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Lee

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(54) **METHODS FOR TREATMENT OF CARDIOVASCULAR DISEASE**

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(58) **Field of Classification Search**

None

See application file for complete search history.

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(57) **ABSTRACT**

This invention pertains to methods and compositions for the diagnosis and treatment of cardiovascular conditions. More specifically, the invention relates to isolated molecules that can be used to diagnose and/or treat cardiovascular conditions including cardiac hypertrophy, myocardial infarction, stroke, arteriosclerosis, and heart failure.

28 Claims, 6 Drawing Sheets

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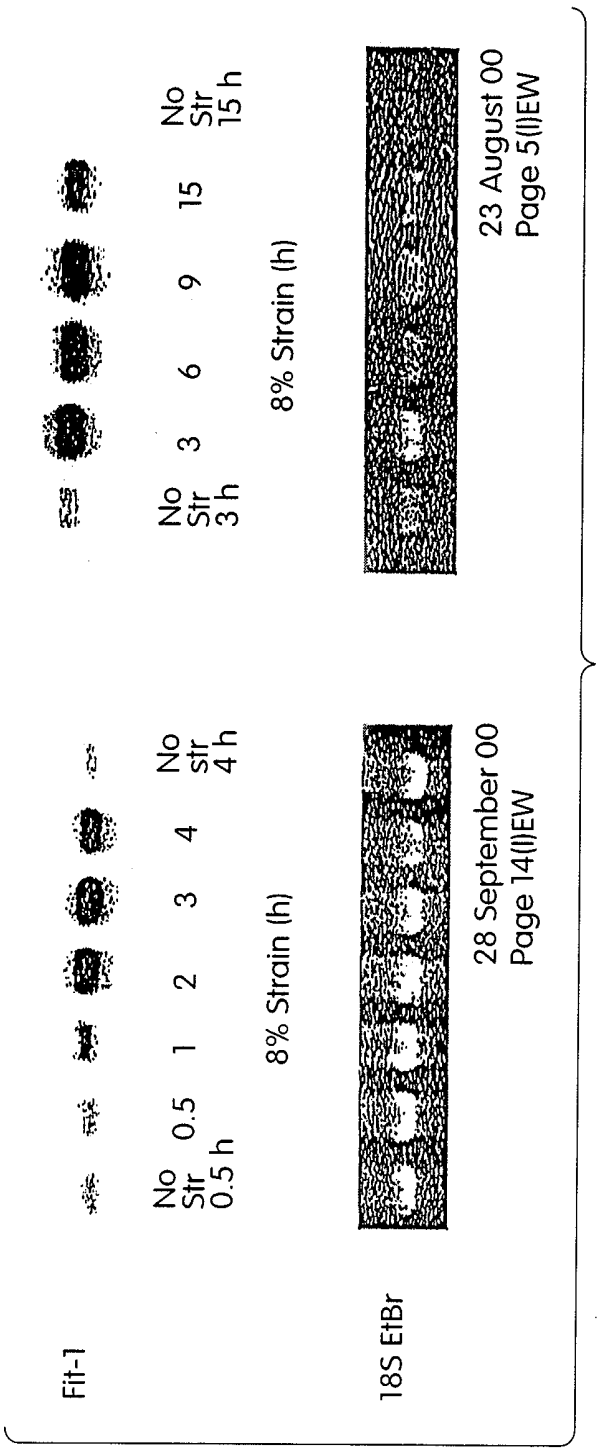
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2
3
4
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No Str 3 h
3
6
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18S EtBr
28 September 00
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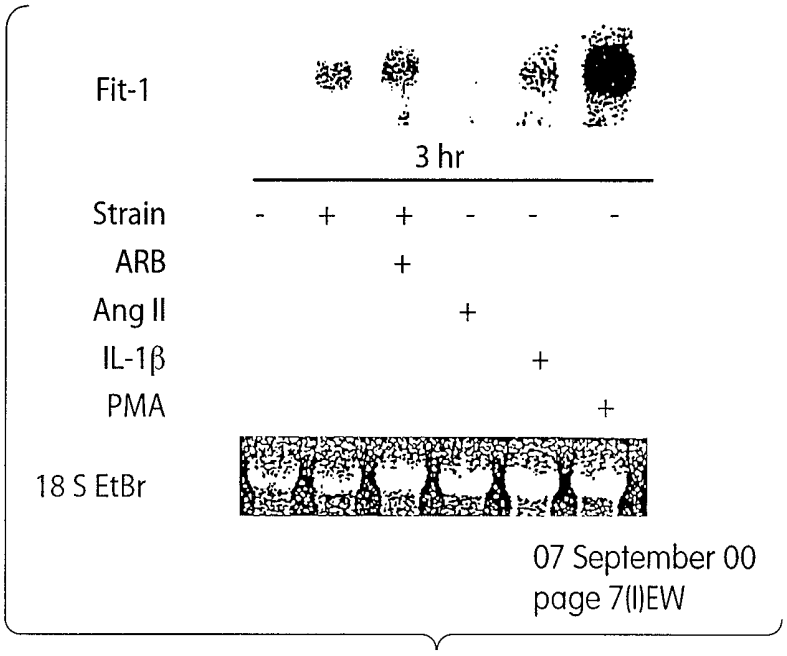
Fig. 1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ARB +
Ang II +
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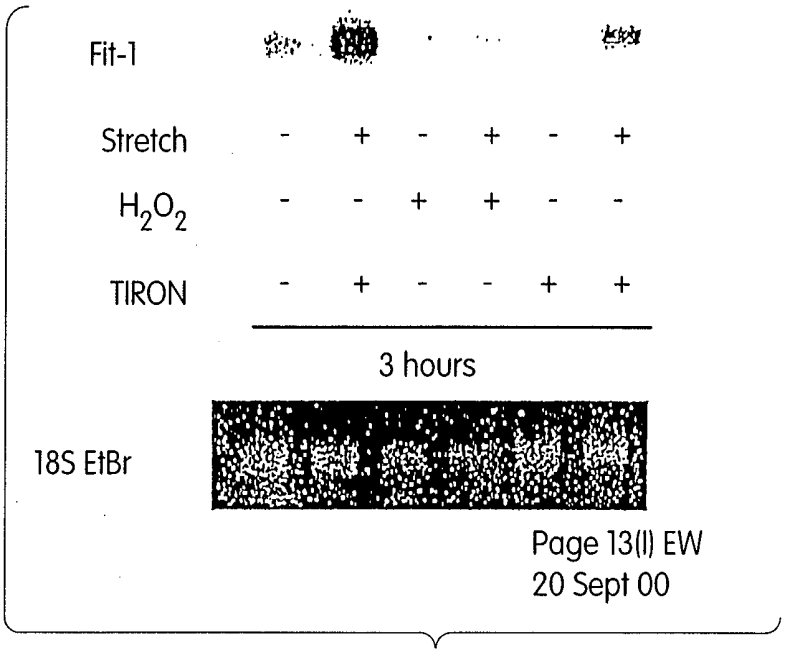
18 S EtBr
07 September 00
page 7(I)EW

Fig. 2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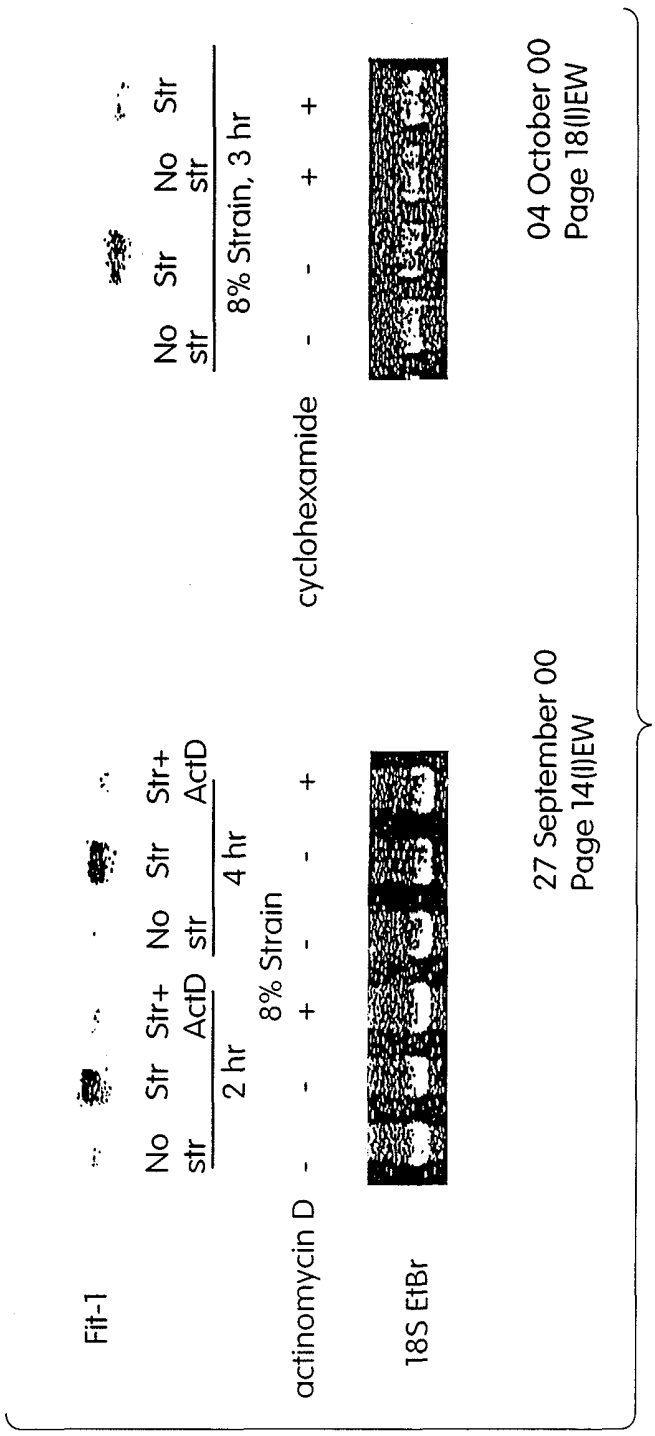
3 hours
18S EtBr
Page 13(I) EW
20 Sept 00

Fig. 3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Str
Str+ ActD
4 hr
actinomycin D
-
-
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-
-
+
18S EtBr
27 September 00
Page 14(I)EW
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Str
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Str
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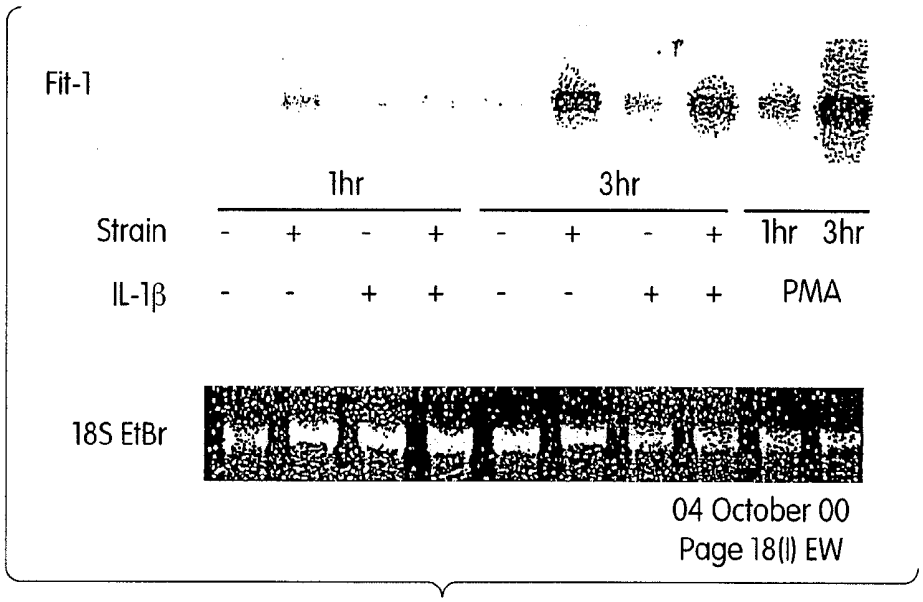
-
-
+
+
04 October 00
Page 18(I)EW

Fig. 4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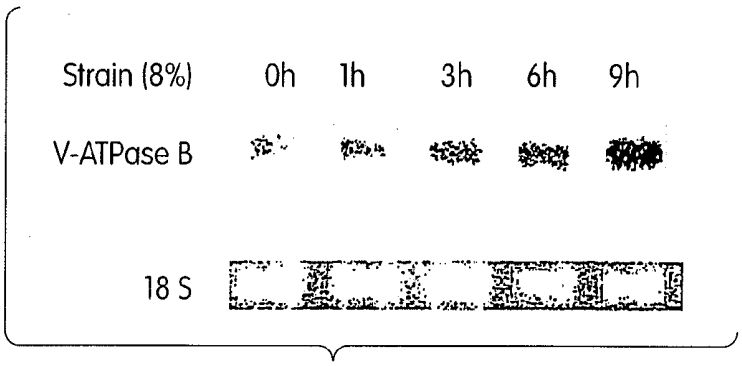
18S EtBr
04 October 00
Page 18(I) EW

Fig. 5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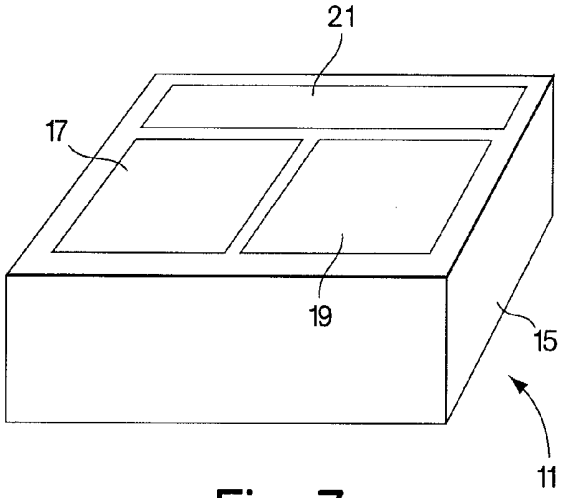
V-ATPase B
18 S

Fig. 6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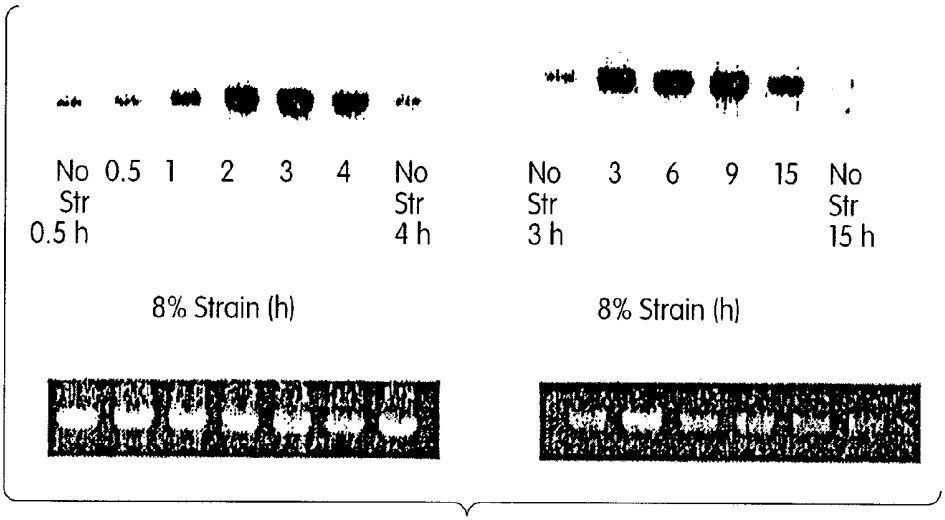
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6
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15
No Str 15 h
8% Strain (h)

Fig. 8

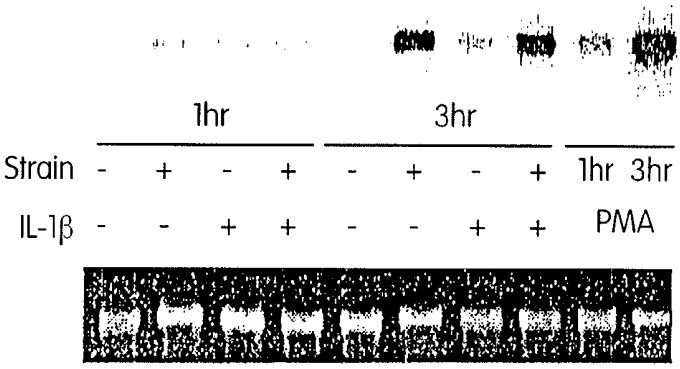

Fig. 9

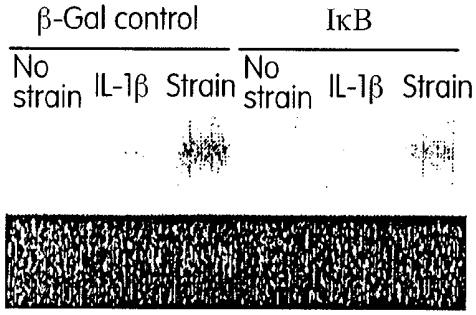

Fig. 10

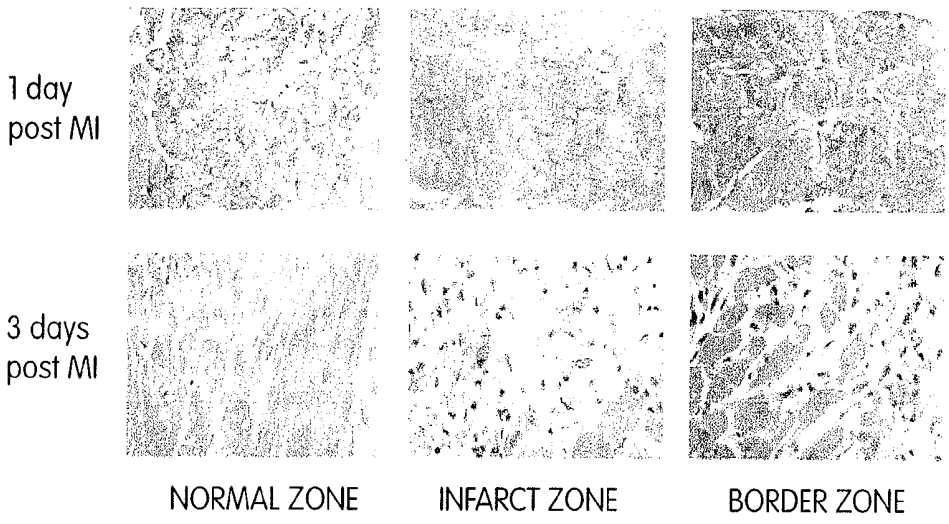

Fig. 11

METHODS FOR TREATMENT OF CARDIOVASCULAR DISEASE

RELATED APPLICATIONS

This application is a continuation application of U.S. patent application Ser. No. 13/151,012, filed on Jun. 1, 2011 (issued as U.S. Pat. No. 8,530,173), which is a continuation application of U.S. patent application Ser. No. 12/167,143, filed on Jul. 2, 2008 (issued as U.S. Pat. No. 7,985,558), which is a continuation application of U.S. patent application Ser. No. 10/024,607, filed on Nov. 8, 2001 (issued as U.S. Pat. No. 7,432,060), which claims priority under 35 U.S.C. §119 (e) from U.S. Provisional Patent Application Ser. No. 60/247,457, filed on Nov. 9, 2000, the entire contents of each of which are herein incorporated by reference.

