well in 12-well tissue culture plates in medium containing serum. 24 hours later the media was changed to serum free media containing
20 insulin, transferin, bovine serum albumin, vitamin B12 and penicillin. Wells were pre-coated with a 0.1% gelatin solution and dried in a hood under UV lights. In addition, all media solutions were supplemented with Hepes (pH 7.5) to a final concentration of 20 mmol/L in order to buffer the pH of the media.

RNase Protection Assay. Total RNA was extracted by TRIZol (Invitrogen) and used
25 in RNase protection assays (RPA). RPAs were performed essentially as described (Patten *et al.*, 1996; Kinugawa *et al.*, 2001). Briefly, 5 µg of total RNA was hybridized against probes specific to skeletal α-actin, SRCA2A, α-MyHC, β-MyHC, ANF, BNP and GAPDH. RNase protection experiments were performed using the RPAII kit (Ambion). RPA experiments were performed from mRNA extracted from a minimum of three different experiments.

ERK1/2 Western Blots. Phosphorylated ERK1/2 protein extraction was performed
30 essentially as described in Headley *et al.* (2004).

Example 2: Results

Role of CaMK in isoproterenol mediated changes in promoter activity and mRNA expression. CaMK has been implicated in the regulation of the hypertrophic markers skeletal α -actin, BNP, ANP and β -MyHC (reviewed in Zhang *et al.*, 2004). To determine if CaMKII plays a role in the changes in gene expression mediated by β_1 -AR in NRVMs, cells were pre-treated with the CaMKII inhibitor KN-93. Inhibition of CaMKII by KN-93 prevented isoproterenol-induced changes in endogenous mRNA fetal gene program induction (FIG. 1). More specifically, KN-93 pre-treatment prevented isoproterenol-associated down-regulation of α -MyHC or up-regulation of skeletal α -actin and natriuretic peptides. However, inhibition of CaMK was not capable of preventing repression of SRCA2a, suggesting that SRCA2a gene repression is regulated by a different pathway. Surprisingly, KN-93 alone resulted in a decrease in ANP, β -MyHC, and SRCA2a mRNA levels.

Inhibition of calcineurin blocks isoproterenol-mediated repression of α -MyHC and SRCA2a. The phosphatase calcineurin has been shown to be both necessary and sufficient for the development of cardiac hypertrophy in a variety of animal models (for review see Wilkins *et al.*, 2004). Calcineurin plays an important role in isoproterenol mediated hypertrophy; in calcineurin deficient animals infused with isoproterenol hypertrophy is inhibited (Bueno *et al.*, 2002). Calcineurin dephosphorylates the transcription factor NFAT, allowing its translocation to the nucleus where NFAT activates a variety of genes involved in the hypertrophic response (Wilkins *et al.*, 2004). The inventors next sought to determine the role of calcineurin in β_1 -AR mediated hypertrophy. As shown in FIG. 2, the calcineurin inhibitor cyclosporine blocks isoproterenol-mediated repression of the adult isoforms, α -MyHC and SRCA2a and induction of the fetal gene ANP. Inhibition of calcineurin with cyclosporine also decreases skeletal α -actin up-regulation.

ERK1/2 inhibition blocks isoproterenol mediated fetal gene induction. The involvement of ERK1/2 in the development of cardiac hypertrophy has been well documented (Bogoyevitch *et al.*, 1996; Yue *et al.*, 2000; Glennon *et al.*, 1996), and over expression of MEK1 (upstream activator of ERK1/2) in a transgenic animal model results in profound cardiac hypertrophy accompanied by repression of α -MyHC and activation of β -MyHC, ANP, BNP and skeletal α -actin (Bueno *et al.*, 2000). It has recently been shown that CaMK is involved in ERK1/2 activation (Illario *et al.*, 2003; Borbiev *et al.*, 2003) in non-myocytes and that calcineurin is involved in ERK activation in cardiac myocytes (Zou *et al.*, 2001), making it a potential candidate for the involvement in the isoproterenol-mediated activation of

the fetal program. In order to determine if the ERK1/2 pathway contributed to the β -AR response, cells were pre-treated with the MEK1/2 inhibitor U0126 one hour prior to isoproterenol stimulation. As shown in FIG. 3, ERK1/2 inhibition does not prevent isoproterenol-associated down-regulation of SRCA2a but blocks isoproterenol-related repression of α -MyHC and up-regulation of ANP, BNP and skeletal α -actin.

ERK1/2 activation is dependent on calcineurin and on CaMKII. In cardiac myocytes ERK1/2 activation has been shown to be dependent on calcineurin (Zou *et al.*, 2001), while in other cell types CaMKII has been shown to be involved in ERK1/2, activation (Zolk *et al.*, 2003). In order to determine which pathway is involved in the activation of ERK1/2, NRVMs were pre-treated with the CaMKII inhibitor KN-93, the calcineurin inhibitor cyclosporine, the PKA inhibitor peptide PKI₁₄₋₂₂ and the MEK inhibitor U0126 followed by 5, 30, 60 minutes and 6, 24, 33 and 48 hours of isoproterenol stimulation. Surprisingly, in NRVMs ERK phosphorylation as a surrogate marker for activation is increased in response to isoproterenol stimulation at three different time points, 5 minutes, 33 hours and 48 hours (FIG. 4A). MEK inhibition completely abrogates ERK activation at all time points (data not shown), and calcineurin inhibition decreased the isoproterenol increase in ERK1/2 activation at the 5 minutes time point only (FIG. 4B). Inhibition of PKA did not prevent isoproterenol activation of ERK1/2 at any time point. Interestingly, CaMKII inhibition blocked activation of ERK1/2 in response to isoproterenol only at the 33 and 48 hour time points (FIG. 4C).

Isoproterenol-activation of the FGP is mediated by the L-Type Ca^{2+} channel (LTCC) and Transient Receptor Potential channels (TRPC). The results presented above suggest that Ca^{2+} is an important mediator of fetal gene activation in response to β_1 -AR stimulation, since calcineurin, CaMKII and ERK1/2 are all activated by Ca^{2+} (Wilkins and Molkentin, 2004; Anderson, 2004; Agell *et al.*, 2002). β_1 -AR signaling has been shown to activate LTCCs through $G_{s\alpha}$ (Lader *et al.*, 1998), and CaMKII has been shown to activate LTCCs (Anderson, 2004), making them a likely candidate for Ca^{2+} regulation in response to β_1 -AR activation. Recently, TRPC3 has been implicated in the calcineurin mediated response in skeletal muscle (Rosenberg *et al.*, 2004), and since the results demonstrated that inhibition of calcineurin also blocks activation of the fetal gene program, the inventors hypothesized that the TRPCs were involved in β_1 -AR mediated gene expression responses. In order to determine if either of these types of channels participates in the isoproterenol-mediated activation of the fetal gene program, NRVMs were pre-treated with the LTCC inhibitor

nicardipine and the TRPC channel inhibitor AFB-30. As shown in FIG. 5, inhibition of LTCCs with nicardipine prevented down-regulation of α -MyHC and SRCA2a and up-regulation of ANP, BNP and skeletal α -actin. Interestingly, inhibition of LTCC increased up-regulation of β -MyHC in response to isoproterenol stimulation. Strikingly, inhibition of TRPC channels completely abrogated all changes in fetal gene expression in response to isoproterenol stimulation (FIG. 6).

Example 3: Materials and Methods

Cell Culture, Transfection and Infection: Neonatal rat cardiac myocytes (NRVMs) were prepared according to the method described in Waspe *et al.* (1990). Briefly, 2,000,000 cells were plated in 100 mm tissue culture plates coated with gelatin. Eighteen hours later, the media was changed to MEM supplemented with Hank's salt and L-glutamine, 20 mM Hepes pH 7.5, Penicillin, Vitamin B12, BSA, insulin and transferin. Cells were infected with an adenovirus expressing YY1-GFP and/or HDAC5-Flag or with a control adenovirus at a MOI of 7 pfu/cell.

RNase Protection Assay: Total RNA was extracted by TRIZol (Invitrogen) and used in RNase protection assays (RPA). RPAs were performed essentially as described (Patten *et al.*, 1996; Kinuguwa *et al.*, 2001). Briefly, 5 μ g of total RNA was hybridized against probes specific to skeletal α -actin, SRCA2A, α -MyHC, β -MyHC, ANF, BNP and GAPDH. RNase protection experiments (RPAs) were performed using the RPAII kit (Ambion). RPA experiments were performed from mRNA extracted from a minimum of three different experiments.

Incorporation of ^3H -leucine: General cell growth was measured by the incorporation of ^{14}C -leucine as described previously (Maass *et al.*, 1995). At the time of treatment of cells with phenylephrine, 1 μCi of ^{14}C -leucine was added to each well (12-well plates) of NRVMs. After 48 hours, plates were washed three times with ice-cold PBS. The cells were precipitated on ice with 5% TCA. Proteins were then solubilized by the addition of 0.1% SDS and incubation at 37°C on a rocker for 20 minutes. ^{14}C -leucine incorporation was measured by scintillation counter.

Nuclear and Cytoplasmic fractionation: Nuclear and cytoplasmic fractionation were performed using the NE-PER kit (Pierce) according to manufacture's recommendation.

Western Blots: Western Blots were performed essentially as described (Sucharov *et al.*, 2003). YY1 or Flag antibody were diluted 1:1000 in 1X TBS (20 mM Tris 500mM NaCl pH 7.5) containing 3% BSA and 0.1% tween and incubated with the blot overnight at 4°C. The mouse secondary antibody conjugated to HRP was diluted 1:10000 in 1X TBS containing 5% low fat dry milk and 0.1% tween and incubated with the blot for 1 hour at room temperature.