GOVERNMENT SUPPORT

This invention was made with government support under grant number HL054759 awarded by The National Institute of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

This invention relates to methods and compositions for the diagnosis and treatment of cardiovascular conditions. More specifically, the invention relates to isolated molecules that can be used to treat cardiovascular conditions including cardiac hypertrophy, myocardial infarction, stroke, arteriosclerosis, and heart failure.

BACKGROUND OF THE INVENTION

Despite significant advances in therapy, cardiovascular disease remains the single most common cause of morbidity and mortality in the developed world. Thus, prevention and therapy of cardiovascular conditions such as myocardial infarction and stroke is an area of major public health importance. Currently, several risk factors for future cardiovascular disorders have been described and are in wide clinical use in the detection of individuals at high risk. Such screening tests include evaluations of total and HDL cholesterol levels. However, a large number of cardiovascular disorders occur in individuals with apparently low to moderate risk profiles, and ability to identify such patients is limited. Moreover, accumulating data suggests that the beneficial effects of certain preventive and therapeutic treatments for patients at risk for or known to have cardiovascular disorders differs in magnitude among different patient groups. At this time, however, data describing diagnostic tests to determine whether certain therapies can be expected to be more or less effective are lacking.

SUMMARY OF THE INVENTION

This invention provides methods and compositions for the diagnosis and treatment of cardiovascular conditions. More specifically, a number of genes were identified that are upregulated in cardiac cells when the cells are subjected to mechanically-induced deformation. In view of these discoveries, it is believed that the molecules of the present invention can be used to treat vascular and cardiovascular conditions including cardiac hypertrophy, myocardial infarction, stroke, arteriosclerosis, and heart failure.

Additionally, methods for using these molecules in the diagnosis of any of the foregoing vascular and cardiovascular conditions, are also provided.

Furthermore, compositions useful in the preparation of therapeutic preparations for the treatment of the foregoing conditions, are also provided.

The present invention thus involves, in several aspects, polypeptides, isolated nucleic acids encoding those polypeptides, functional modifications and variants of the foregoing, useful fragments of the foregoing, as well as therapeutics and diagnostics relating thereto.

According to one aspect of the invention, a method of diagnosing a condition characterized by aberrant expression of a nucleic acid molecule or an expression product thereof (or of unique fragments of the foregoing molecules thereof), is provided. The method involves contacting a biological sample from a subject with an agent, wherein said agent specifically binds to said nucleic acid molecule, an expression product thereof, or a fragment of an expression product thereof, and measuring the amount of bound agent and determining therefrom if the expression of said nucleic acid molecule or of an expression product thereof is aberrant, aberrant expression being diagnostic of the disorder, wherein the nucleic acid molecule is at least one nucleic acid molecule selected from the group consisting of Fit-1 (SEQ ID NOs: 1 and 2 for Fit-1S; SEQ ID NOs: 3 and 4 for Fit-1M), vacuolar ATPase (SEQ ID NOs: 5 and 6), CD44 (SEQ ID NOs: 7 and 8), Lot-1 (SEQ ID NOs: 9 and 10), AA892598 (SEQ ID NO: 11), and Mrg-1 (SEQ ID NO: 12). In some embodiments, the disorder is a cardiovascular condition selected from the group consisting of myocardial infarction, stroke, arteriosclerosis, and heart failure. In one embodiment, the disorder is cardiac hypertrophy. In certain embodiments, biological samples include biopsy samples, and biological fluids such as blood.

According to still another aspect of the invention, a method for determining a stage (e.g., regression, progression or onset) of a cardiovascular condition in a subject characterized by aberrant expression of a nucleic acid molecule or an expression product thereof (or of unique fragments of the foregoing molecules thereof), is provided. The method involves monitoring a sample from a patient for a parameter selected from the group consisting of (i) a nucleic acid molecule selected from the group consisting of Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1 (or a unique fragment thereof), (ii) a polypeptide encoded by the nucleic acid, (iii) a peptide derived from the polypeptide (or of a unique fragment thereof), and (iv) an antibody which selectively binds the polypeptide or peptide (or a unique fragment thereof), as a determination of a stage (e.g., regression, progression or onset) of said cardiovascular condition in the subject. In some embodiments, the sample is a biological fluid or a tissue as described in any of the foregoing embodiments. In certain embodiments, the step of monitoring comprises contacting the sample with a detectable agent selected from the group consisting of (a) an isolated nucleic acid molecule which selectively hybridizes under stringent conditions to the nucleic acid molecule of (i), (b) an antibody which selectively binds the polypeptide of (ii), or the peptide of (iii), and (c) a polypeptide or peptide which binds the antibody of (iv). The antibody, polypeptide, peptide, or nucleic acid can be labeled with a radioactive label or an enzyme. In further embodiments, the method further comprises assaying the sample for the peptide. In still further embodiments, monitoring the sample occurs over a period of time.

According to another aspect of the invention, a kit is provided. The kit comprises a package containing an agent that selectively binds to any of the foregoing isolated nucleic acids

(Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1), or expression products thereof, and a control for comparing to a measured value of binding of said agent any of the foregoing isolated nucleic acids or expression products thereof. In some embodiments, the control is a predetermined value for comparing to the measured value. In certain embodiments, the control comprises an epitope of the expression product of any of the foregoing isolated nucleic acids.

According to one aspect of the invention, a method for treating a cardiovascular condition, is provided. The method involves administering to a subject in need of such treatment a molecule selected from the group consisting of Fit-1 (alternatively referred to herein as T1/ST2), CD44, Lot-1, AA892598, and Mrg-1, in an amount effective to treat the cardiovascular condition. In certain embodiments, the cardiovascular condition is selected from the group consisting of myocardial infarction, stroke, arteriosclerosis, and heart failure. In one embodiment, the molecule administered is vacuolar ATPase. In some embodiments, the method further comprises co-administering an agent selected from the group consisting of an anti-inflammatory agent, an anti-thrombotic agent, an anti-platelet agent, a fibrinolytic agent, a lipid reducing agent, a direct thrombin inhibitor, a glycoprotein IIb/IIIa receptor inhibitor, an agent that binds to cellular adhesion molecules and inhibits the ability of white blood cells to attach to such molecules, a calcium channel blocker, a beta-adrenergic receptor blocker, a cyclooxygenase-2 inhibitor, or an angiotensin system inhibitor.

According to another aspect of the invention, a method for treating cardiac hypertrophy, is provided. The method involves administering to a subject in need of such treatment an agent that increases expression of a nucleic acid molecule selected from the group consisting of Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1, or an expression product thereof, in an amount effective to treat cardiac hypertrophy in the subject.

According to a further aspect of the invention, a method for treating a subject to reduce the risk of a cardiovascular condition developing in the subject, is provided. The method involves administering to a subject that expresses decreased levels of a molecule selected from the group consisting of Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1, an agent for reducing the risk of the cardiovascular disorder in an amount effective to lower the risk of the subject developing a future cardiovascular disorder, wherein the agent is an anti-inflammatory agent, an anti-thrombotic agent, an anti-platelet agent, a fibrinolytic agent, a lipid reducing agent, a direct thrombin inhibitor, a glycoprotein IIb/IIIa receptor inhibitor, an agent that binds to cellular adhesion molecules and inhibits the ability of white blood cells to attach to such molecules, a calcium channel blocker, a beta-adrenergic receptor blocker, a cyclooxygenase-2 inhibitor, or an angiotensin system inhibitor, or an agent that increases expression of a molecule selected from the group consisting of Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1.

According to one aspect of the invention, a method for identifying a candidate agent useful in the treatment of a cardiovascular condition, is provided. The method involves determining expression of a set of nucleic acid molecules in a cardiac cell or tissue under conditions which, in the absence of a candidate agent, permit a first amount of expression of the set of nucleic acid molecules, wherein the set of nucleic acid molecules comprises at least one nucleic acid molecule selected from the group consisting of Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1, contacting the cardiac cell or tissue with the candidate agent, and detecting a test amount of expression of the set of nucleic acid molecules, wherein an increase in the test amount of expression in the presence of the candidate agent relative to the first amount of expression indicates that the candidate agent is useful in the treatment of the cardiovascular condition. In certain embodiments, the cardiovascular condition is selected from the group consisting of cardiac hypertrophy (e.g., maladaptive hypertrophy), myocardial infarction, stroke, arteriosclerosis, and heart failure. In some embodiments, the set of nucleic acid molecules comprises at least two, at least three, at least four, or even at least five nucleic acid molecules, each selected from the group consisting of Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1.

According to another aspect of the invention, a pharmaceutical composition is provided. The composition comprises an agent comprising an isolated nucleic acid molecule selected from the group consisting of Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1, or an expression product thereof, in a pharmaceutically effective amount to treat a cardiovascular condition, and a pharmaceutically acceptable carrier. In some embodiments, the agent is an expression product of the isolated nucleic acid molecule selected from the group consisting of Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1. In certain embodiments, the cardiovascular condition is selected from the group consisting of cardiac hypertrophy, myocardial infarction, stroke, arteriosclerosis, and heart failure.

According to a further aspect of the invention, methods for preparing medicaments useful in the treatment of a cardiovascular condition are also provided.

According to still another aspect of the invention, a solid-phase nucleic acid molecule array, is provided. The array consists essentially of a set of nucleic acid molecules, expression products thereof, or fragments (of either the nucleic acid or the polypeptide molecule) thereof, wherein at least two and as many as all of the nucleic acid molecules selected from the group consisting of Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1 (including expression products thereof, or fragments thereof), are fixed to a solid substrate. In some embodiments, the solid-phase array further comprises at least one control nucleic acid molecule. In certain embodiments, the set of nucleic acid molecules comprises at least three, at least four, or even at least five nucleic acid molecules, each selected from the group consisting of Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1. In preferred embodiments, the set of nucleic acid molecules comprises a maximum number of 100 different nucleic acid molecules. In important embodiments, the set of nucleic acid molecules comprises a maximum number of 10 different nucleic acid molecules.

In certain embodiments, the solid substrate includes a material selected from the group consisting of glass, silica, aluminosilicates, borosilicates, metal oxides such as alumina and nickel oxide, various clays, nitrocellulose, and nylon. Preferably the substrate is glass. In some embodiments, the nucleic acid molecules are fixed to the solid substrate by covalent bonding.

These and other objects of the invention will be described in further detail in connection with the detailed description of the invention.

Brief Description of the Sequences

SEQ ID NO:1 is the nucleotide sequence of the rat Fit-1S cDNA.

SEQ ID NO:2 is the predicted amino acid sequence of the translation product of rat Fit-1S cDNA (SEQ ID NO:1).

SEQ ID NO:3 is the nucleotide sequence of the rat Fit-1M cDNA.

SEQ ID NO:4 is the predicted amino acid sequence of the translation product of the rat Fit-1M cDNA (SEQ ID NO:3).

SEQ ID NO:5 is the nucleotide sequence of the rat vacuolar ATPase cDNA (GenBank Acc. No. Y12635).

SEQ ID NO:6 is the predicted amino acid sequence of the translation product of the rat vacuolar ATPase cDNA (SEQ ID NO:5).

SEQ ID NO:7 is the nucleotide sequence of the rat glycoprotein CD44 cDNA (GenBank Acc. No. M61875).

SEQ ID NO:8 is the predicted amino acid sequence of the translation product of the rat glycoprotein CD44 cDNA (SEQ ID NO:7).

SEQ ID NO:9 is the nucleotide sequence of the rat Lot-1 cDNA (GenBank Acc. No. U72620).

SEQ ID NO:10 is the predicted amino acid sequence of the translation product of the rat Lot-1 cDNA (SEQ ID NO:9).

SEQ ID NO:11 is the nucleotide sequence of the rat AA892598 (EST196401) cDNA.

SEQ ID NO:12 is the nucleotide sequence of the rat Mrg-1 cDNA (GenBank Acc. No. AA900476).

SEQ ID NO:13 is the nucleotide sequence of the mouse ST2 cDNA (GenBank Acc. No. Y07519).

SEQ ID NO:14 is the nucleotide sequence of the mouse ST2L cDNA (GenBank Acc. No. D13695).

SEQ ID NO:15 is the nucleotide sequence of the bovine vacuolar H+-ATPase cDNA (GenBank Acc. No. M88690).

SEQ ID NO:16 is the nucleotide sequence of the human vacuolar H+-ATPase cDNA (GenBank Acc. No. NM_001693).

SEQ ID NO:17 is the nucleotide sequence of the mouse vacuolar H+-ATPase cDNA (GenBank Acc. No. NM_007509).

SEQ ID NO:18 is the nucleotide sequence of the human vacuolar H+-ATPase cDNA (56,000 subunit-HO57) (GenBank Acc. No. L35249).

SEQ ID NO:19 is the nucleotide sequence of the human vacuolar H+-ATPase cDNA (B subunit) (GenBank Acc. No. M60346).

SEQ ID NO:20 is the nucleotide sequence of the bovine vacuolar H+-ATPase cDNA (B subunit) (GenBank Acc. No. M83131).

SEQ ID NO:21 is the nucleotide sequence of the gallus vacuolar H+-ATPase cDNA (GenBank Acc. No. U61724).

SEQ ID NO:22 is the nucleotide sequence of the human CD44R cDNA (GenBank Acc. No. X56794).

SEQ ID NO:23 is the nucleotide sequence of the human CD44 cDNA (GenBank Acc. No. U40373).

SEQ ID NO:24 is the nucleotide sequence of the mouse CD44 cDNA (GenBank Acc. No. M27129).

SEQ ID NO:25 is the nucleotide sequence of the hamster CD44 cDNA (GenBank Acc. No. M33827).

SEQ ID NO:26 is the nucleotide sequence of the human LOT1 cDNA (GenBank Acc. No. U72621).

SEQ ID NO:27 is the nucleotide sequence of the human ZAC zinc finger protein cDNA (GenBank Acc. No. AJ006354).

SEQ ID NO:28 is the nucleotide sequence of the mouse ZAC1 zinc finger protein cDNA (GenBank Acc. No. AF147785).

SEQ ID NO:29 is the nucleotide sequence having GenBank Acc. No. AF191918.1.

SEQ ID NO:30 is the nucleotide sequence of the human putative nucleotide binding protein, estradiol-induced (E2IG3) cDNA (GenBank Acc. No. NM_014366).

SEQ ID NO:31 is the nucleotide sequence of the mouse mrg-1 cDNA (GenBank Acc. No. Y15163).

SEQ ID NO:32 is the nucleotide sequence of the human p35srj cDNA (GenBank Acc. No. AF129290).

SEQ ID NO:33 is the nucleotide sequence of the human p35srj (mrg-1) cDNA (GenBank Acc. No. AF109161).

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** depicts by a Northern Blot the effects of 8% cyclic mechanical strain on the expression of Fit-1 in cultured cardiac myocytes over the course of time.

FIG. **2** depicts by a Northern Blot the effects of 8% cyclic mechanical strain, angiotensin receptor blockade, angiotensin II, IL-1b, and phorbal ester, on the expression of Fit-1 in cultured cardiac myocytes over the course of time.

FIG. **3** depicts by a Northern Blot the effects of 8% cyclic mechanical strain, hydrogen peroxide, and TIRON, on the expression of Fit-1 in cultured cardiac myocytes over the course of time.

FIG. **4** depicts by a Northern Blot the effects of actinomycin D and cyclohexamide on the induction of Fit-1 expression during an 8% cyclic mechanical strain on cardiac myocytes over the course of time.

FIG. **5** depicts by a Northern Blot the effects of 8% cyclic mechanical strain alone and in combination with IL-1b, and phorbal ester in the absence of strain, on the expression of Fit-1 in cultured cardiac myocytes over the course of time.

FIG. **6** depicts by a Northern Blot the effects of an 8% cyclic mechanical strain on the expression of vacuolar ATPase in cultured cardiac myocytes over the course of time.

FIG. **7** depicts a kit embodying features of the present invention.

FIG. **8** depicts early (left) and late (right) time course of the mRNA induction of T2/ST2 by mechanical strain in cardiac myocytes. Maximal induction occurs at 3 hours, is sustained for 9 hours and declines by 15 hours. Top panels, T1/ST2 RNA; bottom panels, ethidium bromide. No str, no strain.

FIG. **9** depicts mRNA induction of T1/ST2 by mechanical strain (8%), interleukin-1 (10 ng/ml) and phorbol ester (PMA, 200 nM) at 1 and 3 hours. PMA>strain>IL-1. Top panel, T1/ST2 mRNA, bottom panel, ethidium bromide.

FIG. **10** depicts T1/ST2 may be a gene induced by NF-κB activation during IL-1/IL-receptor signaling in cardiac myocytes. IL-1 and strain induced T1/ST2 mRNA in the presence of infection with control adenovirus (left). With infection of IκB adenovirus (right), which decreases NF-κB DNA binding activity, the IL-1 induction of T1/ST2 was blocked. The strain induction of T1/ST2 was partially blocked by IκB infection suggesting another pathway for induction of T1/ST2 by strain. Top panel, T1/ST2 mRNA; bottom panel, ethidium bromide.

FIG. **11** shows expression of T1/st2 protein following myocardial infiltration in mice by immunohistochemistry at 1 day but not 3 days after inffarction. 40× magnification.

DETAILED DESCRIPTION OF THE INVENTION

The invention involves the discovery of a number of genes that are upregulated in cardiac cells when the cells are subjected to a mechanically-induced strain deformation. In view of this discovery, it is believed that the molecules of the present invention can be used to treat cardiovascular conditions including cardiac hypertrophy, myocardial infarction, stroke, arteriosclerosis, and/or heart failure.

Additionally, methods for using these molecules in the diagnosis of any of the foregoing cardiovascular conditions, are also provided.

Furthermore, compositions useful in the preparation of therapeutic preparations for the treatment of the foregoing conditions, are also provided.

"Upregulated," as used herein, refers to increased expression of a gene and/or its encoded polypeptide. "Increased expression" refers to increasing (i.e., to a detectable extent) replication, transcription, and/or translation of any of the nucleic acids of the invention (Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1), since upregulation of any of these processes results in concentration/amount increase of the polypeptide encoded by the gene (nucleic acid). Conversely, "downregulation," or "decreased expression" as used herein, refers to decreased expression of a gene and/or its encoded polypeptide. The upregulation or downregulation of gene expression can be directly determined by detecting an increase or decrease, respectively, in the level of mRNA for the gene, or the level of protein expression of the gene-encoded polypeptide, using any suitable means known to the art, such as nucleic acid hybridization or antibody detection methods, respectively, and in comparison to controls.

A "cardiac cell", as used herein, refers to a cardiomyocyte.

A "molecule," as used herein, embraces both "nucleic acids" and "polypeptides."

"Expression," as used herein, refers to nucleic acid and/or polypeptide expression.

As used herein, a "subject" is a mammal or a non-human mammal. In all embodiments human nucleic acids, polypeptides, and human subjects are preferred. Although only rat sequences are exemplified in the Sequence Listing and the Examples section, it is believed that the results obtained using such compositions are predictive of the results that may be obtained using homologous human sequences.

In general human homologs and alleles typically will share at least 80% nucleotide identity and/or at least 85% amino acid identity to the characterized rat sequences of the invention. In further instances, human homologs and alleles typically will share at least 90%, 95%, or even 99% nucleotide identity and/or at least 95%, 98%, or even 99% amino acid identity to the characterized rat sequences, respectively. The homology can be calculated using various, publicly available software tools developed by NCBI (Bethesda, Md.). Exemplary tools include the heuristic algorithm of Altschul S F, et al., (*J Mol Biol,* 1990, 215:403-410), also known as BLAST. Pairwise and ClustalW alignments (BLOSUM30 matrix setting) as well as Kyte-Doolittle hydropathic analysis can be obtained using public (EMBL, Heidelberg, Germany) and commercial (e.g., the MacVector sequence analysis software from Oxford Molecular Group/Genetics Computer Group, Madison, Wis., Accelrys, Inc., San Diego, Calif.). Watson-Crick complements of the foregoing nucleic acids also are embraced by the invention.

In screening for human related genes, such as homologs and alleles of the rat sequences described elsewhere herein, a Southern blot may be performed using stringent conditions, together with a probe. The term "stringent conditions" as used herein refers to parameters with which the art is familiar. Nucleic acid hybridization parameters may be found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, or *Current Protocols in Molecular Biology*, F. M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. For example, stringent conditions may refer to hybridizaotn at 65° C. in 6×SSC. Alternatively, stringent conditions, as used herein, may refer, for example, to hybridization at 65° C. in hybridization buffer (3.5×SSC, 0.02% Ficoll, 0.02% polyvinyl pyrolidone, 0.02% Bovine Serum Albumin, 2.5 mM NaH_2PO_4(pH7), 0.5% SDS, 2 mM EDTA). SSC is 0.15M sodium chloride/0.15M sodium citrate, pH7; SDS is sodium dodecyl sulphate; and EDTA is ethylenediaminetetra acetic acid. After hybridization, the membrane upon which the DNA is transferred is washed at 2×SSC at room temperature and then at 0.1×SSC/0.1×SDS at temperatures up to 68° C. In a further example, an alternative to the use of an aqueous hybridization solution is the use of a formamide hybridization solution. Stringent hybridization conditions can thus be achieved using, for example, a 50% formamide solution and 42° C.

There are other conditions, reagents, and so forth which can be used, and would result in a similar degree of stringency. The skilled artisan will be familiar with such conditions, and thus they are not given here. It will be understood, however, that the skilled artisan will be able to manipulate the conditions in a manner to permit the clear identification of human homologs and alleles of the rat nucleic acids of the invention. The skilled artisan also is familiar with the methodology for screening cells and libraries for expression of such molecules which then are routinely isolated, followed by isolation of the pertinent nucleic acid molecule and sequencing.

Given the teachings herein of full-length rat cDNA clones, other mammalian sequences such as the human (mouse, bovine, etc.) cDNAs corresponding to the related rat nucleic acids can be isolated from cDNA libraries using standard colony hybridization techniques, or can be identified using a homology search, for example, in GenBank using any of the algorithms described elsewhere herein. For example, sequences with GenBank Accession numbers Y07519.1 (SEQ ID NO:13) and D13695.1 (SEQ ID NO:14) for Fit-1 homologs), M88690.1 (SEQ ID NO:15), NM_001693.1 (SEQ ID NO:16), NM_007509.1 (SEQ ID NO:17), L35249.1 (SEQ ID NO:18), M60346.1 (SEQ ID NO:19), M83131.1 (SEQ ID NO:20 and U61724.1 (SEQ ID NO:21) for vacuolar ATPase homologs), X56794.1 (SEQ ID NO:22), U40373.1 (SEQ ID NO:23), M27129.1 (SEQ ID NO:24), and M33827.1 (SEQ ID NO:25) for CD44 homologs), U72621.3 (SEQ ID NO:26), AJ006354.1 (SEQ ID NO:27), and AF147785.1 (SEQ ID NO:28) for Lot-1 homologs), AF191918.1 (SEQ ID NO:29) and NM_014366.1 (SEQ ID NO:30) for AA892598 homologs), and Y15163.1 (SEQ ID NO:31), AF129290.1 (SEQ ID NO:32), and AF109161.1 (SEQ ID NO:33) for Mrg-1 homologs), can be used interchangeably with the homologous rat sequences of the invention, in all aspects of the invention without departing from the essence of the invention.

As used herein with respect to nucleic acids, the term "isolated" means: (i) amplified in vitro by, for example, polymerase chain reaction (PCR); (ii) recombinantly produced by cloning; (iii) purified, as by cleavage and gel separation; or (iv) synthesized by, for example, chemical synthesis. An isolated nucleic acid is one which is readily manipulated by recombinant DNA techniques well known in the art. Thus, a nucleotide sequence contained in a vector in which 5' and 3' restriction sites are known or for which polymerase chain reaction (PCR) primer sequences have been disclosed is considered isolated, but a nucleic acid sequence existing in its native state in its natural host is not. An isolated nucleic acid may be substantially purified, but need not be. For example, a nucleic acid that is isolated within a cloning or expression vector is not pure in that it may comprise only a tiny percentage of the material in the cell in which it resides. Such a

nucleic acid is isolated, however, as the term is used herein because it is readily manipulated by standard techniques known to those of ordinary skill in the art.

According to the invention, expression of any of the foregoing nucleic acids (i.e., Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1), including unique fragments of the foregoing, can be determined using different methodologies. A "unique fragment," as used herein, with respect to a nucleic acid is one that is a "signature" for the larger nucleic acid. For example, the unique fragment is long enough to assure that its precise sequence is not found in molecules within the human genome outside of the sequence for each nucleic acid defined above (Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1, including their human alleles). Those of ordinary skill in the art may apply no more than routine procedures to determine if a fragment is unique within the human genome. Unique fragments, however, exclude fragments completely composed of nucleotide sequences previously published as of the filing date of this application.

Unique fragments can be used as probes in Southern and Northern blot assays to identify such nucleic acids, or can be used in amplification assays such as those employing PCR. As known to those skilled in the art, large probes such as 200, 250, 300 or more nucleotides are preferred for certain uses such as Southern and Northern blots, while smaller fragments will be preferred for other uses such as PCR. Unique fragments also can be used to produce fusion proteins for generating antibodies, or determining binding of the polypeptide fragments, or for generating immunoassay components. Likewise, unique fragments can be employed to produce nonfused fragments of, for example, the Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1 polypeptides, useful, for example, in the preparation of antibodies, immunoassays or therapeutic applications. Unique fragments further can be used as antisense molecules to inhibit the expression of the foregoing nucleic acids and polypeptides respectively.

As will be recognized by those skilled in the art, the size of the unique fragment will depend upon its conservancy in the genetic code. Thus, some regions of SEQ ID NOs: 1, 3, 5, 7, 9, 11 and 12, and complements will require longer segments to be unique while others will require only short segments, typically between 12 and 32 nucleotides long (e.g., 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31 and 32 bases) or more, up to the entire length of each of the disclosed sequences. As mentioned above, this disclosure intends to embrace each and every fragment of each sequence, beginning at the first nucleotide, the second nucleotide and so on, up to 8 nucleotides short of the end, and ending anywhere from nucleotide number 8, 9, 10 and so on for each sequence, up to the very last nucleotide, (provided the sequence is unique as described above). For example, virtually any segment of the region of SEQ ID NO:1 beginning at nucleotide 1 and ending at nucleotide 2586, or SEQ ID NO:3 beginning at nucleotide 1 and ending at nucleotide 2065, or complements thereof, that is 20 or more nucleotides in length will be unique. Those skilled in the art are well versed in methods for selecting such sequences, typically on the basis of the ability of the unique fragment to selectively distinguish the sequence of interest from other sequences in the human genome of the fragment to those on known databases typically is all that is necessary, although in vitro confirmatory hybridization and sequencing analysis may be performed.

As used herein with respect to polypeptides, the term "isolated" means separated from its native environment in sufficiently pure form so that it can be manipulated or used for any one of the purposes of the invention. Thus, isolated means sufficiently pure to be used (i) to raise and/or isolate antibodies, (ii) as a reagent in an assay, (iii) for sequencing, (iv) as a therapeutic, etc.

In certain aspects, the invention embraces antisense oligonucleotides that selectively bind to a nucleic acid molecule encoding a polypeptide, to decrease the polypeptide's activity.

As used herein, the terms "antisense molecules," "antisense oligonucleotide," and "antisense" describe an oligonucleotide that is an oligoribonucleotide, oligodeoxyribonucleotide, modified oligoribonucleotide, or modified oligodeoxyribonucleotide which hybridizes under physiological conditions to DNA comprising a particular gene or to an mRNA transcript of that gene and, thereby, inhibits the transcription of that gene and/or the translation of that mRNA. The antisense molecules are designed so as to interfere with transcription or translation of a target gene upon hybridization with the target gene or transcript. Those skilled in the art will recognize that the exact length of an antisense oligonucleotide and its degree of complementarity with its target will depend upon the specific target selected, including the sequence of the target and the particular bases which comprise that sequence. It is preferred that an antisense oligonucleotide be constructed and arranged so as to bind selectively with a target under physiological conditions, i.e., to hybridize substantially more to the target sequence than to any other sequence in the target cell under physiological conditions. Based upon SEQ ID NOs: 1, 3, 5, 7, 9, 11 and 12, or upon allelic or homologous genomic and/or cDNA sequences, one of skill in the art can easily choose and synthesize any of a number of appropriate antisense molecules for use in accordance with the present invention. In order to be sufficiently selective and potent for inhibition, such antisense oligonucleotides should comprise at least 10 and, more preferably, at least 15 consecutive bases which are complementary to the target, although in certain cases modified oligonucleotides as short as 7 bases in length have been used successfully as antisense oligonucleotides (Wagner et al., *Nat. Med,* 1995, 1(11):1116-1118*; Nat. Biotech.,* 1996, 14:840-844). Most preferably, the antisense oligonucleotides comprise a complementary sequence of 20-30 bases. Although oligonucleotides may be chosen which are antisense to any region of the gene or mRNA transcripts, in preferred embodiments the antisense oligonucleotides correspond to N-terminal or 5' upstream sites such as translation initiation, transcription initiation or promoter sites. In addition, 3'-untranslated regions may be targeted by antisense oligonucleotides. Targeting to mRNA splicing sites has also been used in the art but may be less preferred if alternative mRNA splicing occurs. In addition, the antisense is targeted, preferably, to sites in which mRNA secondary structure is not expected (see, e.g., Sainio et al., *Cell Mol. Neurobiol.* 14(5): 439-457, 1994) and at which proteins are not expected to bind. Finally, although, SEQ ID NOs: 1, 3, 5, 7, 9, 11 and 12 disclose cDNA sequences, one of ordinary skill in the art may easily derive the genomic DNA corresponding to the foregoing sequences. Thus, the present invention also provides for antisense oligonucleotides which are complementary to the genomic DNA corresponding to SEQ ID NOs: 1, 3, 5, 7, 9, 11 and 12. Similarly, antisense to allelic or homologous human cDNAs and genomic DNAs are enabled without undue experimentation.

In one set of embodiments, the antisense oligonucleotides of the invention may be composed of "natural" deoxyribonucleotides, ribonucleotides, or any combination thereof. That is, the 5' end of one native nucleotide and the 3' end of

another native nucleotide may be covalently linked, as in natural systems, via a phosphodiester internucleoside linkage. These oligonucleotides may be prepared by art recognized methods which may be carried out manually or by an automated synthesizer. They also may be produced recombinantly by vectors.

In preferred embodiments, however, the antisense oligonucleotides of the invention also may include "modified" oligonucleotides. That is, the oligonucleotides may be modified in a number of ways which do not prevent them from hybridizing to their target but which enhance their stability or targeting or which otherwise enhance their therapeutic effectiveness.

The term "modified oligonucleotide" as used herein describes an oligonucleotide in which (1) at least two of its nucleotides are covalently linked via a synthetic internucleoside linkage (i.e., a linkage other than a phosphodiester linkage between the 5' end of one nucleotide and the 3' end of another nucleotide) and/or (2) a chemical group not normally associated with nucleic acids has been covalently attached to the oligonucleotide. Preferred synthetic internucleoside linkages are phosphorothioates, alkylphosphonates, phosphorodithioates, phosphate esters, alkylphosphonothioates, phosphoramidates, carbamates, carbonates, phosphate triesters, acetamidates, carboxymethyl esters and peptides.

The term "modified oligonucleotide" also encompasses oligonucleotides with a covalently modified base and/or sugar. For example, modified oligonucleotides include oligonucleotides having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified oligonucleotides may include a 2'-O-alkylated ribose group. In addition, modified oligonucleotides may include sugars such as arabinose in place of ribose. The present invention, thus, contemplates pharmaceutical preparations containing modified antisense molecules that are complementary to and hybridizable with, under physiological conditions, nucleic acids encoding the polypeptides with SEQ ID NOs: 2, 4, 6, 8, and/or 10, together with pharmaceutically acceptable carriers.

Antisense oligonucleotides may be administered as part of a pharmaceutical composition. Such a pharmaceutical composition may include the antisense oligonucleotides in combination with any standard physiologically and/or pharmaceutically acceptable carriers which are known in the art. The compositions should be sterile and contain a therapeutically effective amount of the antisense oligonucleotides in a unit of weight or volume suitable for administration to a patient. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. The term "physiologically acceptable" refers to a non-toxic material that is compatible with a biological system such as a cell, cell culture, tissue, or organism. The characteristics of the carrier will depend on the route of administration. Physiologically and pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials which are well known in the art.

The invention also involves expression vectors coding for proteins encoded by the nucleic acids corresponding to SEQ ID NOs: 1, 3, 5, 7, 9, 11 and/or 12, fragments and variants thereof, and host cells containing those expression vectors. Virtually any cells, prokaryotic or eukaryotic, which can be transformed with heterologous DNA or RNA and which can be grown or maintained in culture, may be used in the practice of the invention. Examples include bacterial cells such as *Escherichia coli* and mammalian cells such as mouse, hamster, pig, goat, primate, etc. They may be of a wide variety of tissue types, including mast cells, fibroblasts, oocytes and lymphocytes, and they may be primary cells or cell lines. Specific examples include CHO cells and COS cells. Cell-free transcription systems also may be used in lieu of cells.

As used herein, a "vector" may be any of a number of nucleic acids into which a desired sequence may be inserted by restriction and ligation for transport between different genetic environments or for expression in a host cell. Vectors are typically composed of DNA although RNA vectors are also available. Vectors include, but are not limited to, plasmids, phagemids and virus genomes. A cloning vector is one which is able to replicate in a host cell, and which is further characterized by one or more endonuclease restriction sites at which the vector may be cut in a determinable fashion and into which a desired DNA sequence may be ligated such that the new recombinant vector retains its ability to replicate in the host cell. In the case of plasmids, replication of the desired sequence may occur many times as the plasmid increases in copy number within the host bacterium or just a single time per host before the host reproduces by mitosis. In the case of phage, replication may occur actively during a lytic phase or passively during a lysogenic phase. An expression vector is one into which a desired DNA sequence may be inserted by restriction and ligation such that it is operably joined to regulatory sequences and may be expressed as an RNA transcript. Vectors may further contain one or more marker sequences suitable for use in the identification of cells which have or have not been transformed or transfected with the vector. Markers include, for example, genes encoding proteins which increase or decrease either resistance or sensitivity to antibiotics or other compounds, genes which encode enzymes whose activities are detectable by standard assays known in the art (e.g., β-galactosidase or alkaline phosphatase), and genes which visibly affect the phenotype of transformed or transfected cells, hosts, colonies or plaques (e.g., green fluorescent protein). Preferred vectors are those capable of autonomous replication and expression of the structural gene products present in the DNA segments to which they are operably joined.

As used herein, a coding sequence and regulatory sequences are said to be "operably joined" when they are covalently linked in such a way as to place the expression or transcription of the coding sequence under the influence or control of the regulatory sequences. If it is desired that the coding sequences be translated into a functional protein, two DNA sequences are said to be operably joined if induction of a promoter in the 5' regulatory sequences results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a promoter region would be operably joined to a coding sequence if the promoter region were capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

The precise nature of the regulatory sequences needed for gene expression may vary between species or cell types, but shall in general include, as necessary, 5' non-transcribed and 5' non-translated sequences involved with the initiation of transcription and translation respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Such 5' non-transcribed regulatory sequences will often include a promoter region which includes a promoter sequence for

transcriptional control of the operably joined gene. Regulatory sequences may also include enhancer sequences or upstream activator sequences as desired. The vectors of the invention may optionally include 5' leader or signal sequences. The choice and design of an appropriate vector is within the ability and discretion of one of ordinary skill in the art.

Expression vectors containing all the necessary elements for expression are commercially available and known to those skilled in the art. See, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, 1989. Cells are genetically engineered by the introduction into the cells of heterologous DNA (RNA) encoding a polypeptide or fragment or variant thereof. That heterologous DNA (RNA) is placed under operable control of transcriptional elements to permit the expression of the heterologous DNA in the host cell.

Preferred systems for mRNA expression in mammalian cells are those such as pRc/CMV (available from Invitrogen, Carlsbad, Calif.) that contain a selectable marker such as a gene that confers G418 resistance (which facilitates the selection of stably transfected cell lines) and the human cytomegalovirus (CMV) enhancer-promoter sequences. Additionally, suitable for expression in primate or canine cell lines is the pCEP4 vector (Invitrogen, Carlsbad, Calif.), which contains an Epstein Barr virus (EBV) origin of replication, facilitating the maintenance of plasmid as a multicopy extrachromosomal element. Another expression vector is the pEF-BOS plasmid containing the promoter of polypeptide Elongation Factor 1α, which stimulates efficiently transcription in vitro. The plasmid is described by Mishizuma and Nagata (*Nuc. Acids Res.* 18:5322, 1990), and its use in transfection experiments is disclosed by, for example, Demoulin (*Mol. Cell. Biol.* 16:4710-4716, 1996). Still another preferred expression vector is an adenovirus, described by Stratford-Perricaudet, which is defective for E1 and E3 proteins (*J. Clin. Invest.* 90:626-630, 1992). The use of the adenovirus as an Adeno.P1A recombinant is disclosed by Warnier et al., in intradermal injection in mice for immunization against P1A (*Int. J. Cancer,* 67:303-310, 1996).