Immunoprecipitation/immunoblotting: Immunoprecipitation experiments were done using Flag antibody. Experiments were done according to Santa Cruz Biotech. recommendations with minor modifications. After 4 washes with 1X RIPA buffer (Calalb *et al.*, 1995), the sample was incubated with 2-3X packed volume with 2X sample buffer (Bio-Rad) at room temperature for 30 minutes. β -mercaptanol was added to the supernatant after centrifugation and samples were loaded without boiling. Western blot experiments were performed as described above.

Immunofluorescence: Immunofluorescence was done according to Harrison *et al.* (2004). Cells were washed with TBST and fixed with 10% formaldehyde for 20 minutes. Cells were again washed with TBST and incubate with 0.1% Triton-X for 30 minutes. Cells were then blocked with 1% BSA in TBST for 1 hour followed by 1 hour incubation with 1:500 dilution of the Flag antibody. Cells were then washed with TBST and incubated with 1:1000 dilution of Alexa 594 anti-mouse antibody and 2 μ g/ml Hoechst staining for 1 hour. Cells were washed three times with TBST and one time with water and sequentially covered with mounting solution (Southern Biotech) and glass coverslips. Images were captured at a 20X magnification with a fluorescence microscope (Nikon E800) equipped with a digital camera (Zeis AxioCam) and Zeis Axiovision ver. 3.0.6.36 imaging software.

Statistical Analysis: All analyses were performed using ANOVA, with a $p < 0.05$ in a two-tailed distribution considered to be statistically significant.

Example 4: Results

Fetal gene expression is regulated by the late ERK1/2 phosphorylation time point. To test the biological significance of the later time point of ERK1/2 phosphorylation, NRVMs were pre-treated with the MEK1 inhibitor U0126 1 hour prior to ISO treatment (FIG. 8A), or with ISO for 1 hour followed by U0126 treatment (FIG. 8B). U0126 prevents up-regulation of the fetal isoforms and down-regulation of α MyHC in NRVMs before and after

ISO treatment. Inhibition of MEK1 1 hour after ISO treatment prevents fetal gene expression, suggesting that the first peak of ERK1/2 activation is not enough to induce ISO-mediated changes in gene expression.

YY1 blocks ERK1/2 phosphorylation at the later time point. Since ISO activation of the fetal gene program is blocked by ERK1/2 inhibition and YY1 up-regulation, the inventors tested if YY1 can prevent ERK1/2 phosphorylation. As shown in FIG. 9, YY1 prevents ERK1/2 phosphorylation in response to ISO but not to PE, suggesting that the mechanisms involved in YY1 repression of β_1 -AR mediated pathologic hypertrophy involve blockade of ERK1/2 phosphorylation. It is interesting that YY1 effect is specific for ISO and does not affect PE-mediated ERK1/2 phosphorylation. ISO stimulation of ERK1/2 at a later time point is far more robust than PE. The inventors also showed that ERK1/2 phosphorylation in response to ISO at a later time point is dependent on CaMKII. The inventors hypothesize that YY1 represses ERK1/2 phosphorylation by down-regulating gene expression of a kinase.

YY1 interaction with GATA4 depends on ERK1/2 phosphorylation status. Interaction of YY1 and GATA4 has been shown by others (Bhalla *et al.*, 2001). Since GATA4 is a transcription activator and YY1 is a transcription repressor, our hypothesis is that in NRVMs, YY1 interaction with GATA4 results in inhibition of GATA4 activation function. GATA4 is phosphorylated/activated by ERK1/2, and the inventors hypothesized that YY1 and GATA4 interact only in the absence of ERK1/2 phosphorylation. ERK1/2 phosphorylation and consequently GATA4 activation would result in dissociation of YY1 from GATA4. To test this hypothesis, the inventors infected COS7 cells with the GATA4 and YY1 adenovirus constructs. Cells were either transfected with a constitutively active MEK1 construct or with a control DNA. As shown in FIG. 10A ERK1/2 phosphorylation is only seen in cells transfected with MEK1 cDNA construct, and in FIG. 10B the inventors show that interaction of YY1 and GATA4 is dependent on ERK1/2 phosphorylation status; YY1 strongly interacts with GATA4 in the absence of ERK1/2 phosphorylation, but this interaction is abolished in the presence of ERK1/2 phosphorylation. These results suggest that activation of ERK1/2 by ISO is an important regulator of GATA4, and that YY1 repression of GATA4 function might occur through interaction with GATA4 and through repression of ERK1/2 phosphorylation.

* * * * *

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods, and in the steps or in the sequence of steps of 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.

XII. 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 4,196,265
U.S. Patent 4,415,723
U.S. Patent 4,458,066
U.S. Patent 4,873,191
U.S. Patent 5,354,855
- 10 U.S. Patent 5,604,251
U.S. Patent 5,795,715
U.S. Patent 5,889,136
U.S. Prov. Appln. Serial No. 60/701,788

- 15 Agell *et al.*, *Cell Signal.*, 14:649-654, 2002.
Anderson, *Trends Cardiovasc. Med.*, 14:152-161, 2004.
Angel *et al.*, *Cell*, 49:729, 1987b.
Angel *et al.*, *Mol. Cell. Biol.*, 7:2256, 1987a.
Atchison and Perry, *Cell*, 46:253, 1986.
- 20 Atchison and Perry, *Cell*, 48:121, 1987.
Backs *et al.*, *J. Clin. Invest.*, 116(7):1853-1864, 2006.
Baichwal and Sugden, In: *Gene Transfer*, Kucherlapati (Ed), NY, Plenum Press, 117-148, 1986.
Banerji *et al.*, *Cell*, 27(2 Pt 1):299-308, 1981.
Banerji *et al.*, *Cell*, 33(3):729-740, 1983.
- 25 Barnes *et al.*, *J. Biol. Chem.*, 272(17):11510-7, 1997.
Benvenisty and Neshif, *Proc. Natl. Acad. Sci. USA*, 83:9551-9555, 1986.
Berkhout *et al.*, *Cell*, 59:273-282, 1989.
Bhalla *et al.*, *J. Biol. Chem.*, 276(14):11439-11445, 2001.
Bhavsar *et al.*, *Genomics*, 35(1):11-23, 1996.
- 30 Blonar *et al.*, *EMBO J.*, 8:1139, 1989.
Bodine and Ley, *EMBO J.*, 6:2997, 1987.
Bogoyevitch *et al.*, *Biochem. J.*, 314:115-121, 1996.
Boluyt *et al.*, *Am. J. Physiol.*, 269:H638-647, 1995.

- Bonow, *Curr. Treat. Options Cardiovasc. Med.*, 2:125-132, 2000.
- Borbiev *et al.*, *Am. JU. Physiol. Lung Cell Mol. Physiol.*, 285:L43-54, 2003.
- Boshart *et al.*, *Cell*, 41:521, 1985.
- Bosher and Labouesse, *Nat. Cell. Biol.*, 2:E31-E36, 2000.
- 5 Bosze *et al.*, *EMBO J.*, 5(7):1615-1623, 1986.
- Braddock *et al.*, *Cell*, 58:269, 1989.
- Brinster *et al.*, *Proc. Natl. Acad. Sci. USA*, 82(13):4438-4442, 1985.
- Bristow, *Am. J. Cardiol.*, 80:261-401, 1997.
- Bristow, *Cardiology*, 92:3-6, 1999.
- 10 Bueno *et al.*, *EMBO J.*, 19:6341-6350, 2000.
- Bueno *et al.*, *Proc. Natl. Acad. Sci. USA*, 99:4586-4591, 2002.
- Bulla and Siddiqui, *J. Virol.*, 62:1437, 1986.
- Calalb *et al.*, *Mol. Cell Biol.*, 15:954-963, 1995.
- Campbell and Villarreal, *Mol. Cell. Biol.*, 8:1993, 1988.
- 15 Campbell *et al.*, *J. Mol. Biol.*, 180:1-19, 1984.
- Campere and Tilghman, *Genes and Dev.*, 3:537, 1989.
- Campo *et al.*, *Nature*, 303:77, 1983.
- Capaldi *et al.*, *Biochem. Biophys. Res. Comm.*, 76:425-433, 1977.
- Caplen *et al.*, *Gene*, 252(1-2):95-105, 2000.
- 20 Celander and Haseltine, *J. Virology*, 61:269, 1987.
- Celander *et al.*, *J. Virology*, 62:1314, 1988.
- Chandler *et al.*, *Cell*, 33:489, 1983.
- Chang *et al.*, *Hepatology*, 14:124A, 1991.
- Chang *et al.*, *Mol. Cell. Biol.*, 9:2153, 1989.
- 25 Chatterjee *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:9114, 1989.
- Chen and Okayama, *Mol. Cell Biol.*, 7:2745-2752, 1987.
- Choi *et al.*, *Cell*, 53:519, 1988.
- Coffin, In: *Virology*, Fields *et al.* (Eds.), Raven Press, NY, 1437-1500, 1990.
- Cohen *et al.*, *J. Cell. Physiol.*, 5:75, 1987.
- 30 Cook *et al.*, *Cell*, 27:487-496, 1981.
- Costa *et al.*, *Mol. Cell. Biol.*, 8:81, 1988.
- Couch *et al.*, *Am. Rev. Resp. Dis.*, 88:394-403, 1963.
- Coupar *et al.*, *Gene*, 68:1-10, 1988.
- Cripe *et al.*, *EMBO J.*, 6:3745, 1987.