The invention also embraces so-called expression kits, which allow the artisan to prepare a desired expression vector or vectors. Such expression kits include at least separate portions of each of the previously discussed coding sequences. Other components may be added, as desired, as long as the previously mentioned sequences, which are required, are included.

It will also be recognized that the invention embraces the use of the above described SEQ ID NOs: 1, 3, 5, 7, 9, 11 and/or 12 cDNA sequence-containing expression vectors, to transfect host cells and cell lines, be these prokaryotic (e.g., *Escherichia coli*), or eukaryotic (e.g., CHO cells, COS cells, yeast expression systems and recombinant baculovirus expression in insect cells). Especially useful are mammalian cells such as mouse, hamster, pig, goat, primate, etc. They may be of a wide variety of tissue types, and include primary cells and cell lines. Specific examples include dendritic cells, U293 cells, peripheral blood leukocytes, bone marrow stem cells and embryonic stem cells.

The invention also provides isolated polypeptides (including whole proteins and partial proteins), encoded by the foregoing nucleic acids (SEQ ID NOs: 1, 3, 5, 7, 9, 11 and 12), and include the polypeptides of SEQ ID NOs: 2, 4, 6, 8, and/or 10, and unique fragments thereof. Such polypeptides are useful, for example, alone or as part of fusion proteins to generate antibodies, as components of an immunoassay, etc. Polypeptides can be isolated from biological samples including tissue or cell homogenates, and can also be expressed recombinantly in a variety of prokaryotic and eukaryotic expression systems by constructing an expression vector appropriate to the expression system, introducing the expression vector into the expression system, and isolating the recombinantly expressed protein. Short polypeptides, including antigenic peptides (such as are presented by MHC molecules on the surface of a cell for immune recognition) also can be synthesized chemically using well-established methods of peptide synthesis.

A unique fragment for each of the foregoing polypeptide, in general, has the features and characteristics of unique fragments as discussed above in connection with nucleic acids. As will be recognized by those skilled in the art, the size of the unique fragment will depend upon factors such as whether the fragment constitutes a portion of a conserved protein domain. Thus, some regions of a polypeptide will require longer segments to be unique while others will require only short segments, typically between 5 and 12 amino acids (e.g. 5, 6, 7, 8, 9, 10, 11 and 12 amino acids long or more, including each integer up to the full length of each polypeptide).

Unique fragments of a polypeptide preferably are those fragments which retain a distinct functional capability of the polypeptide. Functional capabilities which can be retained in a unique fragment of a polypeptide include interaction with antibodies, interaction with other polypeptides or fragments thereof, interaction with other molecules, etc. One important activity is the ability to act as a signature for identifying the polypeptide. Those skilled in the art are well versed in methods for selecting unique amino acid sequences, typically on the basis of the ability of the unique fragment to selectively distinguish the sequence of interest from non-family members. A comparison of the sequence of the fragment to those on known databases typically is all that is necessary.

The invention embraces variants of the polypeptides described above. As used herein, a "variant" of a polypeptide is a polypeptide which contains one or more modifications to the primary amino acid sequence of a natural (e.g., "wild-type": a polypeptide with an amino acid sequence selected from the group consisting of SEQ ID NO: 2, 4, 6, 8, and 10) polypeptide. Modifications which create a polypeptide variant are typically made to the nucleic acid which encodes the polypeptide, and can include deletions, point mutations, truncations, amino acid substitutions and addition of amino acids or non-amino acid moieties to: (1) reduce or eliminate an activity of a polypeptide; (2) enhance a property of a polypeptide, such as protein stability in an expression system or the stability of protein-ligand binding; (3) provide a novel activity or property to a polypeptide, such as addition of an antigenic epitope or addition of a detectable moiety; or (4) to provide equivalent or better binding to a polypeptide receptor or other molecule. Alternatively, modifications can be made directly to the polypeptide, such as by cleavage, addition of a linker molecule, addition of a detectable moiety, such as biotin, addition of a fatty acid, and the like. Modifications also embrace fusion proteins comprising all or part of the polypeptide's amino acid sequence. One of skill in the art will be familiar with methods for predicting the effect on protein conformation of a change in protein sequence, and can thus "design" a variant polypeptide according to known methods. One example of such a method is described by Dahiyat and Mayo in *Science* 278:82-87, 1997, whereby proteins can be designed de novo. The method can be applied to a known protein to vary only a portion of the polypeptide sequence. By applying the computational methods of Dahiyat and Mayo,

specific variants of any of the foregoing polypeptides can be proposed and tested to determine whether the variant retains a desired conformation.

Variants can include polypeptides which are modified specifically to alter a feature of the polypeptide unrelated to its physiological activity. For example, cysteine residues can be substituted or deleted to prevent unwanted disulfide linkages. Similarly, certain amino acids can be changed to enhance expression of a polypeptide by eliminating proteolysis by proteases in an expression system (e.g., dibasic amino acid residues in yeast expression systems in which KEX2 protease activity is present).

Mutations of a nucleic acid which encodes a polypeptide preferably preserve the amino acid reading frame of the coding sequence, and preferably do not create regions in the nucleic acid which are likely to hybridize to form secondary structures, such a hairpins or loops, which can be deleterious to expression of the variant polypeptide.

Mutations can be made by selecting an amino acid substitution, or by random mutagenesis of a selected site in a nucleic acid which encodes the polypeptide. Variant polypeptides are then expressed and tested for one or more activities to determine which mutation provides a variant polypeptide with the desired properties. Further mutations can be made to variants (or to non-variant polypeptides) which are silent as to the amino acid sequence of the polypeptide, but which provide preferred codons for translation in a particular host. The preferred codons for translation of a nucleic acid in, e.g., *Escherichia coli*, are well known to those of ordinary skill in the art. Still other mutations can be made to the noncoding sequences of a gene or cDNA clone to enhance expression of the polypeptide.

The skilled artisan will realize that conservative amino acid substitutions may be made in any of the foregoing polypeptides to provide functionally equivalent variants of the foregoing polypeptides, i.e., the variants retain the functional capabilities of each polypeptide. As used herein, a "conservative amino acid substitution" refers to an amino acid substitution which does not significantly alter the tertiary structure and/or activity of the polypeptide. Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art, and include those that are found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, or *Current Protocols in Molecular Biology*, F. M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. Conservative substitutions of amino acids include substitutions made amongst amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D.

Thus functionally equivalent variants of polypeptides, i.e., variants of polypeptides which retain the function of the natural ("wild-type") polypeptides, are contemplated by the invention. Conservative amino acid substitutions in the amino acid sequence of polypeptides to produce functionally equivalent variants of each polypeptide typically are made by alteration of a nucleic acid encoding the polypeptide. Such substitutions can be made by a variety of methods known to one of ordinary skill in the art. For example, amino acid substitutions may be made by PCR-directed mutation, site-directed mutagenesis according to the method of Kunkel (Kunkel, *Proc. Nat. Acad. Sci. U.S.A.* 82: 488-492, 1985), or by chemical synthesis of a gene encoding a polypeptide. The activity of functionally equivalent fragments of polypeptides can be tested by cloning the gene encoding the altered polypeptide into a bacterial or mammalian expression vector, introducing the vector into an appropriate host cell, expressing the altered polypeptide, and testing for a functional capability of the polypeptides as disclosed herein

The invention as described herein has a number of uses, some of which are described elsewhere herein. First, the invention permits isolation of polypeptides. A variety of methodologies well-known to the skilled artisan can be utilized to obtain isolated molecules. The polypeptide may be purified from cells which naturally produce the polypeptide by chromatographic means or immunological recognition. Alternatively, an expression vector may be introduced into cells to cause production of the polypeptide. In another method, mRNA transcripts may be microinjected or otherwise introduced into cells to cause production of the encoded polypeptide. Translation of mRNA in cell-free extracts such as the reticulocyte lysate system also may be used to produce polypeptides. Those skilled in the art also can readily follow known methods for isolating polypeptides. These include, but are not limited to, immunochromatography, HPLC, size-exclusion chromatography, ion-exchange chromatography and immune-affinity chromatography.

The invention also provides, in certain embodiments, "dominant negative" polypeptides derived from polypeptides. A dominant negative polypeptide is an inactive variant of a protein, which, by interacting with the cellular machinery, displaces an active protein from its interaction with the cellular machinery or competes with the active protein, thereby reducing the effect of the active protein. For example, a dominant negative receptor which binds a ligand but does not transmit a signal in response to binding of the ligand can reduce the biological effect of expression of the ligand. Likewise, a dominant negative catalytically-inactive kinase which interacts normally with target proteins but does not phosphorylate the target proteins can reduce phosphorylation of the target proteins in response to a cellular signal. Similarly, a dominant negative transcription factor which binds to a promoter site in the control region of a gene but does not increase gene transcription can reduce the effect of a normal transcription factor by occupying promoter binding sites without increasing transcription.

The end result of the expression of a dominant negative polypeptide in a cell is a reduction in function of active proteins. One of ordinary skill in the art can assess the potential for a dominant negative variant of a protein, and use standard mutagenesis techniques to create one or more dominant negative variant polypeptides. See, e.g., U.S. Pat. No. 5,580,723 and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, 1989. The skilled artisan then can test the population of mutagenized polypeptides for diminution in a selected activity and/or for retention of such an activity. Other similar methods for creating and testing dominant negative variants of a protein will be apparent to one of ordinary skill in the art.

The isolation of the cDNAs of the invention also makes it possible for the artisan to diagnose a disorder characterized by an aberrant expression of any of the foregoing cDNAs. These methods involve determining expression of each of the identified nucleic acids, and/or polypeptides derived therefrom. In the former situation, such determinations can be carried out via any standard nucleic acid determination assay, including the polymerase chain reaction, or assaying with labeled hybridization probes as exemplified below. In the latter situation, such determination can be carried out via any standard immunological assay using, for example, antibodies which bind to the secreted protein.

The invention also embraces isolated peptide binding agents which, for example, can be antibodies or fragments of antibodies ("binding polypeptides"), having the ability to selectively bind to any of the polypeptides of the invention (e.g., SEQ ID NO: 2, 4, 6, 8, or 10). Antibodies include polyclonal and monoclonal antibodies, prepared according to conventional methodology.

Significantly, as is well-known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W. R. (1986) *The Experimental Foundations of Modern Immunology* Wiley & Sons, Inc., New York; Roitt, I. (1991) *Essential Immunology,* 7th Ed., Blackwell Scientific Publications, Oxford). The pFc' and Fc regions, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated an $F(ab')_2$ fragment, retains both of the antigen binding sites of an intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an Fab fragment, retains one of the antigen binding sites of an intact antibody molecule. Proceeding further, Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

Within the antigen-binding portion of an antibody, as is well-known in the art, there are complementarity determining regions (CDRs), which directly interact with the epitope of the antigen, and framework regions (FRs), which maintain the tertiary structure of the paratope (see, in general, Clark, 1986; Roitt, 1991). In both the heavy chain Fd fragment and the light chain of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated respectively by three complementarity determining regions (CDR1 through CDR3). The CDRs, and in particular the CDR3 regions, and more particularly the heavy chain CDR3, are largely responsible for antibody specificity.

It is now well-established in the art that the non-CDR regions of a mammalian antibody may be replaced with similar regions of conspecific or heterospecific antibodies while retaining the epitopic specificity of the original antibody. This is most clearly manifested in the development and use of "humanized" antibodies in which non-human CDRs are covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody. See, e.g., U.S. Pat. Nos. 4,816, 567; 5,225,539; 5,585,089; 5,693,762 and 5,859,205. Thus, for example, PCT International Publication Number WO 92/04381 teaches the production and use of humanized murine RSV antibodies in which at least a portion of the murine FR regions have been replaced by FR regions of human origin. Such antibodies, including fragments of intact antibodies with antigen-binding ability, are often referred to as "chimeric" antibodies.

Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for $F(ab')_2$, Fab, Fv and Fd fragments; chimeric antibodies in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric $F(ab')_2$ fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric Fab fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; and chimeric Fd fragment antibodies in which the FR and/or CDR1 and/or CDR2 regions have been replaced by homologous human or non-human sequences. The present invention also includes so-called single chain antibodies.

Thus, the invention involves polypeptides of numerous size and type that bind specifically to polypeptides of the invention (e.g., SEQ ID NO: 2, 4, 6, 8, or 10), and complexes of both the polypeptides and their binding partners. These polypeptides may be derived also from sources other than antibody technology. For example, such polypeptide binding agents can be provided by degenerate peptide libraries which can be readily prepared in solution, in immobilized form, as bacterial flagella peptide display libraries or as phage display libraries. Combinatorial libraries also can be synthesized of peptides containing one or more amino acids. Libraries further can be synthesized of peptides and non-peptide synthetic moieties.

The invention further provides efficient methods of identifying agents or lead compounds for agents active at the level of a polypeptide or polypeptide fragment dependent cellular function. In particular, such functions include interaction with other polypeptides or fragments. Generally, the screening methods involve assaying for compounds which interfere with the activity of a polypeptide of the invention, although compounds which enhance such activity also can be assayed using the screening methods. Such methods are adaptable to automated, high throughput screening of compounds. Target indications include cellular processes modulated by such polypeptides, for example, overexpression in cells under mechanical strains.

A wide variety of assays for candidate (pharmacological) agents are provided, including, labeled in vitro protein-ligand binding assays, electrophoretic mobility shift assays, immunoassays, cell-based assays such as two- or three-hybrid screens, expression assays, etc. The transfected nucleic acids can encode, for example, combinatorial peptide libraries or cDNA libraries. Convenient reagents for such assays, e.g., GAL4 fusion proteins, are known in the art. An exemplary cell-based assay involves transfecting a cell with a nucleic acid encoding a polypeptide of the invention fused to a GAL4 DNA binding domain and a nucleic acid encoding a reporter gene operably joined to a gene expression regulatory region, such as one or more GAL4 binding sites. Activation of reporter gene transcription occurs when the reporter fusion polypeptide binds an agent such as to enable transcription of the reporter gene. Agents which modulate polypeptide mediated cell function are then detected through a change in the expression of reporter gene. Methods for determining changes in the expression of a reporter gene are known in the art.

Polypeptide fragments used in the methods, when not produced by a transfected nucleic acid are added to an assay mixture as an isolated polypeptide. Polypeptides preferably are produced recombinantly, although such polypeptides may be isolated from biological extracts. Recombinantly produced polypeptides include chimeric proteins comprising a fusion of a protein of the invention with another polypeptide, e.g., a polypeptide capable of providing or enhancing protein-protein binding, sequence specific nucleic acid binding (such as GAL4), enhancing stability of the polypeptide of the invention under assay conditions, or providing a detectable moiety, such as green fluorescent protein or a Flag epitope.

The assay mixture is comprised of a natural intracellular or extracellular binding target capable of interacting with a

polypeptide of the invention. While natural polypeptide binding targets may be used, it is frequently preferred to use portions (e.g., peptides or nucleic acid fragments) or analogs (i.e., agents which mimic the polypeptide's binding properties of the natural binding target for purposes of the assay) of the polypeptide binding target so long as the portion or analog provides binding affinity and avidity to the polypeptide fragment measurable in the assay.

The assay mixture also comprises a candidate agent. Typically, a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a different response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e., at zero concentration of agent or at a concentration of agent below the limits of assay detection. Candidate agents encompass numerous chemical classes, although typically they are organic compounds. Preferably, the candidate agents are small organic compounds, i.e., those having a molecular weight of more than about 50 yet less than about 2500, preferably less than about 1000 and, more preferably, less than about 500. Candidate agents comprise functional chemical groups necessary for structural interactions with polypeptides and/or nucleic acids, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups and more preferably at least three of the functional chemical groups. The candidate agents can comprise cyclic carbon or heterocyclic structure and/or aromatic or polyaromatic structures substituted with one or more of the above-identified functional groups. Candidate agents also can be biomolecules such as peptides, saccharides, fatty acids, sterols, isoprenoids, purines, pyrimidines, derivatives or structural analogs of the above, or combinations thereof and the like. Where the agent is a nucleic acid, the agent typically is a DNA or RNA molecule, although modified nucleic acids as defined herein are also contemplated.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides, synthetic organic combinatorial libraries, phage display libraries of random peptides, and the like. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural and synthetically produced libraries and compounds can be modified through conventional chemical, physical, and biochemical means. Further, known (pharmacological) agents may be subjected to directed or random chemical modifications such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs of the agents.

A variety of other reagents also can be included in the mixture. These include reagents such as salts, buffers, neutral proteins (e.g., albumin), detergents, etc. which may be used to facilitate optimal protein-protein and/or protein-nucleic acid binding. Such a reagent may also reduce non-specific or background interactions of the reaction components. Other reagents that improve the efficiency of the assay such as protease inhibitors, nuclease inhibitors, antimicrobial agents, and the like may also be used.

The mixture of the foregoing assay materials is incubated under conditions whereby, but for the presence of the candidate agent, the chosen polypeptide of the invention specifically binds a cellular binding target, a portion thereof or analog thereof. The order of addition of components, incubation temperature, time of incubation, and other parameters of the assay may be readily determined. Such experimentation merely involves optimization of the assay parameters, not the fundamental composition of the assay. Incubation temperatures typically are between 4° C. and 40° C. Incubation times preferably are minimized to facilitate rapid, high throughput screening, and typically are between 0.1 and 10 hours.

After incubation, the presence or absence of specific binding between the polypeptide and one or more binding targets is detected by any convenient method available to the user. For cell free binding type assays, a separation step is often used to separate bound from unbound components. The separation step may be accomplished in a variety of ways. Conveniently, at least one of the components is immobilized on a solid substrate, from which the unbound components may be easily separated. The solid substrate can be made of a wide variety of materials and in a wide variety of shapes, e.g., microtiter plate, microbead, dipstick, resin particle, etc. The substrate preferably is chosen to maximize signal to noise ratios, primarily to minimize background binding, as well as for ease of separation and cost.

Separation may be effected for example, by removing a bead or dipstick from a reservoir, emptying or diluting a reservoir such as a microtiter plate well, rinsing a bead, particle, chromatographic column or filter with a wash solution or solvent. The separation step preferably includes multiple rinses or washes. For example, when the solid substrate is a microtiter plate, the wells may be washed several times with a washing solution, which typically includes those components of the incubation mixture that do not participate in specific bindings such as salts, buffer, detergent, a non-specific protein, etc. When the solid substrate is a magnetic bead(s), the bead(s) may be washed one or more times with a washing solution and isolated using a magnet.

Detection may be effected in any convenient way for cell-based assays such as two- or three-hybrid screens. The transcript resulting from a reporter gene transcription assay of a polypeptide interacting with a target molecule typically encodes a directly or indirectly detectable product, e.g., β-galactosidase activity, luciferase activity, and the like. For cell free binding assays, one of the components usually comprises, or is coupled to, a detectable label. A wide variety of labels can be used, such as those that provide direct detection (e.g., radioactivity, luminescence, optical or electron density, etc), or indirect detection (e.g., epitope tag such as the FLAG epitope, enzyme tag such as horseradish peroxidase, etc.). The label may be bound to a binding partner of the polypeptide, or incorporated into the structure of the binding partner.