- Culotta and Hamer, *Mol. Cell. Biol.*, 9:1376, 1989.
- Dandolo *et al.*, *J. Virology*, 47:55-64, 1983.
- De Villiers *et al.*, *Nature*, 312(5991):242-246, 1984.
- Deschamps *et al.*, *Science*, 230:1174-1177, 1985.
- 5 Devereux and Roman, *Hypertens. Res.*, 22:1-9, 1999.
- Dolmetsch *et al.*, *Nature*, 386:855-858, 1997.
- Dubensky *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:7529-7533, 1984.
- Dunnmon *et al.*, *J. Mol. Cell. Cardiol.*, 22:901-10, 1990.
- Durand *et al.*, *Ann. Med.*, 27:311-317, 1995.
- 10 Edbrooke *et al.*, *Mol. Cell. Biol.*, 9:1908, 1989.
- Edlund *et al.*, *Science*, 230:912-916, 1985.
- Eichhorn and Bristow, *Circulation*, 94:2285-2296, 1996.
- Elbashir *et al.*, *Nature*, 411(6836):494-498, 2001.
- Emmel *et al.*, *Science*, 246:1617-1620, 1989.
- 15 EP 0273085
- Esler *et al.*, *Am. J. Cardiol.*, 80:7L-14L, 1997.
- Fechheimer *et al.*, *Proc Natl. Acad. Sci. USA*, 84:8463-8467, 1987.
- Feng and Holland, *Nature*, 334:6178, 1988.
- Ferkol *et al.*, *FASEB J.*, 7:1081-1091, 1993.
- 20 Firak and Subramanian, *Mol. Cell. Biol.*, 6:3667, 1986.
- Fire *et al.*, *Nature*, 391:806-811, 1998.
- Foecking and Hofstetter, *Gene*, 45(1):101-105, 1986.
- Forster and Symons, *Cell*, 49:211-220, 1987.
- Fraley *et al.*, *Proc Natl. Acad. Sci. USA*, 76:3348-3352, 1979
- 25 Franz *et al.*, *Cardoscience*, 5(4):235-43, 1994.
- Friedmann, *Science*, 244:1275-1281, 1989.
- Fujita *et al.*, *Cell*, 49:357, 1987.
- Gefter, *et al.*, *Somatic Cell Genet.*, 3:231-236, 1977.
- Gerlach *et al.*, *Nature (London)*, 328:802-805, 1987.
- 30 Ghosh and Bachhawat, In: *Liver Diseases, Targeted Diagnosis and Therapy Using Specific Receptors and Ligands*, Wu *et al.* (Eds.), Marcel Dekker, NY, 87-104, 1991.
- Ghosh-Choudhury *et al.*, *EMBO J.*, 6:1733-1739, 1987.
- Gilles *et al.*, *Cell*, 33:717, 1983.
- Glennon *et al.*, *Circ. Res.*, 78:954-961, 1996.

- Gloss *et al.*, *EMBO J.*, 6:3735, 1987.
- Godbout *et al.*, *Mol. Cell. Biol.*, 8:1169, 1988.
- Goding, In: *Monoclonal Antibodies: Principles and Practice*, 2d ed., Orlando, Fla., Academic Press, 60-61, 65-66, 71-74, 1986.
- 5 Gomez-Foix *et al.*, *J. Biol. Chem.*, 267:25129-25134, 1992.
- Goodbourn and Maniatis, *Proc. Natl. Acad. Sci. USA*, 85:1447, 1988.
- Goodbourn *et al.*, *Cell*, 45:601, 1986.
- Gopal, *Mol. Cell Biol.*, 5:1188-1190, 1985.
- Gopal-Srivastava *et al.*, *J. Mol. Cell. Biol.*, 15(12):7081-90, 1995.
- 10 Graham and Prevec, In: *Methods in Molecular Biology: Gene Transfer and Expression Protocol*, Murray (Ed.), Humana Press, Clifton, NJ, 7:109-128, 1991.
- Graham and van der Eb, *Virology*, 52:456-467, 1973.
- Graham *et al.*, *J. Gen. Virol.*, 36:59-72, 1977.
- Greene *et al.*, *Immunology Today*, 10:272, 1989
- 15 Grishok *et al.*, *Science*, 287:2494-2497, 2000.
- Grosschedl and Baltimore, *Cell*, 41:885, 1985.
- Grunhaus and Horwitz, *Seminar in Virology*, 3:237-252, 1992.
- Gruver *et al.*, *Endocrinology*, 133:376-388, 1993.
- Harland and Weintraub, *J. Cell Biol.*, 101:1094-1099, 1985.
- 20 Harlow and Lane, *Antibodies: A Laboratory manual*, Cold Spring Harbor Laboratory, 1988.
- Harrison *et al.*, *Mol. Cell Biol.*, 24:10636-10649, 2004.
- Haslinger and Karin, *Proc. Natl. Acad. Sci. USA*, 82:8572, 1985.
- Hauber and Cullen, *J. Virology*, 62:673, 1988.
- Headley *et al.*, *Mol. Cell Biochem.*, 258:109-119, 2004.
- 25 Hen *et al.*, *Nature*, 321:249, 1986.
- Hensel *et al.*, *Lymphokine Res.*, 8:347, 1989.
- Hermonat and Muzyczska, *Proc. Nat'l Acad. Sci. USA*, 81:6466-6470, 1984.
- Herr and Clarke, *Cell*, 45:461, 1986.
- Hersdorffer *et al.*, *DNA Cell Biol.*, 9:713-723, 1990.
- 30 Hirochika *et al.*, *J. Virol.*, 61:2599, 1987.
- Hirsch *et al.*, *Mol. Cell. Biol.*, 10:1959, 1990.
- Holbrook *et al.*, *Virology*, 157:211, 1987.
- Horlick and Benfield, *Mol. Cell. Biol.*, 9:2396, 1989.
- Horwich *et al.*, *J. Virol.*, 64:642-650, 1990.

- Huang *et al.*, *Cell*, 27:245, 1981.
- Hug *et al.*, *Mol. Cell. Biol.*, 8:3065, 1988.
- Hwang *et al.*, *Mol. Cell. Biol.*, 10:585, 1990.
- Illario *et al.*, *J. Biol. Chem.*, 278:45101-45108, 2003.
- 5 Imagawa *et al.*, *Cell*, 51:251, 1987.
- Imbra and Karin, *Nature*, 323:555, 1986.
- Imler *et al.*, *Mol. Cell. Biol.*, 7:2558, 1987.
- Imperiale and Nevins, *Mol. Cell. Biol.*, 4:875, 1984.
- Iwanaga *et al.*, *Circulation*, 104:606-612, 2001.
- 10 Jakobovits *et al.*, *Mol. Cell. Biol.*, 8:2555, 1988.
- Jameel and Siddiqui, *Mol. Cell. Biol.*, 6:710, 1986.
- Jaynes *et al.*, *Mol. Cell. Biol.*, 8:62, 1988.
- Johnson *et al.*, *Mol. Cell. Biol.*, 9:3393, 1989.
- Jones and Shenk, *Cell*, 13:181-188, 1978.
- 15 Joyce, *Nature*, 338:217-244, 1989.
- Kadesch and Berg, *Mol. Cell. Biol.*, 6:2593, 1986.
- Kaneda *et al.*, *Science*, 243:375-378, 1989.
- Karin *et al.*, *Mol. Cell. Biol.*, 7:606, 1987.
- Karlsson *et al.*, *EMBO J.*, 5:2377-2385, 1986.
- 20 Katinka *et al.*, *Cell*, 20:393, 1980.
- Katinka *et al.*, *Nature*, 290:720, 1981.
- Kato *et al.*, *J Biol Chem.*, 266(6):3361-3364, 1991.
- Kawamoto *et al.*, *Mol. Cell. Biol.*, 8:267, 1988.
- Kelly *et al.*, *J. Cell Biol.*, 129(2):383-96, 1995.
- 25 Ketting *et al.*, *Cell*, 99:133-141, 1999.
- Kiledjian *et al.*, *Mol. Cell. Biol.*, 8:145, 1988.
- Kim and Cook, *Proc. Natl. Acad. Sci. USA*, 84:8788-8792, 1987.
- Kimura *et al.*, *Dev. Growth Differ.*, 39(3):257-65, 1997.
- Kinugawa *et al.*, *Circ. Res.*, 89:591-598, 2001.
- 30 Klaassen, In: *The Pharmacological Basis of Therapeutics*, Goodman and Gilman, Eds.,
Pergamon Press, 8th Ed., 49-61, 1990.
- Klamut *et al.*, *Mol. Cell. Biol.*, 10:193, 1990.
- Klein *et al.*, *Nature*, 327:70-73, 1987.
- Koch *et al.*, *Mol. Cell. Biol.*, 9:303, 1989.

- Kohler and Milstein, *Eur. J. Immunol.*, 6:511-519, 1976.
- Kohler and Milstein, *Nature*, 256:495-497, 1975.
- Kono *et al.*, *J. Am. Coll. Cardiol.*, 20:1594-1598, 1992.
- Kriegler and Botchan, In: *Eukaryotic Viral Vectors*, Gluzman (Ed.), Cold Spring Harbor:
5 Cold Spring Harbor Laboratory, NY, 1982.
- Kriegler and Botchan, *Mol. Cell. Biol.*, 3:325, 1983a.
- Kriegler *et al.*, *Cell*, 38:483, 1984.
- Kriegler *et al.*, *Cell*, 53:45, 1988.
- Kriegler *et al.*, In: *Gene Expression*, Alan Liss (Ed.), Hamer and Rosenberg, New York,
10 1983b.
- Kudoh *et al.*, *Circ. Res.*, 80:139-146, 1997.
- Kuhl *et al.*, *Cell*, 50:1057, 1987.
- Kunz *et al.*, *Nucl. Acids Res.*, 17:1121, 1989.
- Lader *et al.*, *Proc. Natl. Acad. Sci. USA*, 95:9669-9674, 1998.
- 15 LaPointe *et al.*, *J. Biol. Chem.*, 263(19):9075-8, 1988.
- Larsen *et al.*, *Proc. Natl. Acad. Sci. USA*, 83:8283, 1986.
- Laspia *et al.*, *Cell*, 59:283, 1989.
- Latimer *et al.*, *Mol. Cell. Biol.*, 10:760, 1990.
- Le Gal La Salle *et al.*, *Science*, 259:988-990, 1993.
- 20 Lee *et al.*, *Nature*, 294:228, 1981.
- Levinson *et al.*, *Nature*, 295:79, 1982.
- Levrero *et al.*, *Gene*, 101:195-202, 1991.
- Lin and Avery, *Nature*, 402:128-129, 1999.
- Lin *et al.*, *Mol. Cell. Biol.*, 10:850, 1990.
- 25 Lompre *et al.*, *Nature*, 282:105-107, 1979.
- Long *et al.*, *Basic Res. Cardiol.*, 87(2):19-31, 1992.
- Lowes *et al.*, *N. Engl. J. Med.*, 346:1357-1365, 2002.
- Luria *et al.*, *EMBO J.*, 6:3307, 1987.
- Lusky and Botchan, *Proc. Natl. Acad. Sci. USA*, 83:3609, 1986.
- 30 Lusky *et al.*, *Mol. Cell. Biol.*, 3:1108, 1983.
- Maass *et al.*, *Eur. Heart J.*, 16(C):12-14, 1995.
- Macejak and Sarnow, *Nature*, 353:90-94, 1991.
- Majors and Varmus, *Proc. Natl. Acad. Sci. USA*, 80:5866, 1983.
- Mann *et al.*, *Cell*, 33:153-159, 1983.

- Markowitz *et al.*, *J. Virol.*, 62:1120-1124, 1988.
- McNeall *et al.*, *Gene*, 76:81, 1989.
- Michel and Westhof, *J. Mol. Biol.*, 216:585-610, 1990.
- Miksicek *et al.*, *Cell*, 46:203, 1986.
- 5 Montgomery *et al.*, *Proc. Natl. Acad. Sci. USA*, 95:1552-15507, 1998.
- Mordacq and Linzer, *Genes and Dev.*, 3:760, 1989.
- Moreau *et al.*, *Nucl. Acids Res.*, 9:6047, 1981.
- Moss *et al.*, *J. Gen. Physiol.*, 108(6):473-84, 1996.
- Muesing *et al.*, *Cell*, 48:691, 1987.
- 10 Ng *et al.*, *Nuc. Acids Res.*, 17:601, 1989.
- Nicolas and Rubinstein, In: *Vectors: A survey of molecular cloning vectors and their uses*, Rodriguez and Denhardt (Eds.), Stoneham: Butterworth, 494-513, 1988.
- Nicolau and Sene, *Biochim. Biophys. Acta*, 721:185-190, 1982.
- Nicolau *et al.*, *Methods Enzymol.*, 149:157-176, 1987.
- 15 Olson and Williams, *Cell*, 101:689-692, 2000.
- Ondek *et al.*, *EMBO J.*, 6:1017, 1987.
- Ornitz *et al.*, *Mol. Cell. Biol.*, 7:3466, 1987.
- Palmiter *et al.*, *Cell*, 29:701, 1982.
- Palmiter *et al.*, *Nature*, 300:611, 1982.
- 20 Paskind *et al.*, *Virology*, 67:242-248, 1975.
- Patten *et al.*, *J. Biol. Chem.*, 271:21134-21141, 1996.
- Pech *et al.*, *Mol. Cell. Biol.*, 9:396, 1989.
- Pelletier and Sonenberg, *Nature*, 334:320-325, 1988.
- Perales *et al.*, *Proc. Natl. Acad. Sci. USA*, 91(9):4086-4090, 1994.
- 25 Perez-Stable and Constantini, *Mol. Cell. Biol.*, 10:1116, 1990.
- Physicians Desk Reference.
- Picard and Schaffner, *Nature*, 307:83, 1984.
- Pinkert *et al.*, *Genes and Dev.*, 1:268, 1987.
- Ponta *et al.*, *Proc. Natl. Acad. Sci. USA*, 82:1020, 1985.
- 30 Porton *et al.*, *Mol. Cell. Biol.*, 10:1076, 1990.
- Potter *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:7161-7165, 1984.
- Queen and Baltimore, *Cell*, 35:741, 1983.
- Quinn *et al.*, *Mol. Cell. Biol.*, 9:4713, 1989.
- Racher *et al.*, *Biotech. Techniques*, 9:169-174, 1995.

- Ragot *et al.*, *Nature*, 361:647-650, 1993.
- Redondo *et al.*, *Science*, 247:1225, 1990.
- Reinhold-Hurek and Shub, *Nature*, 357:173-176, 1992.
- Reisman and Rotter, *Mol. Cell. Biol.*, 9:3571, 1989.
- 5 Remington's Pharmaceutical Sciences, 15th ed., 1035-1038 and 1570-1580, Mack Publishing Company, PA, 1980.
- Renan, *Radiother. Oncol.*, 19:197-218, 1990.
- Resendez Jr. *et al.*, *Mol. Cell. Biol.*, 8:4579, 1988.
- Rich *et al.*, *Hum. Gene Ther.*, 4:461-476, 1993.
- 10 Ridgeway, In: *Vectors: A survey of molecular cloning vectors and their uses*, Stoneham: Butterworth, 467-492, 1988.
- Ripe *et al.*, *Mol. Cell. Biol.*, 9:2224, 1989.
- Rippe *et al.*, *Mol. Cell Biol.*, 10:689-695, 1990.
- Rittling *et al.*, *Nuc. Acids Res.*, 17:1619, 1989.
- 15 Rockman *et al.*, *J. Mol. Med.*, 74:489-495, 1996.
- Rosen *et al.*, *Cell*, 41:813, 1988.
- Rosenberg *et al.*, *Proc. Natl. Acad. Sci. USA*, 101:9387-9392, 2004.
- Rosenfeld *et al.*, *Cell*, 68:143-155, 1992.
- Rosenfeld *et al.*, *Science*, 252:431-434, 1991.
- 20 Rothermel *et al.*, *Proc. Natl. Sci. USA*, 98:3328-3333, 2001.
- Roux *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:9079-9083, 1989.
- Sadoshima and Izumo, *Circ. Res.*, 73:424-438, 1993.
- Sadoshima *et al.*, *Cell*, 75:977-984, 1993.
- Sakai *et al.*, *Genes and Dev.*, 2:1144, 1988.
- 25 Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY, 2001.
- Sarver *et al.*, *Science*, 247:1222-1225, 1990.
- Satake *et al.*, *J. Virology*, 62:970, 1988.
- Scanlon *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:10591-10595, 1991.
- 30 Schaffner *et al.*, *J. Mol. Biol.*, 201:81, 1988.
- Searle *et al.*, *Mol. Cell. Biol.*, 5:1480, 1985.
- Sharp and Marciniak, *Cell*, 59:229, 1989.
- Sharp and Zamore, *Science*, 287:2431-2433, 2000.
- Sharp, *Genes Dev.*, 13:139-141, 1999.

- Shaul and Ben-Levy, *EMBO J.*, 6:1913, 1987.
- Sherman *et al.*, *Mol. Cell. Biol.*, 9:50, 1989.
- Simpson and Savion, *Circ. Res.*, 50:101-116, 1982.
- Simpson *et al.*, *Cir. Res.*, 51:787-801, 1982.
- 5 Sleigh and Lockett, *J. EMBO*, 4:3831, 1985.
- Spalholz *et al.*, *Cell*, 42:183, 1985.
- Spandau and Lee, *J. Virology*, 62:427, 1988.
- Spandidos and Wilkie, *EMBO J.*, 2:1193, 1983.
- Stemmer and Klee, *Biochemistry*, 33:6859-6866, 1994.
- 10 Stephens and Hentschel, *Biochem. J.*, 248:1, 1987.
- Stratford-Perricaudet and Perricaudet, *In: Human Gene Transfer*, Cohen-Haguenauer and Boiron (Eds.), John Libbey Eurotext, France, 51-61, 1991.
- Stratford-Perricaudet *et al.*, *Hum. Gene. Ther.*, 1:241-256, 1990.
- Stuart *et al.*, *Nature*, 317:828, 1985.
- 15 Su *et al.*, *Eur. J. Biochem.*, 230:469-474, 1995.
- Sucharov *et al.*, *J. Biol. Chem.*, 278:31233-31239, 2003.
- Sullivan and Peterlin, *Mol. Cell. Biol.*, 7:3315, 1987.
- Swartzendruber and Lehman, *J. Cell. Physiology*, 85:179, 1975.
- Tabara *et al.*, *Cell*, 99:123-132, 1999.
- 20 Takebe *et al.*, *Mol. Cell. Biol.*, 8:466, 1988.
- Tavernier *et al.*, *Nature*, 301:634, 1983.
- Taylor and Kingston, *Mol. Cell. Biol.*, 10:165, 1990a.
- Taylor and Kingston, *Mol. Cell. Biol.*, 10:176, 1990b.
- Taylor *et al.*, *J. Biol. Chem.*, 264:15160, 1989.
- 25 Temin, *In: Gene Transfer*, Kucherlapati (Ed.), NY, Plenum Press, 149-188, 1986.
- The Merck Index, 11th Edition.
- Thiesen *et al.*, *J. Virology*, 62:614, 1988.
- Top *et al.*, *J. Infect. Dis.*, 124:155-160, 1971.
- Treisman, *Cell*, 46(4):567-174, 1986.
- 30 Tronche *et al.*, *Mol. Biol. Med.*, 7:173, 1990.
- Tronche *et al.*, *Mol. Cell Biol.*, 9(11):4759-4766, 1989.
- Trudel and Constantini, *Genes and Dev.* 6:954, 1987.
- Tur-Kaspa *et al.*, *Mol. Cell Biol.*, 6:716-718, 1986.
- Tyndell *et al.*, *Nuc. Acids. Res.*, 9:6231, 1981.