A variety of methods may be used to detect the label, depending on the nature of the label and other assay components. For example, the label may be detected while bound to the solid substrate or subsequent to separation from the solid substrate. Labels may be directly detected through optical or electron density, radioactive emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, streptavidin-biotin conjugates, etc. Methods for detecting the labels are well known in the art.

The invention provides polypeptide-specific binding agents, methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, polypeptide-specific pharmacological agents are useful in a variety of diagnostic and therapeutic applications, especially where disease or disease prognosis is associated with altered polypeptide binding characteristics. Novel polypeptide-specific binding agents include polypeptide-specific antibodies, cell surface receptors, and other natural intracellular and extracellular binding agents identified with assays such as two hybrid screens, and non-natural

intracellular and extracellular binding agents identified in screens of chemical libraries and the like.

In general, the specificity of polypeptide binding to a specific molecule is determined by binding equilibrium constants. Targets which are capable of selectively binding a polypeptide preferably have binding equilibrium constants of at least about 10^7 M^{-1}, more preferably at least about 10^8 M^{-1}, and most preferably at least about 10^9 M^{-1}. A wide variety of cell based and cell free assays may be used to demonstrate polypeptide-specific binding. Cell based assays include one, two and three hybrid screens, assays in which polypeptide-mediated transcription is inhibited or increased, etc. Cell free assays include protein binding assays, immunoassays, etc. Other assays useful for screening agents which bind polypeptides of the invention include fluorescence resonance energy transfer (FRET), and electrophoretic mobility shift analysis (EMSA).

According to still another aspect of the invention, a method of diagnosing a disorder characterized by aberrant expression of a nucleic acid molecule, an expression product thereof, or a fragment of an expression product thereof, is provided. The method involves contacting a biological sample isolated from a subject with an agent that specifically binds to the nucleic acid molecule, an expression product thereof, or a fragment of an expression product thereof, and determining the interaction between the agent and the nucleic acid molecule or the expression product as a determination of the disorder, wherein the nucleic acid molecule is selected from the group consisting of Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1. In some embodiments, the disorder is a cardiovascular condition selected from the group consisting of myocardial infarction, stroke, arteriosclerosis, and heart failure. In one embodiment, the disorder is cardiac hypertrophy.

In the case where the molecule is a nucleic acid molecule, such determinations can be carried out via any standard nucleic acid determination assay, including the polymerase chain reaction, or assaying with labeled hybridization probes as exemplified herein. In the case where the molecule is an expression product of the nucleic acid molecule, or a fragment of an expression product of the nucleic acid molecule, such determination can be carried out via any standard immunological assay using, for example, antibodies which bind to any of the polypeptide expression products.

"Aberrant expression" refers to decreased expression (underexpression) or increased expression (overexpression) of any of the foregoing molecules (Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1, nucleic acids and/or polypeptides) in comparison with a control (i.e., expression of the same molecule in a healthy or "normal" subject). A "healthy subject," as used herein, refers to a subject who is not at risk for developing a future cardiovascular condition (see earlier discussion and Harrison's Principles of Experimental Medicine, 13th Edition, McGraw-Hill, Inc., N.Y.—hereinafter "Harrison's"). Healthy subjects also do not otherwise exhibit symptoms of disease. In other words, such subjects, if examined by a medical professional, would be characterized as healthy and free of symptoms of a cardiovascular disorder or at risk of developing a cardiovascular disorder.

When the disorder is a cardiovascular condition selected from the group consisting of cardiac hypertrophy, myocardial infarction, stroke, arteriosclerosis, and heart failure, decreased expression of any of the foregoing molecules in comparison with a control (e.g., a healthy individual) is indicative of the presence of the disorder, or indicative of the risk for developing such disorder in the future.

The invention also provides novel kits which could be used to measure the levels of the nucleic acids of the invention, or expression products of the invention.

In one embodiment, a kit comprises a package containing an agent that selectively binds to an isolated nucleic acid selected from the group consisting of Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1, or expression products thereof, and a control for comparing to a measured value of binding of said agent any of the foregoing isolated nucleic acids or expression products thereof. Kits are generally comprised of the following major elements: packaging, an agent of the invention, a control agent, and instructions. Packaging may be a box-like structure for holding a vial (or number of vials) containing an agent of the invention, a vial (or number of vials) containing a control agent, and instructions. Individuals skilled in the art can readily modify the packaging to suit individual needs. In some embodiments, the control is a predetermined value for comparing to the measured value. In certain embodiments, the control comprises an epitope of the expression product of any of the foregoing isolated nucleic acids.

In the case of nucleic acid detection, pairs of primers for amplifying a nucleic acid molecule of the invention can be included. The preferred kits would include controls such as known amounts of nucleic acid probes, epitopes (such as Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1 expression products) or anti-epitope antibodies, as well as instructions or other printed material. In certain embodiments the printed material can characterize risk of developing a cardiovascular condition based upon the outcome of the assay. The reagents may be packaged in containers and/or coated on wells in predetermined amounts, and the kits may include standard materials such as labeled immunological reagents (such as labeled anti-IgG antibodies) and the like. One kit is a packaged polystyrene microtiter plate coated with any of the foregoing proteins of the invention and a container containing labeled anti-human IgG antibodies. A well of the plate is contacted with, for example, a biological fluid, washed and then contacted with the anti-IgG antibody. The label is then detected. A kit embodying features of the present invention, generally designated by the numeral **11**, is illustrated in FIG. **7**. Kit **11** is comprised of the following major elements: packaging **15**, an agent of the invention **17**, a control agent **19**, and instructions **21**. Packaging **15** is a box-like structure for holding a vial (or number of vials) containing an agent of the invention **17**, a vial (or number of vials) containing a control agent **19**, and instructions **21**. Individuals skilled in the art can readily modify packaging **15** to suit individual needs.

The invention also embraces methods for treating a cardiovascular condition. In some embodiments, the method involves administering to a subject in need of such treatment a molecule selected from the group consisting of Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1, in an amount effective to treat the cardiovascular condition. In certain embodiments, the method involves administering to a subject in need of such treatment an agent that increases expression of any of the foregoing molecules (Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1), in an amount effective to treat the cardiovascular condition.

"Agents that increase expression" of a nucleic acid or a polypeptide, as used herein, are known in the art, and refer to sense nucleic acids, polypeptides encoded by the nucleic acids, and other agents that enhance expression of such molecules (e.g., transcription factors specific for the nucleic acids that enhance their expression). Any agents that increase

expression of a molecule (and as described herein, increase its activity), are useful according to the invention.

In certain embodiments, the molecule is a nucleic acid. In some embodiments the nucleic acid is operatively coupled to a gene expression sequence which directs the expression of the nucleic acid molecule within a cardiomyocyte. The "gene expression sequence" is any regulatory nucleotide sequence, such as a promoter sequence or promoter-enhancer combination, which facilitates the efficient transcription and translation of the nucleic acid to which it is operably joined. The gene expression sequence may, for example, be a mammalian or viral promoter, such as a constitutive or inducible promoter. Constitutive mammalian promoters include, but are not limited to, the promoters for the following genes: hypoxanthine phosphoribosyl transferase (HPTR), adenosine deaminase, pyruvate kinase, α-actin promoter and other constitutive promoters. Exemplary viral promoters which function constitutively in eukaryotic cells include, for example, promoters from the simian virus, papilloma virus, adenovirus, human immunodeficiency virus (HIV), Rous sarcoma virus, cytomegalovirus, the long terminal repeats (LTR) of Moloney leukemia virus and other retroviruses, and the thymidine kinase promoter of herpes simplex virus. Other constitutive promoters are known to those of ordinary skill in the art. The promoters useful as gene expression sequences of the invention also include inducible promoters. Inducible promoters are activated in the presence of an inducing agent. For example, the metallothionein promoter is activated to increase transcription and translation in the presence of certain metal ions. Other inducible promoters are known to those of ordinary skill in the art.

In general, the gene expression sequence shall include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with the initiation of transcription and translation, respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribing sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined nucleic acid. The gene expression sequences optionally includes enhancer sequences or upstream activator sequences as desired.

Preferably, any of the nucleic acid molecules of the invention (e.g., Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1) is linked to a gene expression sequence which permits expression of the nucleic acid molecule in a cell such as a cardiomyocyte and/or a vascular endothelial cell (including a smooth muscle cell). More preferably, the gene expression sequence permits expression of the nucleic acid molecule in a cardiomyocyte, and does not permit expression of the molecule in a cell selected from the group consisting of a neuronal cell, a fibroblast, and a cell of hematopoietic origin. A sequence which permits expression of the nucleic acid molecule in a cardiomyocyte, is one which is selectively active in such a cell type, thereby causing expression of the nucleic acid molecule in the cell. The cardiac myosin heavy chain gene promoter, for example, can be used to express any of the foregoing nucleic acid molecules of the invention in a cardiomyocyte. Those of ordinary skill in the art will be able to easily identify alternative promoters that are capable of expressing a nucleic acid molecule in a cardiomyocyte.

The nucleic acid sequence and the gene expression sequence are said to be "operably joined" when they are covalently linked in such a way as to place the transcription and/or translation of the nucleic acid coding sequence under the influence or control of the gene expression sequence. If it is desired that the nucleic acid sequence be translated into a functional protein, two DNA sequences are said to be operably joined if induction of a promoter in the 5' gene expression sequence results in the transcription of the nucleic acid sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the nucleic acid sequence, and/or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a gene expression sequence would be operably linked to a nucleic acid sequence if the gene expression sequence were capable of effecting transcription of that nucleic acid sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

The molecules of the invention can be delivered to the preferred cell types of the invention alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating: (1) delivery of a molecule to a target cell and/or (2) uptake of the molecule by a target cell. Preferably, the vectors transport the molecule into the target cell with reduced degradation relative to the extent of degradation that would result in the absence of the vector. Optionally, a "targeting ligand" can be attached to the vector to selectively deliver the vector to a cell which expresses on its surface the cognate receptor for the targeting ligand. In this manner, the vector (containing a nucleic acid or a protein) can be selectively delivered to a cardiomyocyte cell in, e.g., the myocardium. Methodologies for targeting include conjugates, such as those described in U.S. Pat. No. 5,391,723 to Priest. Another example of a well-known targeting vehicle is a liposome. Liposomes are commercially available from Gibco BRL (Life Technologies Inc., Rockville, Md.). Numerous methods are published for making targeted liposomes. Preferably, the molecules of the invention are targeted for delivery to cardiomyocytes, and/or vascular endothelial cells.

In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the nucleic acid sequences of the invention, and additional nucleic acid fragments (e.g., enhancers, promoters) which can be attached to the nucleic acid sequences of the invention. Viral vectors are a preferred type of vector and include, but are not limited to, nucleic acid sequences from the following viruses: adenovirus; adeno-associated virus; retrovirus, such as Moloney murine leukemia virus; Harvey murine sarcoma virus; murine mammary tumor virus; rouse sarcoma virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA viruses such as a retrovirus. One can readily employ other vectors not named but known in the art.

A particularly preferred virus for certain applications is the adeno-associated virus, a double-stranded DNA virus. The adeno-associated virus is capable of infecting a wide range of cell types and species and can be engineered to be replication-deficient i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle. It further has advantages, such as heat and lipid solvent stability, high transduction frequencies in cells of diverse lineages, including hematopoietic cells, and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus

genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

In general, other preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses, the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Adenoviruses and retroviruses have been approved for human gene therapy trials. In general, the retroviruses are replication-deficient. Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell line with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, M., "Gene Transfer and Expression, A Laboratory Manual," W.H. Freeman C.O., New York (1990) and Murry, E. J. Ed. "Methods in Molecular Biology," vol. 7, Humana Press, Inc., Cliffton, N.J. (1991).

Another preferred retroviral vector is the vector derived from the Moloney murine leukemia virus, as described in Nabel, E. G., et al., *Science,* 1990, 249:1285-1288. These vectors reportedly were effective for the delivery of genes to all three layers of the arterial wall, including the media. Other preferred vectors are disclosed in Flugelman, et al., *Circulation,* 1992, 85:1110-1117. Additional vectors that are useful for delivering molecules of the invention are described in U.S. Pat. No. 5,674,722 by Mulligan, et. al.

In addition to the foregoing vectors, other delivery methods may be used to deliver a molecule of the invention to a cell such as a cardiomyocyte and/or a vascular endothelial cell, and facilitate uptake thereby.

A preferred such delivery method of the invention is a colloidal dispersion system. Colloidal dispersion systems include lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. A preferred colloidal system of the invention is a liposome. Liposomes are artificial membrane vessels which are useful as a delivery vector in vivo or in vitro. It has been shown that large unilamellar vessels (LUV), which range in size from 0.2-4.0 μm can encapsulate large macromolecules. RNA, DNA, and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, et al., *Trends Biochem. Sci.,* 1981, 6:77). In order for a liposome to be an efficient gene transfer vector, one or more of the following characteristics should be present: (1) encapsulation of the gene of interest at high efficiency with retention of biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information.

Liposomes may be targeted to a particular tissue, such as the myocardium or the vascular cell wall, by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein. Ligands which may be useful for targeting a liposome to the vascular wall include, but are not limited to, the viral coat protein of the Hemagglutinating virus of Japan. Additionally, the vector may be coupled to a nuclear targeting peptide, which will direct the nucleic acid to the nucleus of the host cell.

Liposomes are commercially available from Gibco BRL, for example, as LIPOFECTIN™ and LIPOFECTACE™, which are formed of cationic lipids such as N-[1-(2,3-dioleyloxy)-propyl]-N,N,N-trimethylammonium chloride (DOTMA) and dimethyl dioctadecylammonium bromide (DDAB). Methods for making liposomes are well known in the art and have been described in many publications. Liposomes also have been reviewed by Gregoriadis, G. in *Trends in Biotechnology*, V. 3, p. 235-241 (1985). Novel liposomes for the intracellular delivery of macromolecules, including nucleic acids, are also described in PCT International application no. PCT/US96/07572 (Publication No. WO 96/40060, entitled "Intracellular Delivery of Macromolecules").

In one particular embodiment, the preferred vehicle is a biocompatible micro particle or implant that is suitable for implantation into the mammalian recipient. Exemplary bioerodible implants that are useful in accordance with this method are described in PCT International application no. PCT/US/03307 (Publication No. WO 95/24929, entitled "Polymeric Gene Delivery System", which claims priority to U.S. patent application Ser. No. 213,668, filed Mar. 15, 1994). PCT/US/03307 describes a biocompatible, preferably biodegradable polymeric matrix for containing an exogenous gene under the control of an appropriate promoter. The polymeric matrix is used to achieve sustained release of the exogenous gene in the patient. In accordance with the instant invention, the nucleic acids described herein are encapsulated or dispersed within the biocompatible, preferably biodegradable polymeric matrix disclosed in PCT/US/03307. The polymeric matrix preferably is in the form of a micro particle such as a micro sphere (wherein a nucleic acid is dispersed throughout a solid polymeric matrix) or a microcapsule (wherein a nucleic acid is stored in the core of a polymeric shell). Other forms of the polymeric matrix for containing the nucleic acids of the invention include films, coatings, gels, implants, and stents. The size and composition of the polymeric matrix device is selected to result in favorable release kinetics in the tissue into which the matrix device is implanted. The size of the polymeric matrix device further is selected according to the method of delivery which is to be used, typically injection into a tissue or administration of a suspension by aerosol into the nasal and/or pulmonary areas. The polymeric matrix composition can be selected to have both favorable degradation rates and also to be formed of a material which is bioadhesive, to further increase the effectiveness of transfer when the device is administered to a vascular surface. The matrix composition also can be selected not to degrade, but rather, to release by diffusion over an extended period of time.

Both non-biodegradable and biodegradable polymeric matrices can be used to deliver the nucleic acids of the invention to the subject. Biodegradable matrices are preferred. Such polymers may be natural or synthetic polymers. Synthetic polymers are preferred. The polymer is selected based on the period of time over which release is desired, generally in the order of a few hours to a year or longer. Typically, release over a period ranging from between a few hours and three to twelve months is most desirable. The polymer optionally is in the form of a hydrogel that can absorb up to about 90% of its weight in water and further, optionally is cross-linked with multi-valent ions or other polymers.

In general, the nucleic acids of the invention are delivered using the bioerodible implant by way of diffusion, or more preferably, by degradation of the polymeric matrix. Exemplary synthetic polymers which can be used to form the biodegradable delivery system include: polyamides, polycarbonates, polyalkylenes, polyalkylene glycols, polyalkylene

oxides, polyalkylene terepthalates, polyvinyl alcohols, polyvinyl ethers, polyvinyl esters, polyvinyl halides, polyglycolides, polysiloxanes, polyurethanes and co-polymers thereof, alkyl cellulose, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, nitrocelluloses, polymers of acrylic and methacrylic esters, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxy-propyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, carboxylethyl cellulose, cellulose triacetate, cellulose sulphate sodium salt, poly(methylmethacrylate), poly (ethylmethacrylate), poly(butylmethacrylate), poly(isobutylmethacrylate), poly(hexylmethacrylate), poly (isodecylmethacrylate), poly(laurylmethacrylate), poly (phenylmethacrylate), poly(methylacrylate), poly (isopropylacrylate), poly(isobutylacrylate), poly (octadecylacrylate), polyethylene, polypropylene, poly (ethyleneglycol), poly(ethyleneoxide), poly (ethyleneterephthalate), poly(vinyl alcohols), polyvinyl acetate, poly vinyl chloride, polystyrene and polyvinylpyrrolidone.

Examples of non-biodegradable polymers include ethylene vinyl acetate, poly(meth)acrylic acid, polyamides, copolymers and mixtures thereof.

Examples of biodegradable polymers include synthetic polymers such as polymers of lactic acid and glycolic acid, polyanhydrides, poly(ortho)esters, polyurethanes, poly(butic acid), poly(valeric acid), and poly(lactide-cocaprolactone), and natural polymers such as alginate and other polysaccharides including dextran and cellulose, collagen, chemical derivatives thereof (substitutions, additions of chemical groups, for example, alkyl, alkylene, hydroxylations, oxidations, and other modifications routinely made by those skilled in the art), albumin and other hydrophilic proteins, zein and other prolamines and hydrophobic proteins, copolymers and mixtures thereof. In general, these materials degrade either by enzymatic hydrolysis or exposure to water in vivo, by surface or bulk erosion.