- Vannice and Levinson, *J. Virology*, 62:1305, 1988.
- Varmus *et al.*, *Cell*, 25:23-36, 1981.
- Vasseur *et al.*, *Proc Natl. Acad. Sci. U.S.A.*, 77:1068, 1980.
- Wagner *et al.*, *Proc. Natl. Acad. Sci. USA*, 87(9):3410-3414, 1990.
- 5 Wang and Calame, *Cell*, 47:241, 1986.
- Wang *et al.*, *Circ. Res.*, 95:798-806, 2004.
- Waspe *et al.*, *J. Clin. Invest.*, 85:1206-1214, 1990.
- Weber *et al.*, *Cell*, 36:983, 1984.
- Weinberger *et al.* *Mol. Cell. Biol.*, 8:988, 1984.
- 10 Wilkins and Molkentin, *Biochem. Biophys. Res. Commun.*, 322:1178-1191, 2004.
- Wincott *et al.*, *Nucleic Acids Res.*, 23(14):2677-2684, 1995.
- Winoto and Baltimore, *Cell*, 59:649, 1989.
- WO 00/44914
- WO 01/36646
- 15 WO 01/68836
- WO 84/03564
- WO 98/33791
- WO 99/32619
- Wong *et al.*, *Gene*, 10:87-94, 1980.
- 20 Wu and Wu, *Adv. Drug Delivery Rev.*, 12:159-167, 1993.
- Wu and Wu, *Biochemistry*, 27:887-892, 1988.
- Wu and Wu, *J. Biol. Chem.*, 262:4429-4432, 1987.
- Yamauchi-Takahara *et al.*, *Proc. Natl. Acad. Sci. USA*, 86(10):3504-3508, 1989.
- Yamazaki *et al.*, *Circulation*, 95:1260-1268, 1997.
- 25 Yang *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:9568-9572, 1990.
- Young *et al.*, In: *Handbook of Applied Therapeutics*, 7.1-7.12 and 9.1-9.10, 1989.
- Yue *et al.*, *J. Biol. Chem.*, 275:37895-37901, 2000.
- Yutzey *et al.* *Mol. Cell. Biol.*, 9:1397, 1989.
- Zelenin *et al.*, *FEBS Lett.*, 280:94-96, 1991.
- 30 Zhang *et al.*, *Cardiovasc. Res.*, 63:476-486, 2004.
- Zhu *et al.*, *J. Clin. Invest.*, 111:617-625, 2003.
- Ziober and Kramer, *J. Bio. Chem.*, 271(37):22915-22922, 1996.
- Zolk *et al.*, *Cardiovasc. Res.*, 59:563-572, 2003.
- Zou *et al.*, *Circulation*, 104:102-108, 2001.

Zou *et al.*, *J. Biol. Chem.*, 271(52):33592-33597, 1996.

CLAIMS

1. A method of treating pathologic cardiac hypertrophy or heart failure comprising:
 - 5 (a) identifying a patient having cardiac hypertrophy or heart failure; and
 - (b) administering to said patient an inhibitor of ERK1/2.
2. The method of claim 1, wherein said inhibitor of ERK1/2 is selected from the group consisting of a ERK1/2 RNAi molecule, a ERK1/2 antisense molecule, a ERK1/2
10 ribozyme molecule or a ERK1/2-binding single-chain antibody, an expression construct that encodes a ERK1/2-binding single-chain antibody, an inhibitor of ERK1/2 phosphorylation, YY1 and an expression construct encoding YY1.
3. The method of claim 1, wherein administering the inhibitor of ERK1/2 is performed
15 intravenously or by direct injection into cardiac tissue.
4. The method of claim 1, wherein administering comprises oral, transdermal, sustained release, controlled release, delayed release, suppository, or sublingual administration.
- 20 5. The method of claim 1, further comprising administering to said patient a second cardiac hypertrophic therapy.
6. The method of claim 5, wherein said second therapy is selected from the group consisting of a beta blocker, an ionotrope, a diuretic, ACE-I, AII antagonist, BNP, a
25 Ca^{++} -blocker, an inhibitor of calcineurin, an inhibitor of CamKII or an HDAC inhibitor.
7. The method of claim 5, wherein said second therapy is administered at the same time as said inhibitor of ERK1/2.
30
8. The method of claim 5, wherein said second therapy is administered either before or after said inhibitor of ERK1/2.

9. The method of claim 1, wherein treating comprises improving one or more symptoms of pathologic cardiac hypertrophy.
10. The method of claim 1, wherein treating comprises improving one or more symptoms of heart failure.
11. The method of claim 9, wherein said one or more improved symptoms comprises increased exercise capacity, increased cardiac ejection volume, decreased left ventricular end diastolic pressure, decreased pulmonary capillary wedge pressure, increased cardiac output, or cardiac index, lowered pulmonary artery pressures, decreased left ventricular end systolic and diastolic dimensions, decreased left and right ventricular wall stress, decreased wall tension, increased quality of life, and decreased disease related morbidity or mortality.
12. A method of preventing pathologic hypertrophy or heart failure comprising:
- (a) identifying a patient at risk of developing pathologic cardiac hypertrophy or heart failure; and
 - (b) administering to said patient an inhibitor of ERK1/2.
13. The method of claim 12, wherein said inhibitor of ERK1/2 is selected from the group consisting of a ERK1/2 RNAi molecule, a ERK1/2 antisense molecule, a ERK1/2 ribozyme molecule or a ERK1/2-binding single-chain antibody, or expression construct that encodes a ERK1/2-binding single-chain antibody, an inhibitor of ERK1/2 phosphorylation, YY1 and an expression construct encoding YY1.
14. The method of claim 12, wherein administering the inhibitor of ERK1/2 is performed intravenously or by direct injection into cardiac tissue.
15. The method of claim 12, wherein administering comprises oral, transdermal, sustained release, controlled release, delayed release, suppository, or sublingual administration.
16. The method of claim 12, wherein the patient at risk exhibits one or more of a list of risk factors comprising long standing uncontrolled hypertension, uncorrected valvular

disease, chronic angina, recent myocardial infarction, congenital predisposition to heart disease or pathological hypertrophy.

- 5 17. The method of claim 12, wherein the patient at risk is diagnosed as having a genetic predisposition to cardiac hypertrophy.
18. The method of claim 12, wherein the patient at risk has a familial history of cardiac hypertrophy.
- 10 19. A method of assessing an inhibitor of ERK1/2 for efficacy in treatment of cardiac hypertrophy or heart failure comprising:
- (a) providing an inhibitor of ERK1/2;
 - (b) treating a cell with said inhibitor of ERK1/2; and
 - 15 (c) measuring the expression of one or more cardiac hypertrophy parameters,
- wherein a change in said one or more cardiac hypertrophy parameters, as compared to one or more cardiac hypertrophy parameters in a cell not treated with said inhibitor of ERK1/2, identifies said inhibitor of ERK1/2 as an inhibitor of cardiac hypertrophy or
- 20 heart failure.
20. The method of claim 19, wherein said cell is a myocyte.
21. The method of claim 19, wherein said cell is an isolated myocyte.
- 25 22. The method of claim 21, wherein said myocyte is a cardiomyocyte
23. The method of claim 20, wherein said myocyte is comprised in isolated intact tissue.
- 30 24. The method of claim 20, wherein said myocyte is a neonatal rat ventricular myocyte.
25. The method of claim 19, wherein said cell is an H9C2 cell.

26. The method of claim 22, wherein said cardiomyocyte is located *in vivo* in a functioning intact heart muscle.
27. The method of claim 26, wherein said functioning intact heart muscle is subjected to a stimulus that triggers a hypertrophic response in one or more cardiac hypertrophy parameters.
28. The method of claim 27, wherein said stimulus is aortic banding, rapid cardiac pacing, induced myocardial infarction, or transgene expression.
29. The method of claim 27, wherein said stimulus is a chemical or pharmaceutical agent.
30. The method of claim 29, wherein said chemical or pharmaceutical agent comprises angiotensin II, isoproterenol, phenylephrine, endothelin-I, vasoconstrictors, antidiuretics.
31. The method of claim 27, wherein said one or more cardiac hypertrophy parameters comprises right ventricular ejection fraction, left ventricular ejection fraction, ventricular wall thickness, heart weight/body weight ratio, right or left ventricular weight/body weight ratio, or cardiac weight normalization measurement.
32. The method of claim 20, wherein said myocyte is subjected to a stimulus that triggers a hypertrophic response in said one or more cardiac hypertrophy parameters.
33. The method of claim 32, wherein said stimulus is expression of a transgene.
34. The method of claim 32, wherein said stimulus is treatment with a drug.
35. The method of claim 19, wherein said one or more cardiac hypertrophy parameters comprises the expression level of one or more target genes in said myocyte, wherein expression level of said one or more target genes is indicative of cardiac hypertrophy.
36. The method of claim 35, wherein said one or more target genes is selected from the group consisting of ANP, β -MyHC, BNP, and α -skeletal actin.

37. The method of claim 35, wherein the expression level is measured using a reporter protein coding region operably linked to a target gene promoter.
- 5 38. The method of claim 37, wherein said reporter protein is luciferase, β -gal, or green fluorescent protein.
39. The method of claim 35, wherein the expression level is measured using hybridization of a nucleic acid probe to a target mRNA or amplified nucleic acid product.
- 10 40. The method of claim 19, wherein said one or more cardiac hypertrophy parameters comprises one or more aspects of cellular morphology.
41. The method of claim 40, wherein said one or more aspects of cellular morphology
15 comprises sarcomere assembly, cell size, or cell contractility.
42. The method of claim 19, wherein said one or more cardiac hypertrophy parameters comprises total protein synthesis
- 20 43. The method of claim 19, wherein said one or more cardiac hypertrophy parameters comprises ERK1/2 phosphorylation.
44. The method of claim 19, further comprising measuring cell toxicity.
- 25 45. The method of claim 19, wherein said treating is performed *in vitro*.
46. The method of claim 19, wherein said treating is performed *in vivo*.
47. The method of claim 19, wherein said cell is part of a transgenic, non-human mammal.
- 30 48. A method of identifying an inhibitor of cardiac hypertrophy or heart failure comprising:
- (a) providing a ERK1/2;

- (b) contacting the ERK1/2 with a candidate inhibitor substance; and
- (c) assessing the phosphorylation of said ERK1/2,

wherein a decrease in the phosphorylation of the ERK1/2 identifies said candidate inhibitor substance as an inhibitor of cardiac hypertrophy or heart failure.