Bioadhesive polymers of particular interest include bioerodible hydrogels described by H. S. Sawhney, C. P. Pathak and J. A. Hubell in Macromolecules, 1993, 26, 581-587, the teachings of which are incorporated herein, polyhyaluronic acids, casein, gelatin, glutin, polyanhydrides, polyacrylic acid, alginate, chitosan, poly(methyl methacrylates), poly (ethyl methacrylates), poly(butylmethacrylate), poly(isobutyl methacrylate), poly(hexylmethacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), and poly(octadecyl acrylate). Thus, the invention provides a composition of the above-described molecules of the invention for use as a medicament, methods for preparing the medicament and methods for the sustained release of the medicament in vivo.

Compaction agents also can be used in combination with a vector of the invention. A "compaction agent", as used herein, refers to an agent, such as a histone, that neutralizes the negative charges on the nucleic acid and thereby permits compaction of the nucleic acid into a fine granule. Compaction of the nucleic acid facilitates the uptake of the nucleic acid by the target cell. The compaction agents can be used alone, e.g., to deliver an isolated nucleic acid of the invention in a form that is more efficiently taken up by the cell or, more preferably, in combination with one or more of the above-described vectors.

Other exemplary compositions that can be used to facilitate uptake by a target cell of the nucleic acids of the invention include calcium phosphate and other chemical mediators of intracellular transport, microinjection compositions, electroporation and homologous recombination compositions (e.g., for integrating a nucleic acid into a preselected location within the target cell chromosome).

The invention also provides methods for the diagnosis and therapy of vascular and cardiovascular disorders. Such disorders include myocardial infarction, stroke, arteriosclerosis, heart failure, and cardiac hypertrophy.

The methods of the invention are useful in both the acute and the prophylactic treatment of any of the foregoing conditions. As used herein, an acute treatment refers to the treatment of subjects having a particular condition. Prophylactic treatment refers to the treatment of subjects at risk of having the condition, but not presently having or experiencing the symptoms of the condition.

In its broadest sense, the terms "treatment" or "to treat" refer to both acute and prophylactic treatments. If the subject in need of treatment is experiencing a condition (or has or is having a particular condition), then treating the condition refers to ameliorating, reducing or eliminating the condition or one or more symptoms arising from the condition. In some preferred embodiments, treating the condition refers to ameliorating, reducing or eliminating a specific symptom or a specific subset of symptoms associated with the condition. If the subject in need of treatment is one who is at risk of having a condition, then treating the subject refers to reducing the risk of the subject having the condition.

Stroke (also referred to herein as ischemic stroke and/or cerebrovascular ischemia) is often cited as the third most common cause of death in the industrial world, ranking behind ischemic heart disease and cancer. Strokes are responsible for about 300,000 deaths annually in the United States and are a leading cause of hospital admissions and long-term disabilities. Accordingly, the socioeconomic impact of stroke and its attendant burden on society is practically immeasurable.

"Stroke" is defined by the World Health Organization as a rapidly developing clinical sign of focal or global disturbance of cerebral function with symptoms lasting at least 24 hours. Strokes are also implicated in deaths where there is no apparent cause other than an effect of vascular origin.

Strokes are typically caused by blockages or occlusions of the blood vessels to the brain or within the brain. With complete occlusion, arrest of cerebral circulation causes cessation of neuronal electrical activity within seconds. Within a few minutes after the deterioration of the energy state and ion homeostasis, depletion of high energy phosphates, membrane ion pump failure, efflux of cellular potassium, influx of sodium chloride and water, and membrane depolarization occur. If the occlusion persists for more than five to ten minutes, irreversible damage results. With incomplete ischemia, however, the outcome is difficult to evaluate and depends largely on residual perfusion and the availability of oxygen. After a thrombotic occlusion of a cerebral vessel, ischemia is rarely total. Some residual perfusion usually persists in the ischemic area, depending on collateral blood flow and local perfusion pressure.

Cerebral blood flow can compensate for drops in mean arterial blood pressure from 90 to 60 mm Hg by autoregulation. This phenomenon involves dilatation of downstream resistant vessels. Below the lower level of autoregulation (about 60 mm Hg), vasodilatation is inadequate and the cerebral blood flow falls. The brain, however, has perfusion reserves that can compensate for the fall in cerebral blood flow. This reserve exists because under normal conditions only about 35% of the oxygen delivered by the blood is extracted. Therefore, increased oxygen extraction can take

place, provided that normoxia and normocapnea exist. When distal blood pressure falls below approximately 30 mm Hg, the two compensatory mechanisms (autoregulation and perfusion reserve) are inadequate to prevent failure of oxygen delivery.

As blood flow drops below the ischemic threshold of 23 ml/100 g/minute, symptoms of tissue hypoxia develop. Severe ischemia may be lethal. When the ischemia is moderate, it will result in "penumbra." In the neurological context, penumbra refers to a zone of brain tissue with moderate ischemia and paralyzed neuronal function, which is reversible with restoration of adequate perfusion. The penumbra forms a zone of collaterally perfused tissue surrounding a core of severe ischemia in which an infarct has developed. In other words, the penumbra is the tissue area that can be saved, and is essentially in a state between life and death.

Although an ischemic event can occur anywhere in the vascular system, the carotid artery bifurcation and the origin of the internal carotid artery are the most frequent sites for thrombotic occlusions of cerebral blood vessels, which result in cerebral ischemia. The symptoms of reduced blood flow due to stenosis or thrombosis are similar to those caused by middle cerebral artery disease. Flow through the ophthalmic artery is often affected sufficiently to produce amaurosis fugax or transient monocular blindness. Severe bilateral internal carotid artery stenosis may result in cerebral hemispheric hypoperfusion. This manifests with acute headache ipsilateral to the acutely ischemic hemisphere. Occlusions or decrease of the blood flow with resulting ischemia of one anterior cerebral artery distal to the anterior communicating artery produces motor and cortical sensory symptoms in the contralateral leg and, less often, proximal arm. Other manifestations of occlusions or underperfusion of the anterior cerebral artery include gait ataxia and sometimes urinary incontinence due to damage to the parasagital frontal lobe. Language disturbances manifested as decreased spontaneous speech may accompany generalized depression of psychomotor activity.

Most ischemic strokes involve portions or all of the territory of the middle cerebral artery with emboli from the heart or extracranial carotid arteries accounting for most cases. Emboli may occlude the main stem of the middle cerebral artery, but more frequently produce distal occlusion of either the superior or the inferior branch. Occlusions of the superior branch cause weakness and sensory loss that are greatest in the face and arm. Occlusions of the posterior cerebral artery distal to its penetrating branches cause complete contralateral loss of vision. Difficulty in reading (dyslexia) and in performing calculations (dyscalculia) may follow ischemia of the dominant posterior cerebral artery. Proximal occlusion of the posterior cerebral artery causes ischemia of the branches penetrating to calamic and limbic structures. The clinical results are hemisensory disturbances that may chronically change to intractable pain of the defective side (thalamic pain).

A subject having a stroke is so diagnosed by symptoms experienced and/or by a physical examination including interventional and non-interventional diagnostic tools such as CT and MR imaging. The methods of the invention are advantageous for the treatment of various clinical presentations of stroke subjects. A subject having a stroke may present with one or more of the following symptoms: paralysis, weakness, decreased sensation and/or vision, numbness, tingling, aphasia (e.g., inability to speak or slurred speech, difficulty reading or writing), agnosia (i.e., inability to recognize or identify sensory stimuli), loss of memory, co-ordination difficulties, lethargy, sleepiness or unconsciousness, lack of bladder or bowel control and cognitive decline (e.g., dementia, limited attention span, inability to concentrate). Using medical imaging techniques, it may be possible to identify a subject having a stroke as one having an infarct or one having hemorrhage in the brain.

An important embodiment of the invention is treatment of a subject with an abnormally elevated risk of an ischemic stroke. As used herein, subjects having an abnormally elevated risk of an ischemic stroke are a category determined according to conventional medical practice (see earlier discussion); such subjects may also be identified in conventional medical practice as having known risk factors for stroke or having increased risk of cerebrovascular events. This category includes, for example, subjects which are having elected vascular surgery. Typically, the risk factors associated with cardiac disease are the same as are associated with stroke. The primary risk factors include hypertension, hypercholesterolemia, and smoking. Atrial fibrillation or recent myocardial infarction are also important risk factors. In addition, modified levels of expression of a nucleic acid molecule selected from the group consisting of Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1, or an expression product thereof, are also, according to the present invention, important risk factors.

As used herein, subjects having an abnormally elevated risk of an ischemic stroke also include individuals undergoing surgical or diagnostic procedures which risk release of emboli, lowering of blood pressure or decrease in blood flow to the brain, such as carotid endarterectomy, brain angiography, neurosurgical procedures in which blood vessels are compressed or occluded, cardiac catheterization, angioplasty, including balloon angioplasty, coronary by-pass surgery, or similar procedures. Subjects having an abnormally elevated risk of an ischemic stroke also include individuals having any cardiac condition that may lead to decreased blood flow to the brain, such as atrial fibrillation, ventrical tachycardia, dilated cardiomyopathy and other cardiac conditions requiring anticoagulation. Subjects having an abnormally elevated risk of an ischemic stroke also include individuals having conditions including arteriopathy or brain vasculitis, such as that caused by lupus, congenital diseases of blood vessels, such as CADASIL syndrome, or migraine, especially prolonged episodes.

The treatment of stroke can be for patients who have experienced a stroke or can be a prophylactic treatment. Short term prophylactic treatment is indicated for subjects having surgical or diagnostic procedures which risk release of emboli, lowering of blood pressure or decrease in blood flow to the brain, to reduce the injury due to any ischemic event that occurs as a consequence of the procedure. Longer term or chronic prophylactic treatment is indicated for subjects having cardiac conditions that may lead to decreased blood flow to the brain, or conditions directly affecting brain vasculature. If prophylactic, then the treatment is for subjects having an abnormally elevated risk of an ischemic stroke, as described above. If the subject has experienced a stroke, then the treatment can include acute treatment. Acute treatment for stroke subjects means administration of an agent of the invention at the onset of symptoms of the condition or within 48 hours of the onset, preferably within 24 hours, more preferably within 12 hours, more preferably within 6 hours, and even more preferably within 3 hours of the onset of symptoms of the condition.

Criteria for defining hypercholesterolemic and/or hypertriglyceridemic subjects are well known in the art (see, e.g., "Harrison's"). Hypercholesterolemic subjects and hypertriglyceridemic subjects are associated with increased incidence

of premature coronary heart disease. A hypercholesterolemic subject has an LDL level of >160 mg/dL or >130 mg/dL and at least two risk factors selected from the group consisting of male gender, family history of premature coronary heart disease, cigarette smoking (more than 10 per day), hypertension, low HDL (<35 mg/dL), diabetes mellitus, hyperinsulinemia, abdominal obesity, high lipoprotein (a), and personal history of cerebrovascular disease or occlusive peripheral vascular disease. A hypertriglyceridemic subject has a triglyceride (TG) level of >250 mg/dL. Thus, a hyperlipidemic subject is defined as one whose cholesterol and triglyceride levels equal or exceed the limits set as described above for both the hypercholesterolemic and hypertriglyceridemic subjects.

"Myocardial infarction" is a focus of necrosis resulting from inadequate perfusion of the cardiac tissue. Myocardial infarction generally occurs with the abrupt decrease in coronary blood flow that follows a thrombotic occlusion of a coronary artery previously narrowed by atherosclerosis. Generally, infarction occurs when an atherosclerotic plaque fissures, ruptures, or ulcerates, and a mural thrombus forms leading to coronary artery occlusion.

The diagnosis of myocardial infarction in a subject determines the need for treating the subject according to the methods of the invention. A number of laboratory tests, well known in the art, are described, for example, in Harrison's. Generally, the tests may be divided into four main categories: (1) nonspecific indexes of tissue necrosis and inflammation, (2) electrocardiograms, (3) serum enzyme changes (e.g., creatine phosphokinase levels), and (4) cardiac imaging. A person of ordinary skill in the art could easily apply any of the foregoing tests to determine when a subject is at risk, is suffering, or has suffered, a myocardial infarction. In addition, decreased levels of expression of a nucleic acid molecule selected from the group consisting of Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1, or an expression product thereof, are also, according to the present invention, important risk factors. A positively identified subject would thus benefit from a method of treatment of the invention.

According to the invention, the method involves administering to a subject having a myocardial infarction any of the foregoing molecules (Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1) in an amount effective to treat the cardiovascular disorder in the subject. By "having a myocardial infarction" it is meant that the subject is at risk of developing, is currently having, or has suffered a myocardial infarction. It is believed that immediate administration of the molecule would greatly benefit the subject by inhibiting apoptotic cell-death of cardiomyocytes (the cells mostly affected by the infarct) prior to, or following the infarct. By "immediate" it is meant that administration occurs before (if it is diagnosed in time), or within 48 hours from the myocardial infarct, although administration up to 14 days after the episode may also be beneficial to the subject.

Another important embodiment of the invention is the treatment of ischemic injury resulting from arteriosclerosis. Arteriosclerosis is a term used to describe a thickening and hardening of the arterial wall. It is believed to be responsible for the majority of deaths in the United States and in most westernized societies. Atherosclerosis is one type of arteriosclerosis that is believed to be the cause of most coronary artery disease, aortic aneurysm and arterial disease of the lower extremities (including peripheral vascular arteriopathy), as well as contributing to cerebrovascular disease. Atherosclerosis is the leading cause of death in the United States.

A normal artery typically is lined on its inner-side only by a single layer of endothelial cells, the intima. The intima overlays the media, which contains only a single cell type, the smooth muscle cell. The outer-most layer of the artery is the adventitia. With aging, there is a continuous increase in the thickness of the intima, believed to result in part from migration and proliferation of smooth muscle cells from the media. A similar increase in the thickness of the intima also occurs as a result of various traumatic events or interventions, such as occurs when, for example, a balloon dilatation procedure causes injury to the vessel wall. The invention is used in connection with treating ischemic injury resulting from arteriosclerotic conditions. An arteriosclerotic condition as used herein means classical atherosclerosis, accelerated atherosclerosis, atherosclerosis lesions and any other arteriosclerotic conditions characterized by undesirable endothelial and/or vascular smooth muscle cell proliferation, including vascular complications of diabetes.

Another important embodiment of the invention is the treatment of heart failure. Heart failure is a clinical syndrome of diverse etiologies linked by the common denominator of impaired heart pumping and is characterized by the failure of the heart to pump blood commensurate with the requirements of the metabolizing tissues, or to do so only from an elevating filling pressure.

Another important embodiment of the invention is the treatment of cardiac hypertrophy. This condition is typically characterized by left ventricular hypertrophy, usually of a nondilated chamber, without obvious antecedent cause. Current methods of diagnosis include the electrocardiogram and the echocardiogram. Many patients, however, are asymptomatic and may be relatives of patients with known disease. Unfortunately, the first manifestation of the disease may be sudden death, frequently occurring in children and young adults, often during or after physical exertion.

Agents for reducing the risk of or treating a cardiovascular disorder include those selected from the group consisting of anti-inflammatory agents, anti-thrombotic agents, anti-platelet agents, fibrinolytic agents, lipid reducing agents, direct thrombin inhibitors, glycoprotein IIb/IIIa receptor inhibitors, agents that bind to cellular adhesion molecules and inhibit the ability of white blood cells to attach to such molecules (e.g. anti-cellular adhesion molecule antibodies), calcium channel blockers, beta-adrenergic receptor blockers, cyclooxygenase-2 inhibitors, angiotensin system inhibitors, and/or any combinations thereof. One preferred agent is aspirin.

The mode of administration and dosage of a therapeutic agent of the invention will vary with the particular stage of the condition being treated, the age and physical condition of the subject being treated, the duration of the treatment, the nature of the concurrent therapy (if any), the specific route of administration, and the like factors within the knowledge and expertise of the health practitioner.

As described herein, the agents of the invention are administered in effective amounts to treat any of the foregoing cardiovascular disorders. In general, an effective amount is any amount that can cause a beneficial change in a desired tissue of a subject. Preferably, an effective amount is that amount sufficient to cause a favorable phenotypic change in a particular condition such as a lessening, alleviation or elimination of a symptom or of a condition as a whole.

In general, an effective amount is that amount of a pharmaceutical preparation that alone, or together with further doses, produces the desired response. This may involve only slowing the progression of the condition temporarily, although more preferably, it involves halting the progression of the condition permanently or delaying the onset of or preventing the condition from occurring. This can be monitored by routine methods. Generally, doses of active compounds would be from about 0.01 mg/kg per day to 1000

mg/kg per day. It is expected that doses ranging from 50-500 mg/kg will be suitable, preferably orally and in one or several administrations per day.

Such amounts will depend, of course, on the particular condition being treated, the severity of the condition, the individual patient parameters including age, physical condition, size and weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. Lower doses will result from certain forms of administration, such as intravenous administration. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits. Multiple doses per day are contemplated to achieve appropriate systemic levels of compounds. It is preferred generally that a maximum dose be used, that is, the highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any other reasons.

The agents of the invention may be combined, optionally, with a pharmaceutically-acceptable carrier to form a pharmaceutical preparation. The term "pharmaceutically-acceptable carrier," as used herein, means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy. In some aspects, the pharmaceutical preparations comprise an agent of the invention in an amount effective to treat a disorder.

The pharmaceutical preparations may contain suitable buffering agents, including: acetic acid in a salt; citric acid in a salt; boric acid in a salt; or phosphoric acid in a salt. The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens or thimerosal.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular drug selected, the severity of the condition being treated and the dosage required for therapeutic efficacy. The methods of the invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal, topical, nasal, intradermal, transdermal, or parenteral routes. The term "parenteral" includes subcutaneous, intravenous, intramuscular, or infusion. Intravenous or intramuscular routes are not particularly suitable for long-term therapy and prophylaxis. As an example, pharmaceutical compositions for the acute treatment of subjects having a migraine headache may be formulated in a variety of different ways and for a variety of administration modes including tablets, capsules, powders, suppositories, injections and nasal sprays.

The pharmaceutical preparations may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active compound. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, elixir or an emulsion.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of an agent of the invention, which is preferably isotonic with the blood of the recipient. This aqueous preparation may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono or di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Formulations suitable for oral, subcutaneous, intravenous, intramuscular, etc. administrations can be found in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, Pa.

The term "permit entry" of a molecule into a cell according to the invention has the following meanings depending upon the nature of the molecule. For an isolated nucleic acid it is meant to describe entry of the nucleic acid through the cell membrane and into the cell nucleus, where upon the "nucleic acid transgene" can utilize the cell machinery to produce functional polypeptides encoded by the nucleic acid. By "nucleic acid transgene" it is meant to describe all of the nucleic acids of the invention with or without the associated vectors. For a polypeptide, it is meant to describe entry of the polypeptide through the cell membrane and into the cell cytoplasm, and if necessary, utilization of the cell cytoplasmic machinery to functionally modify the polypeptide (e.g., to an active form).