49. The method of claim 48, where said ERK1/2 is purified away from whole cells.

50. The method of claim 49, wherein said cells are heart cells.

51. The method of claim 48, wherein said ERK1/2 is located in an intact cell.

52. The method of claim 51, wherein said intact cell is a myocyte.

53. The method of claim 52, wherein said myocyte is a cardiomyocyte.

54. The method of claim 48, wherein the candidate inhibitor substance is an interfering RNA.

55. The method of claim 48, wherein the candidate inhibitor substance is an antibody preparation.

56. The method of claim 55, wherein the antibody preparation comprises single chain antibodies.

57. The method of claim 48, wherein the candidate inhibitor substance is an antisense construct.

58. The method of claim 48, wherein said inhibitor is an enzyme, chemical, pharmaceutical, or small compound.

59. The method of claim 48, wherein said inhibitor of ERK1/2 is selected from the group consisting of a ERK1/2 RNAi molecule, a ERK1/2 antisense molecule, a ERK1/2 ribozyme molecule or a ERK1/2-binding single-chain antibody, or an expression

construct that encodes a ERK1/2-binding single-chain antibody, YY1 and an expression construct encoding YY1.

- 5 60. A method of preventing cardiac hypertrophy and dilated cardiomyopathy comprising decreasing ERK1/2 activity in heart cells of a subject.
61. A method of inhibiting progression of cardiac hypertrophy comprising decreasing ERK1/2 activity in heart cells of a subject.
- 10 62. A method of treating heart failure comprising decreasing ERK1/2 activity in heart cells of a subject.
63. A method of inhibiting progression of heart failure comprising decreasing ERK1/2 activity in heart cells of a subject.
- 15 64. A method of increasing exercise tolerance in a subject with heart failure or cardiac hypertrophy comprising decreasing ERK1/2 activity in heart cells of a subject.
65. A method of reducing hospitalization in a subject with heart failure or cardiac hypertrophy comprising decreasing ERK1/2 activity in heart cells of a subject.
- 20 66. A method of improving quality of life in a subject with heart failure or cardiac hypertrophy comprising decreasing ERK1/2 activity in heart cells of a subject.
- 25 67. A method of decreasing morbidity in a subject with heart failure or cardiac hypertrophy comprising decreasing ERK1/2 activity in heart cells of a subject.
68. A method of decreasing mortality in a subject with heart failure or cardiac hypertrophy comprising decreasing ERK1/2 activity in heart cells of a subject.



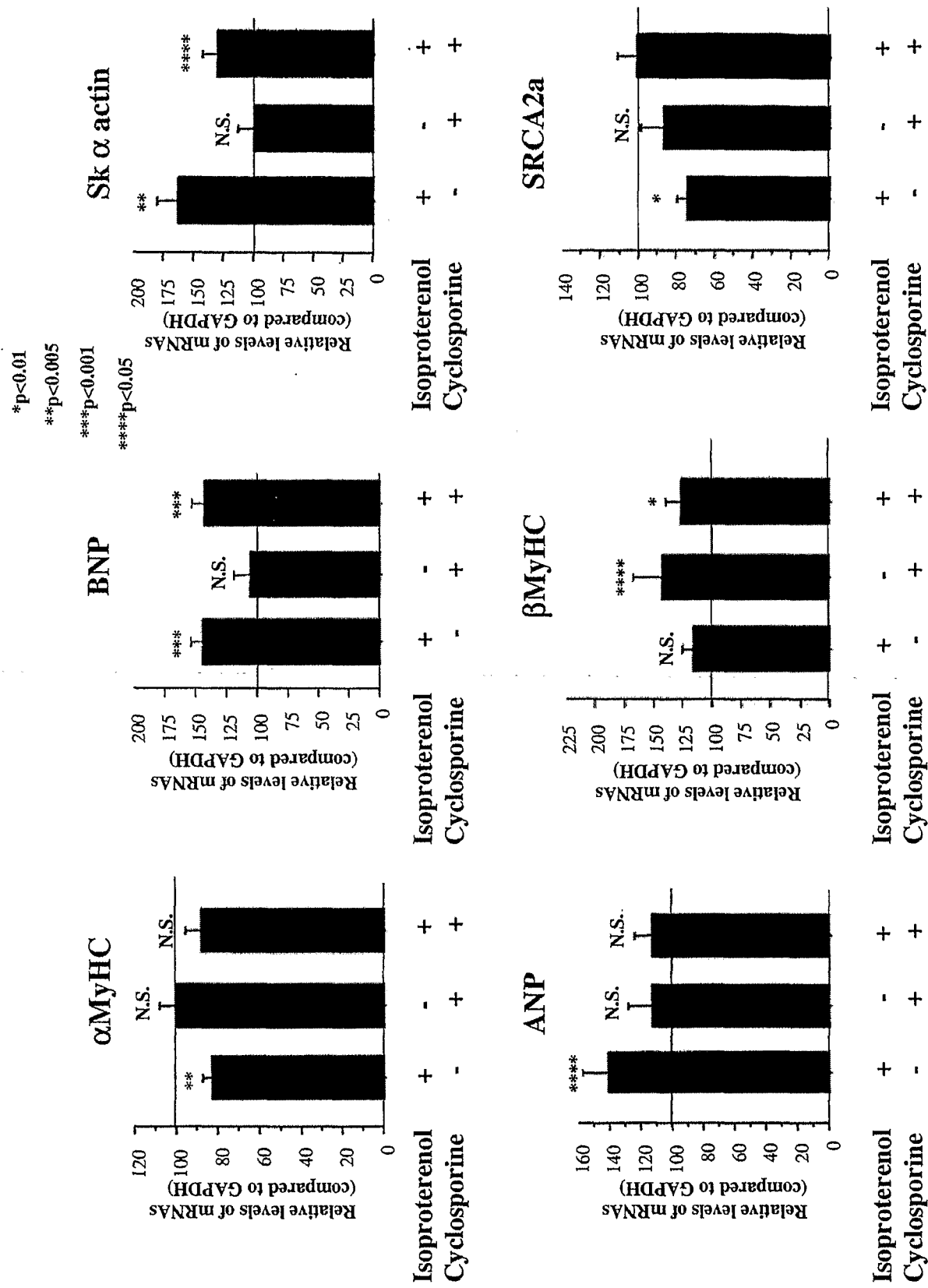


FIG. 2

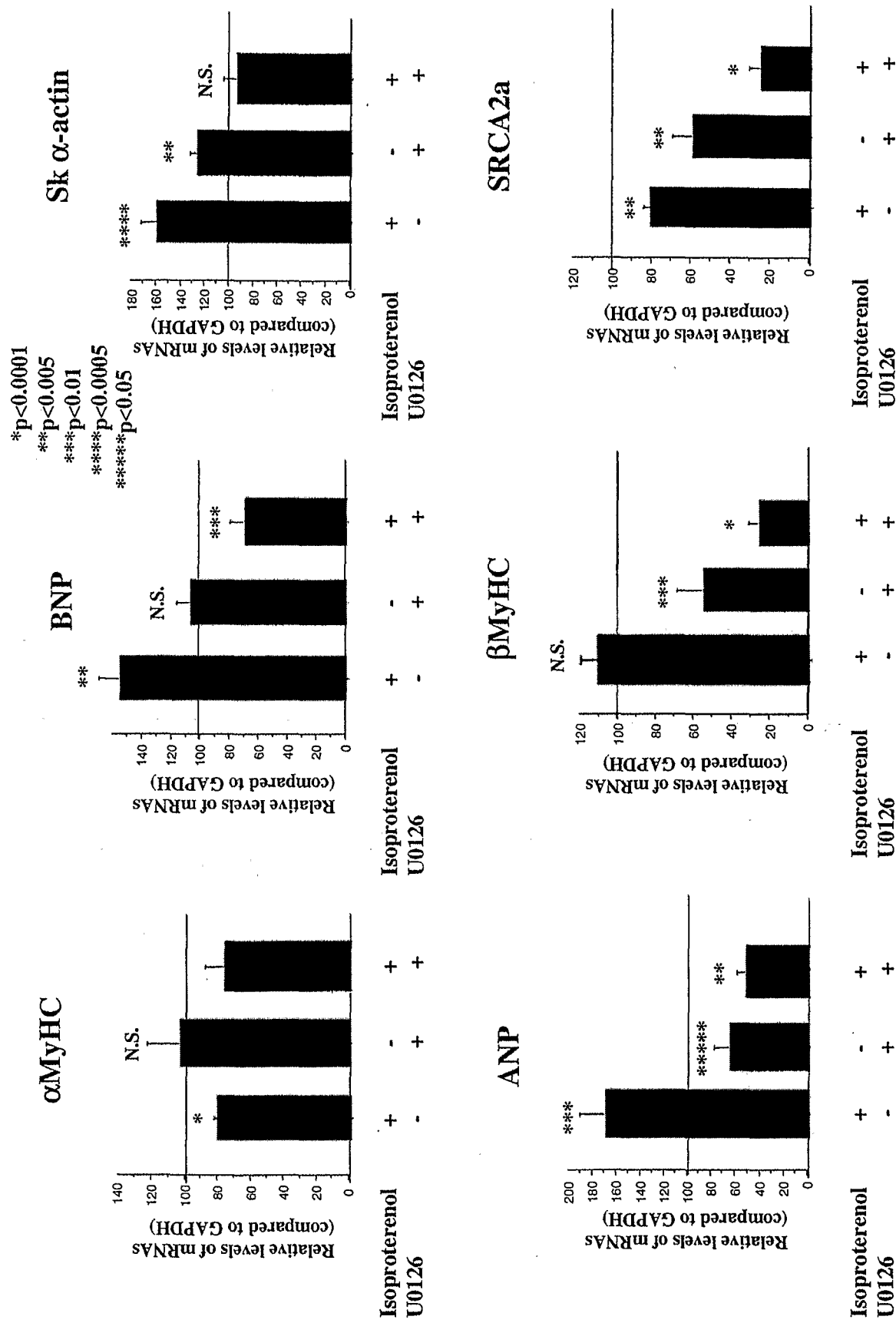


FIG.3

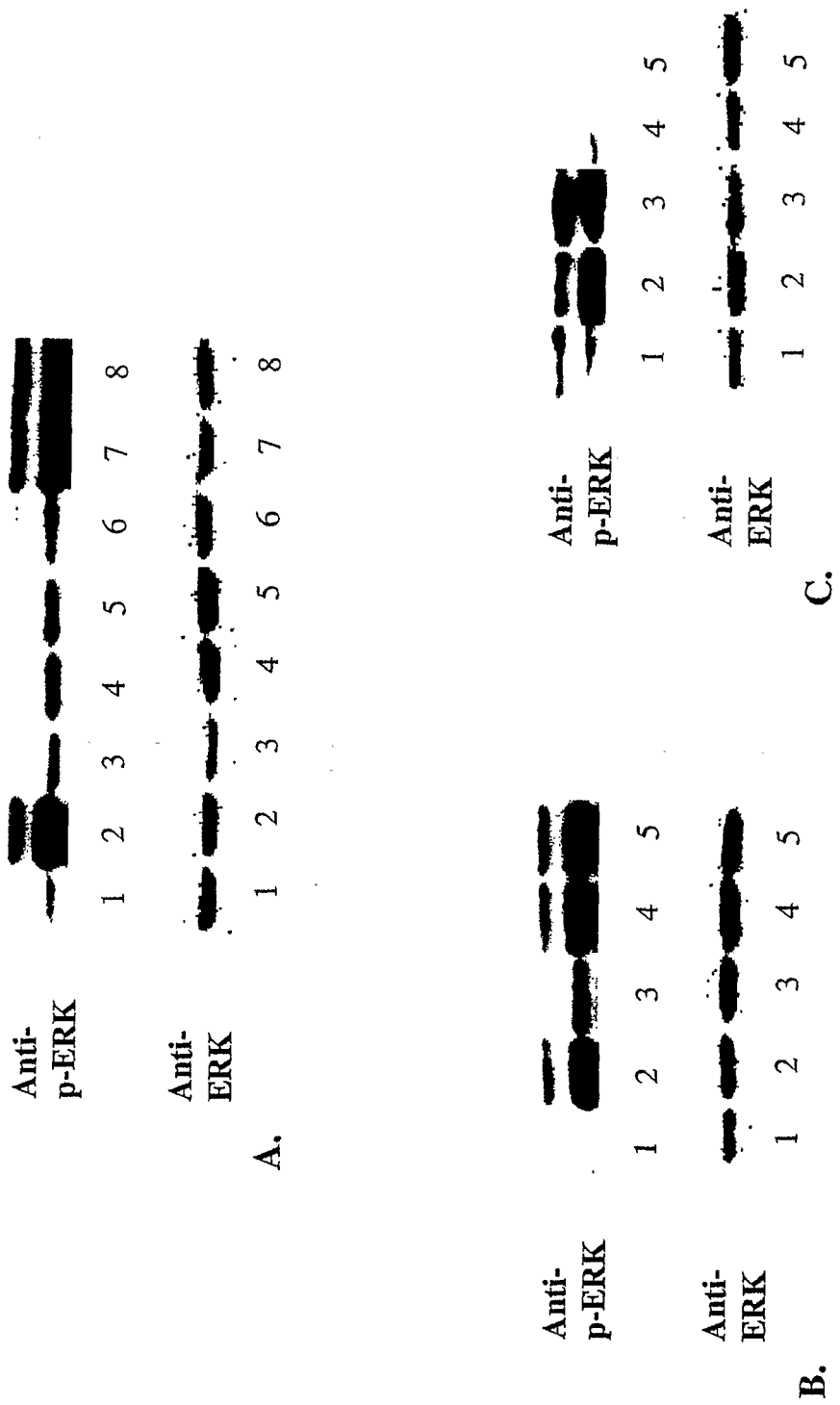


FIG. 4A-C

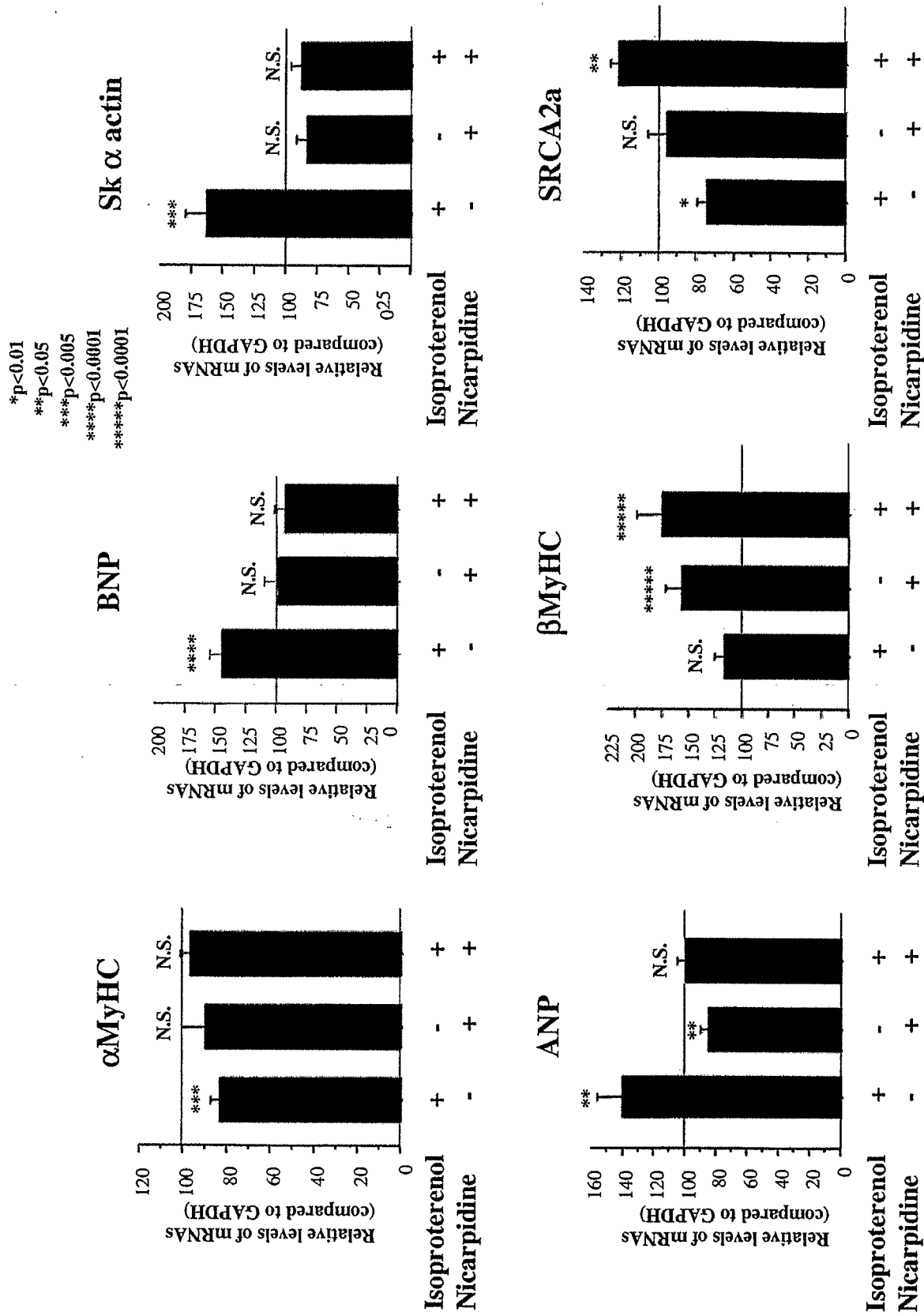
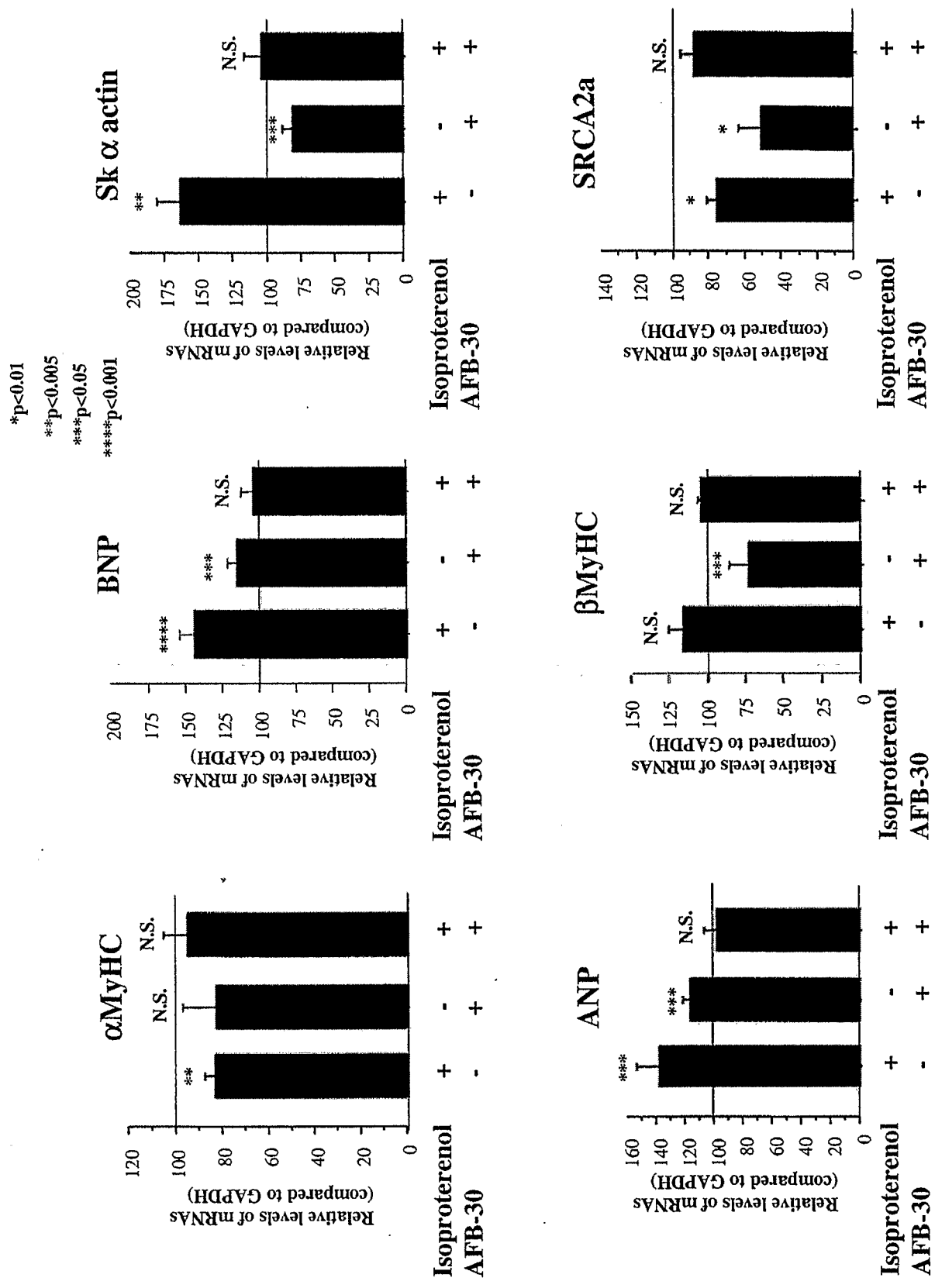