Various techniques may be employed for introducing nucleic acids of the invention into cells, depending on whether the nucleic acids are introduced in vitro or in vivo in a host. Such techniques include transfection of nucleic acid-$CaPO_4$ precipitates, transfection of nucleic acids associated with DEAE, transfection with a retrovirus including the nucleic acid of interest, liposome mediated transfection, and the like. For certain uses, it is preferred to target the nucleic acid to particular cells. In such instances, a vehicle used for delivering a nucleic acid of the invention into a cell (e.g., a liposome, a retrovirus, or other virus) can have a targeting molecule attached thereto. For example, a molecule such as an antibody specific for a surface membrane protein on the target cell or a ligand for a receptor on the target cell can be bound to or incorporated within the nucleic acid delivery vehicle. For example, where liposomes are employed to deliver the nucleic acids of the invention, proteins which bind to a surface membrane protein associated with endocytosis may be incorporated into the liposome formulation for targeting and/or to facilitate uptake. Such proteins include capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, proteins that target intracellular localization and enhance intracellular half life, and the like. Polymeric

delivery systems also have been used successfully to deliver nucleic acids into cells, as is known by those skilled in the art. Such systems even permit oral delivery of nucleic acids.

Other delivery systems can include time release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of an agent of the present invention, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. They include polymer base systems such as poly(lactide-glycolide), copolyoxalates, polycaprolactones, polyesteramides, polyorthoesters, polyhydroxybutyric acid, and polyanhydrides. Microcapsules of the foregoing polymers containing drugs are described in, for example, U.S. Pat. No. 5,075,109. Delivery systems also include non-polymer systems that are: lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono-, di-, and tri-glycerides; hydrogel release systems; sylastic systems; peptide based systems; wax coatings; compressed tablets using conventional binders and excipients; partially fused implants; and the like. Specific examples include, but are not limited to: (a) erosional systems in which an agent of the invention is contained in a form within a matrix such as those described in U.S. Pat. Nos. 4,452,775; 4,675,189; and 5,736,152; and (b) diffusional systems in which an active component permeates at a controlled rate from a polymer such as described in U.S. Pat. Nos. 3,854,480; 5,133,974: and 5,407,686. In addition, pump-based hardware delivery systems can be used, some of which are adapted for implantation.

Use of a long-term sustained release implant may be desirable. Long-term release, as used herein, means that the implant is constructed and arranged to deliver therapeutic levels of the active ingredient for at least 30 days, and preferably 60 days. Long-term sustained release implants are well-known to those of ordinary skill in the art and include some of the release systems described above. Specific examples include, but are not limited to, long-term sustained release implants described in U.S. Pat. No. 4,748,024, and Canadian Patent No. 1330939.

The invention also involves the administration, and in some embodiments co-administration, of agents other than the molecules of the invention (Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1, nucleic acids and polypeptides, and/or fragments thereof) that when administered in effective amounts can act cooperatively, additively or synergistically with a molecule of the invention to: (i) modulate cardiac cell anti-apoptotic activity, and (ii) treat any of the conditions in which cardiac cell anti-apoptotic activity of a molecule of the invention is involved. Agents other than the molecules of the invention include anti-inflammatory agents, anti-thrombotic agents, anti-coagulants, anti-platelet agents, fibrinolytic agents, lipid reducing agents, direct thrombin inhibitors, glycoprotein IIb/IIIa receptor inhibitors, agents that bind to cellular adhesion molecules and inhibit the ability of white blood cells to attach to such molecules, calcium channel blockers, beta-adrenergic receptor blockers, cyclooxygenase-2 inhibitors, angiotensin system inhibitors, anti-hypertensive agents, and/or combinations thereof.

"Anti-inflammatory" agents include Alclofenac; Alclometasone Dipropionate; Algestone Acetonide; Alpha Amylase; Amcinafal; Amcinafide; Amfenac Sodium; Amiprilose Hydrochloride; Anakinra; Anirolac; Anitrazafen; Apazone; Balsalazide Disodium; Bendazac; Benoxaprofen; Benzydamine Hydrochloride; Bromelains; Broperamole; Budesonide; Carprofen; Cicloprofen; Cintazone; Cliprofen; Clobetasol Propionate; Clobetasone Butyrate; Clopirac; Cloticasone Propionate; Cormethasone Acetate; Cortodoxone; Deflazacort; Desonide; Desoximetasone; Dexamethasone Dipropionate; Diclofenac Potassium; Diclofenac Sodium; Diflorasone Diacetate; Diflumidone Sodium; Diflunisal; Difluprednate; Diftalone; Dimethyl Sulfoxide; Drocinonide; Endrysone; Enlimomab; Enolicam Sodium; Epirizole; Etodolac; Etofenamate; Felbinac; Fenamole; Fenbufen; Fenclofenac; Fenclorac; Fendosal; Fenpipalone; Fentiazac; Flazalone; Fluazacort; Flufenamic Acid; Flumizole; Flunisolide Acetate; Flunixin; Flunixin Meglumine; Fluocortin Butyl; Fluorometholone Acetate; Fluquazone; Flurbiprofen; Fluretofen; Fluticasone Propionate; Furaprofen; Furobufen; Halcinonide; Halobetasol Propionate; Halopredone Acetate; Ibufenac; Ibuprofen; Ibuprofen Aluminum; Ibuprofen Piconol; Ilonidap; Indomethacin; Indomethacin Sodium; Indoprofen; Indoxole; Intrazole; Isoflupredone Acetate; Isoxepac; Isoxicam; Ketoprofen; Lofemizole Hydrochloride; Lornoxicam; Loteprednol Etabonate; Meclofenamate Sodium; Meclofenamic Acid; Meclorisone Dibutyrate; Mefenamic Acid; Mesalamine; Meseclazone; Methylprednisolone Suleptanate; Morniflumate; Nabumetone; Naproxen; Naproxen Sodium; Naproxol; Nimazone; Olsalazine Sodium; Orgotein; Orpanoxin; Oxaprozin; Oxyphenbutazone; Paranyline Hydrochloride; Pentosan Polysulfate Sodium; Phenbutazone Sodium Glycerate; Pirfenidone; Piroxicam; Piroxicam Cinnamate; Piroxicam Olamine; Pirprofen; Prednazate; Prifelone; Prodolic Acid; Proquazone; Proxazole; Proxazole Citrate; Rimexolone; Romazarit; Salcolex; Salnacedin; Salsalate; Salycilates; Sanguinarium Chloride; Seclazone; Sermetacin; Sudoxicam; Sulindac; Suprofen; Talmetacin; Talniflumate; Talosalate; Tebufelone; Tenidap; Tenidap Sodium; Tenoxicam; Tesicam; Tesimide; Tetrydamine; Tiopinac; Tixocortol Pivalate; Tolmetin; Tolmetin Sodium; Triclonide; Triflumidate; Zidometacin; Glucocorticoids; and Zomepirac Sodium. One preferred anti-inflammatory agent is aspirin.

"Anti-thrombotic" and/or "fibrinolytic" agents include plasminogen (to plasmin via interactions of prekallikrein, kininogens, Factors XII, XIIIa, plasminogen proactivator, and tissue plasminogen activator[TPA]) Streptokinase; Urokinase: Anisoylated Plasminogen-Streptokinase Activator Complex; Pro-Urokinase; (Pro-UK); rTPA (alteplase or activase; "r" denotes recombinant); rPro-UK; Abbokinase; Eminase; Sreptase Anagrelide Hydrochloride; Bivalirudin; Dalteparin Sodium; Danaparoid Sodium; Dazoxiben Hydrochloride; Efegatran Sulfate; Enoxaparin Sodium; Ifetroban; Ifetroban Sodium; Tinzaparin Sodium; Retaplase; Trifenagrel; Warfarin; and Dextrans.

"Anti-platelet" agents include Clopridogrel; Sulfinpyrazone; Aspirin; Dipyridamole; Clofibrate; Pyridinol Carbamate; PGE; Glucagon; Antiserotonin drugs; Caffeine; Theophyllin Pentoxifyllin; Ticlopidine; and Anagrelide.

"Lipid reducing" agents include gemfibrozil, cholystyramine, colestipol, nicotinic acid, probucol lovastatin, fluvastatin, simvastatin, atorvastatin, pravastatin, and cirivastatin.

"Direct thrombin inhibitors" include hirudin, hirugen, hirulog, agatroban, PPACK, and thrombin aptamers.

"Glycoprotein IIb/IIIa receptor inhibitors" embraces both antibodies and non-antibodies, and include, but are not limited, to ReoPro (abcixamab), lamifiban, and tirofiban.

"Calcium channel blockers" are a chemically diverse class of compounds having important therapeutic value in the control of a variety of diseases including several cardiovascular disorders, such as hypertension, angina, and cardiac arrhythmias (Fleckenstein, *Cir. Res.* v. 52, (suppl. 1), p. 13-16 (1983); Fleckenstein, *Experimental Facts and Therapeutic Prospects*, John Wiley, New York (1983); McCall, D., *Curr Pract*

Cardiol, v. 10, p. 1-11 (1985)). Calcium channel blockers are a heterogeneous group of drugs that prevent or slow the entry of calcium into cells by regulating cellular calcium channels. (Remington, *The Science and Practice of Pharmacy*, Nineteenth Edition, Mack Publishing Company, Eaton, Pa., p. 963 (1995)). Most of the currently available calcium channel blockers, and useful according to the present invention, belong to one of three major chemical groups of drugs, the dihydropyridines, such as nifedipine, the phenyl alkyl amines, such as verapamil, and the benzothiazepines, such as diltiazem. Other calcium channel blockers useful according to the invention, include, but are not limited to, amrinone, amlodipine, bencyclane, felodipine, fendiline, flunarizine, isradipine, nicardipine, nimodipine, perhexilene, gallopamil, tiapamil and tiapamil analogues (such as 1993RO-11-2933), phenyloin, barbiturates, and the peptides dynorphin, omega-conotoxin, and omega-agatoxin, and the like and/or pharmaceutically acceptable salts thereof.

"Beta-adrenergic receptor blocking agents" are a class of drugs that antagonize the cardiovascular effects of catecholamines in angina pectoris, hypertension, and cardiac arrhythmias. Beta-adrenergic receptor blockers include, but are not limited to, atenolol, acebutolol, alprenolol, befunolol, betaxolol, bunitrolol, carteolol, celiprolol, hydroxalol, indenolol, labetalol, levobunolol, mepindolol, methypranol, metindol, metoprolol, metrizoranolol, oxprenolol, pindolol, propranolol, practolol, practolol, sotalolnadolol, tiprenolol, tomalolol, timolol, bupranolol, penbutolol, trimepranol, 2-(3-(1,1-dimethylethyl)-amino-2-hydroxypropoxy)-3-pyridenecarbonitrilHCl, 1-butylamino-3-(2,5-dichlorophenoxy)-2-propanol, 1-isopropylamino-3-(4-(2-cyclopropylmethoxyethyl)phenoxy)-2-propanol, 3-isopropylamino-1-(7-methylindan-4-yloxy)-2-butanol, 2-(3-t-butylamino-2-hydroxy-propylthio)-4-(5-carbamoyl-2-thienyl)thiazol, 7-(2-hydroxy-3-t-butylaminpropoxy)phthalide. The above-identified compounds can be used as isomeric mixtures, or in their respective levorotating or dextrorotating form.

Cyclooxygenase-2 (COX-2) is a recently identified form of a cyclooxygenase. "Cyclooxygenase" is an enzyme complex present in most tissues that produces various prostaglandins and thromboxanes from arachidonic acid. Non-steroidal, anti-inflammatory drugs exert most of their anti-inflammatory, analgesic and antipyretic activity and inhibit hormone-induced uterine contractions and certain types of cancer growth through inhibition of the cyclooxygenase (also known as prostaglandin G/H synthase and/or prostaglandin-endoperoxide synthase). Initially, only one form of cyclooxygenase was known, the "constitutive enzyme" or cyclooxygenase-1 (COX-1). It and was originally identified in bovine seminal vesicles.

Cyclooxygenase-2 (COX-2) has been cloned, sequenced and characterized initially from chicken, murine and human sources (see, e.g., U.S. Pat. No. 5,543,297, issued Aug. 6, 1996 to Cromlish et al., and assigned to Merck Frosst Canada, Inc., Kirkland, Calif., entitled: "Human cyclooxygenase-2 cDNA and assays for evaluating cyclooxygenase-2 activity"). This enzyme is distinct from COX-1. COX-2 is rapidly and readily inducible by a number of agents including mitogens, endotoxin, hormones, cytokines and growth factors. As prostaglandins have both physiological and pathological roles, the constitutive enzyme, COX-1, is responsible, in large part, for endogenous basal release of prostaglandins and hence is important in their physiological functions such as the maintenance of gastrointestinal integrity and renal blood flow. By contrast, it is believed that the inducible form, COX-2, is mainly responsible for the pathological effects of prostaglandins where rapid induction of the enzyme would occur in response to such agents as inflammatory agents, hormones, growth factors, and cytokines. Therefore, it is believed that a selective inhibitor of COX-2 has similar anti-inflammatory, antipyretic and analgesic properties to a conventional non-steroidal anti-inflammatory drug, and in addition inhibits hormone-induced uterine contractions and also has potential anti-cancer effects, but with reduced side effects. In particular, such COX-2 inhibitors are believed to have a reduced potential for gastrointestinal toxicity, a reduced potential for renal side effects, a reduced effect on bleeding times and possibly a decreased potential to induce asthma attacks in aspirin-sensitive asthmatic subjects, and are therefore useful according to the present invention.

A number of selective "COX-2 inhibitors" are known in the art. These include, but are not limited to, COX-2 inhibitors described in U.S. Pat. No. 5,474,995 "Phenyl heterocycles as COX-2 inhibitors"; U.S. Pat. No. 5,521,213 "Diaryl bicyclic heterocycles as inhibitors of cyclooxygenase-2"; U.S. Pat. No. 5,536,752 "Phenyl heterocycles as COX-2 inhibitors"; U.S. Pat. No. 5,550,142 "Phenyl heterocycles as COX-2 inhibitors"; U.S. Pat. No. 5,552,422 "Aryl substituted 5,5 fused aromatic nitrogen compounds as anti-inflammatory agents"; U.S. Pat. No. 5,604,253 "N-Benzylindol-3-yl propanoic acid derivatives as cyclooxygenase inhibitors"; U.S. Pat. No. 5,604,260 "5-Methanesulfonamido-1-indanones as an inhibitor of cyclooxygenase-2"; U.S. Pat. No. 5,639,780 N-Benzyl indol-3-yl butanoic acid derivatives as cyclooxygenase inhibitors"; U.S. Pat. No. 5,677,318 Diphenyl-1,2-3-thiadiazoles as anti-inflammatory agents"; U.S. Pat. No. 5,691,374 "Diaryl-5-oxygenated-2-(5H)-furanones as COX-2 inhibitors"; U.S. Pat. No. 5,698,584 "3,4-Diaryl-2-hydroxy-2,5-dihydrofurans as prodrugs to COX-2 inhibitors"; U.S. Pat. No. 5,710,140 "Phenyl heterocycles as COX-2 inhibitors"; U.S. Pat. No. 5,733,909 "Diphenyl stilbenes as prodrugs to COX-2 inhibitors"; U.S. Pat. No. 5,789,413 "Alkylated styrenes as prodrugs to COX-2 inhibitors"; U.S. Pat. No. 5,817,700 "Bisaryl cyclobutenes derivatives as cyclooxygenase inhibitors"; U.S. Pat. No. 5,849,943 "Stilbene derivatives useful as cyclooxygenase-2 inhibitors"; U.S. Pat. No. 5,861,419 "Substituted pyridines as selective cyclooxygenase-2 inhibitors"; U.S. Pat. No. 5,922,742 "Pyridinyl-2-cyclopenten-1-ones as selective cyclooxygenase-2 inhibitors"; U.S. Pat. No. 5,925,631 "Alkylated styrenes as prodrugs to COX-2 inhibitors"; all of which are commonly assigned to Merck Frosst Canada, Inc. (Kirkland, Calif. or Merck & Co., Inc. (Rahway, N.J.). Additional COX-2 inhibitors are also described in U.S. Pat. No. 5,643,933, assigned to G. D. Searle & Co. (Skokie, Ill.), entitled: "Substituted sulfonylphenylheterocycles as cyclooxygenase-2 and 5-lipoxygenase inhibitors."

A number of the above-identified COX-2 inhibitors are prodrugs of selective COX-2 inhibitors, and exert their action by conversion in vivo to the active and selective COX-2 inhibitors. The active and selective COX-2 inhibitors formed from the above-identified COX-2 inhibitor prodrugs are described in detail in WO 95/00501, published Jan. 5, 1995, WO 95/18799, published Jul. 13, 1995 and U.S. Pat. No. 5,474,995, issued Dec. 12, 1995. Given the teachings of U.S. Pat. No. 5,543,297, entitled: "Human cyclooxygenase-2 cDNA and assays for evaluating cyclooxygenase-2 activity," a person of ordinary skill in the art would be able to determine whether an agent is a selective COX-2 inhibitor or a precursor of a COX-2 inhibitor, and therefore part of the present invention.

An "angiotensin system inhibitor" is an agent that interferes with the function, synthesis or catabolism of angiotensin

II. These agents include, but are not limited to, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II antagonists, angiotensin II receptor antagonists, agents that activate the catabolism of angiotensin II, and agents that prevent the synthesis of angiotensin I from which angiotensin II is ultimately derived. The renin-angiotensin system is involved in the regulation of hemodynamics and water and electrolyte balance. Factors that lower blood volume, renal perfusion pressure, or the concentration of Na^+ in plasma tend to activate the system, while factors that increase these parameters tend to suppress its function.

Angiotensin I and angiotensin II are synthesized by the enzymatic renin-angiotensin pathway. The synthetic process is initiated when the enzyme renin acts on angiotensinogen, a pseudoglobulin in blood plasma, to produce the decapeptide angiotensin I. Angiotensin I is converted by angiotensin converting enzyme (ACE) to angiotensin II (angiotensin-[1-8] octapeptide). The latter is an active pressor substance which has been implicated as a causative agent in several forms of hypertension in various mammalian species, e.g., humans.

Angiotensin (renin-angiotensin) system inhibitors are compounds that act to interfere with the production of angiotensin II from angiotensinogen or angiotensin I or interfere with the activity of angiotensin II. Such inhibitors are well known to those of ordinary skill in the art and include compounds that act to inhibit the enzymes involved in the ultimate production of angiotensin II, including renin and ACE. They also include compounds that interfere with the activity of angiotensin II, once produced. Examples of classes of such compounds include antibodies (e.g., to renin), amino acids and analogs thereof (including those conjugated to larger molecules), peptides (including peptide analogs of angiotensin and angiotensin I), pro-renin related analogs, etc. Among the most potent and useful renin-angiotensin system inhibitors are renin inhibitors, ACE inhibitors, and angiotensin II antagonists. In a preferred embodiment of the invention, the renin-angiotensin system inhibitors are renin inhibitors, ACE inhibitors, and angiotensin II antagonists.