FIG. 5



FIG, 6

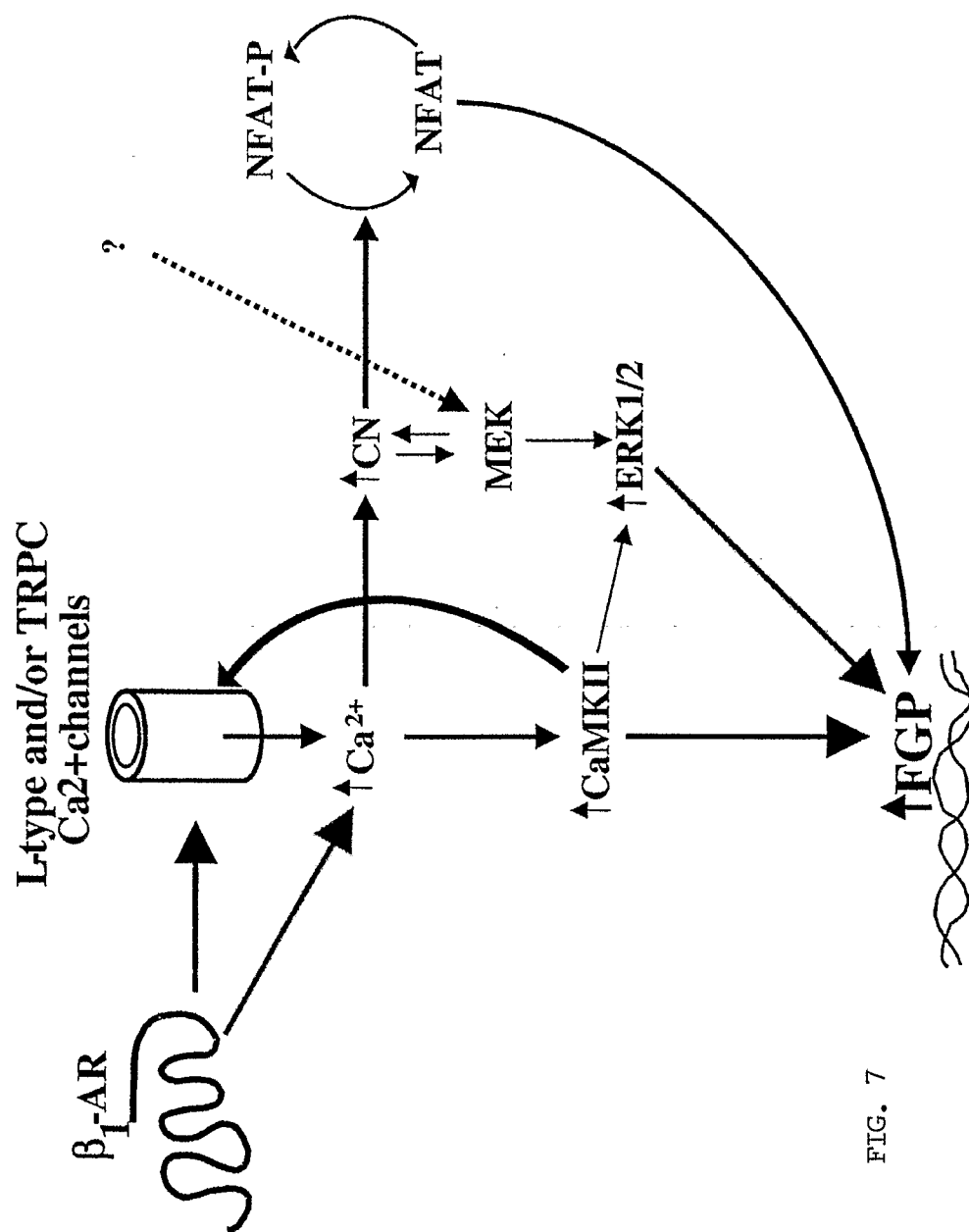


FIG. 7

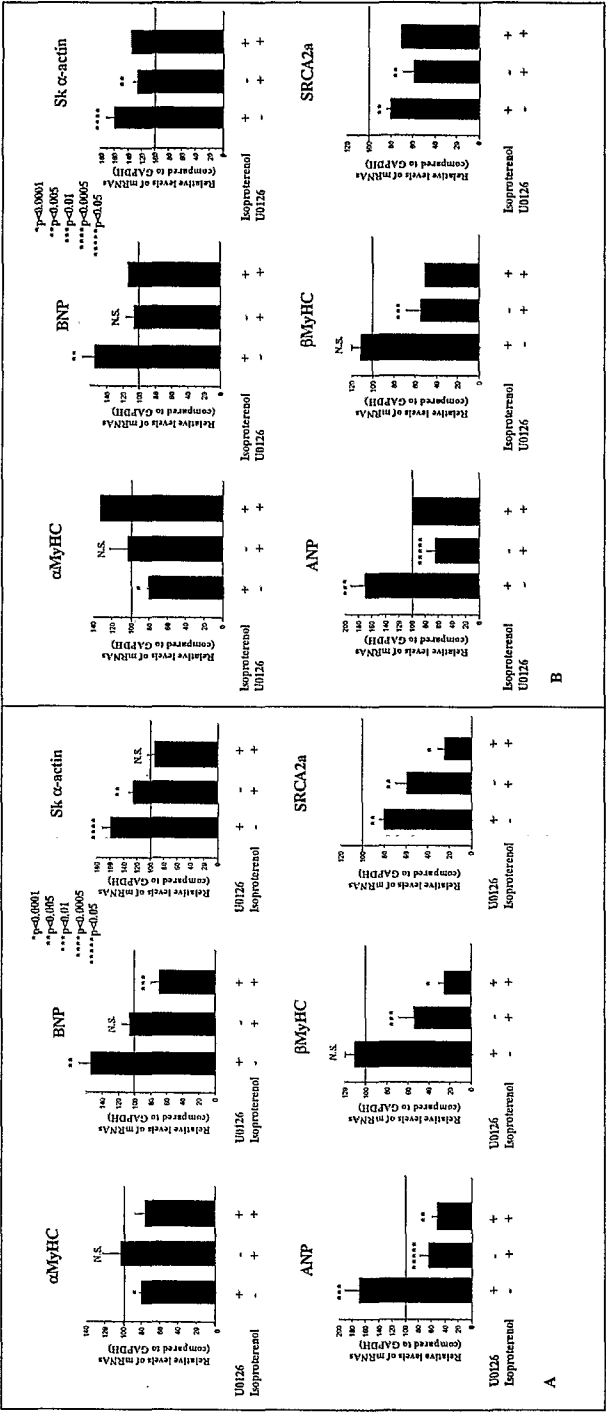


FIG. 8A-B

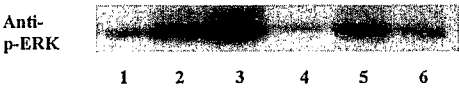


FIG. 9

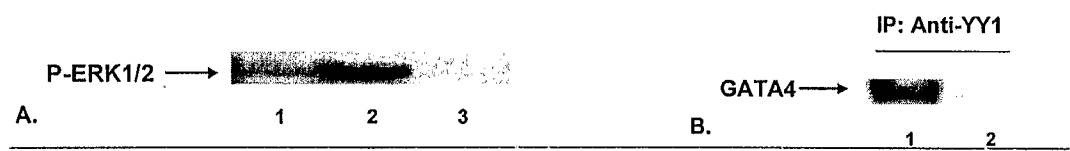


FIG. 10