"Angiotensin II antagonists" are compounds which interfere with the activity of angiotensin II by binding to angiotensin II receptors and interfering with its activity. Angiotensin II antagonists are well known and include peptide compounds and non-peptide compounds. Most angiotensin II antagonists are slightly modified congeners in which agonist activity is attenuated by replacement of phenylalanine in position 8 with some other amino acid; stability can be enhanced by other replacements that slow degeneration in vivo. Examples of angiotensin II antagonists include: peptidic compounds (e.g., saralasin, [(San^1)(Val^5)(Ala^8)] angiotensin-(1-8) octapeptide and related analogs); N-substituted imidazole-2-one (U.S. Pat. No. 5,087,634); imidazole acetate derivatives including 2-N-butyl-4-chloro-1-(2-chlorobenzile), imidazole-5-acetic acid (see Long et al., *J. Pharmacol. Exp. Ther.* 247(1), 1-7 (1988)); 4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid and analog derivatives (U.S. Pat. No. 4,816,463); N2-tetrazole beta-glucuronide analogs (U.S. Pat. No. 5,085,992); substituted pyrroles, pyrazoles, and tryazoles (U.S. Pat. No. 5,081,127); phenol and heterocyclic derivatives such as 1,3-imidazoles (U.S. Pat. No. 5,073,566); imidazo-fused 7-member ring heterocycles (U.S. Pat. No. 5,064,825); peptides (e.g., U.S. Pat. No. 4,772,684); antibodies to angiotensin II (e.g., U.S. Pat. No. 4,302,386); and aralkyl imidazole compounds such as biphenyl-methyl substituted imidazoles (e.g., EP Number 253,310, Jan. 20, 1988); ES8891 (N-morpholinoacetyl-(−1-naphthyl)-L-alanyl-(4, thiazolyl)-L-alanyl (35, 45)-4-amino-3-hydroxy-5-cyclo-hexapentanoyl-N-hexylamide, Sankyo Company, Ltd., Tokyo, Japan); SKF108566 (E-alpha-2-[2-butyl-1-(carboxyphenyl)methyl]1H-imidazole-5-yl[methylane]-2-thiophenepropanoic acid, Smith Kline Beecham Pharmaceuticals, PA); Losartan (DUP753/MK954, DuPont Merck Pharmaceutical Company); Remikirin (RO42-5892, F. Hoffman LaRoche AG); A_2 agonists (Marion Merrill Dow) and certain non-peptide heterocycles (G.D. Searle and Company).

"Angiotensin converting enzyme," (ACE), is an enzyme which catalyzes the conversion of angiotensin Ito angiotensin II. ACE inhibitors include amino acids and derivatives thereof, peptides, including di- and tripeptides and antibodies to ACE which intervene in the renin-angiotensin system by inhibiting the activity of ACE thereby reducing or eliminating the formation of pressor substance angiotensin II. ACE inhibitors have been used medically to treat hypertension, congestive heart failure, myocardial infarction and renal disease. Classes of compounds known to be useful as ACE inhibitors include acylmercapto and mercaptoalkanoyl prolines such as captopril (U.S. Pat. No. 4,105,776) and zofenopril (U.S. Pat. No. 4,316,906), carboxyalkyl dipeptides such as enalapril (U.S. Pat. No. 4,374,829), lisinopril (U.S. Pat. No. 4,374,829), quinapril (U.S. Pat. No. 4,344,949), ramipril (U.S. Pat. No. 4,587,258), and perindopril (U.S. Pat. No. 4,508,729), carboxyalkyl dipeptide mimics such as cilazapril (U.S. Pat. No. 4,512,924) and benazapril (U.S. Pat. No. 4,410, 520), phosphinylalkanoyl prolines such as fosinopril (U.S. Pat. No. 4,337,201) and trandolopril.

"Renin inhibitors" are compounds which interfere with the activity of renin. Renin inhibitors include amino acids and derivatives thereof, peptides and derivatives thereof, and antibodies to renin. Examples of renin inhibitors that are the subject of United States patents are as follows: urea derivatives of peptides (U.S. Pat. No. 5,116,835); amino acids connected by nonpeptide bonds (U.S. Pat. No. 5,114,937); di- and tri-peptide derivatives (U.S. Pat. No. 5,106,835); amino acids and derivatives thereof (U.S. Pat. Nos. 5,104,869 and 5,095,119); diol sulfonamides and sulfinyls (U.S. Pat. No. 5,098,924); modified peptides (U.S. Pat. No. 5,095,006); peptidyl beta-aminoacyl aminodiol carbamates (U.S. Pat. No. 5,089,471); pyrolimidazolones (U.S. Pat. No. 5,075,451); fluorine and chlorine statine or statone containing peptides (U.S. Pat. No. 5,066,643); peptidyl amino diols (U.S. Pat. Nos. 5,063,208 and 4,845,079); N-morpholino derivatives (U.S. Pat. No. 5,055,466); pepstatin derivatives (U.S. Pat. No. 4,980,283); N-heterocyclic alcohols (U.S. Pat. No. 4,885, 292); monoclonal antibodies to renin (U.S. Pat. No. 4,780, 401); and a variety of other peptides and analogs thereof (U.S. Pat. Nos. 5,071,837, 5,064,965, 5,063,207, 5,036,054, 5,036, 053, 5,034,512, and 4,894,437).

Agents that bind to cellular adhesion molecules and inhibit the ability of white blood cells to attach to such molecules include polypeptide agents. Such polypeptides include polyclonal and monoclonal antibodies, prepared according to conventional methodology. Such antibodies already are known in the art and include anti-ICAM 1 antibodies as well as other such antibodies described above.

Anticoagulant agents include, but are not limited to, Ancrod; Anticoagulant Citrate Dextrose Solution; Anticoagulant Citrate Phosphate Dextrose Adenine Solution; Anticoagulant Citrate Phosphate Dextrose Solution; Anticoagulant Heparin Solution; Anticoagulant Sodium Citrate Solution; Ardeparin Sodium; Bivalirudin; Bromindione; Dalteparin Sodium; Desirudin; Dicumarol; Heparin Calcium; Heparin Sodium; Lyapolate Sodium; Nafamostat Mesylate; Phenprocoumon; Tinzaparin Sodium; and Warfarin Sodium.

Heparin may stabilize symptoms in evolving stroke, but anticoagulants are useless (and possibly dangerous) in acute completed stroke, and are contraindicated in hypertensives because of the increased possibility of hemorrhage into the brain or other organs. Although the timing is controversial, anticoagulants may be started to prevent recurrent cardiogenic emboli. Clot lysing agents, including tissue plasminogen activator and streptokinase, are being evaluated for the very early treatment of acute stroke. Nimodipine has recently been shown to improve survival and clinical outcome after ischemic stroke.

Other than aspirin, ticlopidine is another antiplatelet agent that has been shown to be beneficial for stroke treatment. Endarterectomy may be indicated in patients with 70 to 99 percent narrowing of a symptomatic internal carotid artery. However, most authorities agree that carotid endarterectomy is not indicated in patients with TIAs that are referable to the basilar-vertebral system, in patients with significant deficits from prior strokes, or in patients in whom a stroke is evolving.

HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase is the microsomal enzyme that catalyzes the rate limiting reaction in cholesterol biosynthesis (HMG-CoA6Mevalonate). An HMG-CoA reductase inhibitor inhibits HMG-CoA reductase, and as a result inhibits the synthesis of cholesterol. A number of HMG-CoA reductase inhibitors has been used to treat individuals with hypercholesterolemia. More recently, HMG-CoA reductase inhibitors have been shown to be beneficial in the treatment of stroke (Endres M, et al., *Proc Natl Acad Sci USA,* 1998, 95:8880-5).

HMG-CoA reductase inhibitors useful for co-administration with the agents of the invention include, but are not limited to, simvastatin (U.S. Pat. No. 4,444,784); lovastatin (U.S. Pat. No. 4,231,938); pravastatin sodium (U.S. Pat. No. 4,346,227); fluvastatin (U.S. Pat. No. 4,739,073); atorvastatin (U.S. Pat. No. 5,273,995); cerivastatin, and numerous others described in U.S. Pat. No. 5,622,985; U.S. Pat. No. 5,135,935; U.S. Pat. No. 5,356,896; U.S. Pat. No. 4,920,109; U.S. Pat. No. 5,286,895; U.S. Pat. No. 5,262,435; U.S. Pat. No. 5,260,332; U.S. Pat. No. 5,317,031; U.S. Pat. No. 5,283,256; U.S. Pat. No. 5,256,689; U.S. Pat. No. 5,182,298; U.S. Pat. No. 5,369,125; U.S. Pat. No. 5,302,604; U.S. Pat. No. 5,166,171; U.S. Pat. No. 5,202,327; U.S. Pat. No. 5,276,021; U.S. Pat. No. 5,196,440; U.S. Pat. No. 5,091,386; U.S. Pat. No. 5,091,378; U.S. Pat. No. 4,904,646; U.S. Pat. No. 5,385,932; U.S. Pat. No. 5,250,435; U.S. Pat. No. 5,132,312; U.S. Pat. No. 5,130,306; U.S. Pat. No. 5,116,870; U.S. Pat. No. 5,112,857; U.S. Pat. No. 5,102,911; U.S. Pat. No. 5,098,931; U.S. Pat. No. 5,081,136; U.S. Pat. No. 5,025,000; U.S. Pat. No. 5,021,453; U.S. Pat. No. 5,017,716; U.S. Pat. No. 5,001,144; U.S. Pat. No. 5,001,128; U.S. Pat. No. 4,997,837; U.S. Pat. No. 4,996,234; U.S. Pat. No. 4,994,494; U.S. Pat. No. 4,992,429; U.S. Pat. No. 4,970,231; U.S. Pat. No. 4,968,693; U.S. Pat. No. 4,963,538; U.S. Pat. No. 4,957,940; U.S. Pat. No. 4,950,675; U.S. Pat. No. 4,946,864; U.S. Pat. No. 4,946,860; U.S. Pat. No. 4,940,800; U.S. Pat. No. 4,940,727; U.S. Pat. No. 4,939,143; U.S. Pat. No. 4,929,620; U.S. Pat. No. 4,923,861; U.S. Pat. No. 4,906,657" U.S. Pat. No. 4,906,624; and U.S. Pat. No. 4,897,402, the disclosures of which patents are incorporated herein by reference.

Nitric oxide (NO) has been recognized as a messenger molecule with many physiologic roles, in the cardiovascular, neurologic and immune systems (Griffith, T M et al., *J Am Coll Cardiol,* 1988, 12:797-806). It mediates blood vessel relaxation, neurotransmission and pathogen suppression. NO is produced from the guanidino nitrogen of L-arginine by NO Synthase (Moncada, S and Higgs, E A, *Eur J Clin Invest,* 1991, 21:361-374). Agents that upregulate endothelial cell Nitric Oxide Synthase include, but are not limited to, L-arginine, rho GTPase function inhibitors (see International Application WO 99/47153, the disclosure of which is incorporated herein by reference), and agents that disrupt actin cytoskeletal organization (see International Application WO 00/03746, the disclosure of which is incorporated herein by reference).

"Co-administering," as used herein, refers to administering simultaneously two or more compounds of the invention (e.g., a Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and/or Mrg-1, nucleic acid and/or polypeptide, and an agent known to be beneficial in the treatment of, for example, a cardiovascular condition, e.g., an anticoagulant-), as an admixture in a single composition, or sequentially, close enough in time so that the compounds may exert an additive or even synergistic effect, i.e., on reducing cardiomyocyte cell-death in a cardiovascular condition.

The invention also embraces solid-phase nucleic acid molecule arrays. The array consists essentially of a set of nucleic acid molecules, expression products thereof, or fragments (of either the nucleic acid or the polypeptide molecule) thereof, each nucleic acid molecule selected from the group consisting of Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1, fixed to a solid substrate. In some embodiments, the solid-phase array further comprises at least one control nucleic acid molecule. In certain embodiments, the set of nucleic acid molecules comprises at least one, at least two, at least three, at least four, or even at least five nucleic acid molecules, each selected from the group consisting of Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and Mrg-1, provided that when only one nucleic acid molecule is present on the array, the nucleic acid molecule is not vacuolar ATPase. In preferred embodiments, the set of nucleic acid molecules comprises a maximum number of 100 different nucleic acid molecules. In important embodiments, the set of nucleic acid molecules comprises a maximum number of 10 different nucleic acid molecules.

According to the invention, standard hybridization techniques of microarray technology are utilized to assess patterns of nucleic acid expression and identify nucleic acid expression. Microarray technology, which is also known by other names including: DNA chip technology, gene chip technology, and solid-phase nucleic acid array technology, is well known to those of ordinary skill in the art and is based on, but not limited to, obtaining an array of identified nucleic acid probes (e.g., molecules described elsewhere herein such as Fit-1, vacuolar ATPase, CD44, Lot-1, AA892598, and/or Mrg-1) on a fixed substrate, labeling target molecules with reporter molecules (e.g., radioactive, chemiluminescent, or fluorescent tags such as fluorescein, Cye3-dUTP, or Cye5-dUTP), hybridizing target nucleic acids to the probes, and evaluating target-probe hybridization. A probe with a nucleic acid sequence that perfectly matches the target sequence will, in general, result in detection of a stronger reporter-molecule signal than will probes with less perfect matches. Many components and techniques utilized in nucleic acid microarray technology are presented in Nature Genetics, Vol. 21, January 1999, the entire contents of which is incorporated by reference herein.

According to the present invention, microarray substrates may include but are not limited to glass, silica, aluminosilicates, borosilicates, metal oxides such as alumina and nickel oxide, various clays, nitrocellulose, or nylon. In all embodiments a glass substrate is preferred. According to the invention, probes are selected from the group of nucleic acids including, but not limited to: DNA, genomic DNA, cDNA, and oligonucleotides; and may be natural or synthetic. Oligonucleotide probes preferably are 20 to 25-mer oligonucle-

otides and DNA/cDNA probes preferably are 500 to 5000 bases in length, although other lengths may be used. Appropriate probe length may be determined by one of ordinary skill in the art by following art-known procedures. In one embodiment, preferred probes are sets of two or more of the nucleic acid molecules set forth as SEQ ID NOs: 1, 3, 5, 7, 9, 11 and/or 12. Probes may be purified to remove contaminants using standard methods known to those of ordinary skill in the art such as gel filtration or precipitation.

In one embodiment, the microarray substrate may be coated with a compound to enhance synthesis of the probe on the substrate. Such compounds include, but are not limited to, oligoethylene glycols. In another embodiment, coupling agents or groups on the substrate can be used to covalently link the first nucleotide or olignucleotide to the substrate. These agents or groups may include, but are not limited to: amino, hydroxy, bromo, and carboxy groups. These reactive groups are preferably attached to the substrate through a hydrocarbyl radical such as an alkylene or phenylene divalent radical, one valence position occupied by the chain bonding and the remaining attached to the reactive groups. These hydrocarbyl groups may contain up to about ten carbon atoms, preferably up to about six carbon atoms. Alkylene radicals are usually preferred containing two to four carbon atoms in the principal chain. These and additional details of the process are disclosed, for example, in U.S. Pat. No. 4,458,066, which is incorporated by reference in its entirety.

In one embodiment, probes are synthesized directly on the substrate in a predetermined grid pattern using methods such as light-directed chemical synthesis, photochemical deprotection, or delivery of nucleotide precursors to the substrate and subsequent probe production.

In another embodiment, the substrate may be coated with a compound to enhance binding of the probe to the substrate. Such compounds include, but are not limited to: polylysine, amino silanes, amino-reactive silanes (Nature Genetics, Vol. 21, January 1999) or chromium (Gwynne and Page, 2000). In this embodiment, presynthesized probes are applied to the substrate in a precise, predetermined volume and grid pattern, utilizing a computer-controlled robot to apply probe to the substrate in a contact-printing manner or in a non-contact manner such as ink jet or piezo-electric delivery. Probes may be covalently linked to the substrate with methods that include, but are not limited to, UV-irradiation and heat.

Targets are nucleic acids selected from the group, including but not limited to, DNA, genomic DNA, cDNA, RNA, mRNA and may be natural or synthetic. In all embodiments, nucleic acid molecules from subjects suspected of developing or having a cardiovascular condition, are preferred. In certain embodiments of the invention, one or more control nucleic acid molecules are attached to the substrate. Preferably, control nucleic acid molecules allow determination of factors including but not limited to: nucleic acid quality and binding characteristics; reagent quality and effectiveness; hybridization success; and analysis thresholds and success. Control nucleic acids may include, but are not limited to, expression products of genes such as housekeeping genes or fragments thereof.

To select a set of cardiovascular disease markers, the expression data generated by, for example, microarray analysis of gene expression, is preferably analyzed to determine which genes in different categories of patients (each category of patients being a different cardiovascular disorder), are significantly differentially expressed. The significance of gene expression can be determined using Permax computer software, although any standard statistical package that can discriminate significant differences is expression may be used. Permax performs permutation 2-sample t-tests on large arrays of data. For high dimensional vectors of observations, the Permax software computes t-statistics for each attribute, and assesses significance using the permutation distribution of the maximum and minimum overall attributes. The main use is to determine the attributes (genes) that are the most different between two groups (e.g., control healthy subject and a subject with a particular cardiovascular disorder), measuring "most different" using the value of the t-statistics, and their significance levels.

Expression of cardiovascular disease nucleic acid molecules can also be determined using protein measurement methods to determine expression of SEQ ID NOs: 2, 4, 6, 8, and/or 10, e.g., by determining the expression of polypeptides encoded by SEQ ID NOs: 1, 3, 5, 7, and/or 9, respectively. Preferred methods of specifically and quantitatively measuring proteins include, but are not limited to: mass spectroscopy-based methods such as surface enhanced laser desorption ionization (SELDI; e.g., Ciphergen ProteinChip System), non-mass spectroscopy-based methods, and immunohistochemistry-based methods such as 2-dimensional gel electrophoresis.

SELDI methodology may, through procedures known to those of ordinary skill in the art, be used to vaporize microscopic amounts of tumor protein and to create a "fingerprint" of individual proteins, thereby allowing simultaneous measurement of the abundance of many proteins in a single sample. Preferably SELDI-based assays may be utilized to characterize cardiovascular conditions as well as stages of such conditions. Such assays preferably include, but are not limited to the following examples. Gene products discovered by RNA microarrays may be selectively measured by specific (antibody mediated) capture to the SELDI protein disc (e.g., selective SELDI). Gene products discovered by protein screening (e.g., with 2-D gels), may be resolved by "total protein SELDI" optimized to visualize those particular markers of interest from among SEQ ID NOs: 1, 3, 5, 7, and/or 9. Predictive models of tumor classification from SELDI measurement of multiple markers from among SEQ ID NOs: 1, 3, 5, 7, and/or 9 may be utilized for the SELDI strategies.

The use of any of the foregoing microarray methods to determine expression of cardiovascular disease nucleic acids can be done with routine methods known to those of ordinary skill in the art and the expression determined by protein measurement methods may be correlated to predetermined levels of a marker used as a prognostic method for selecting treatment strategies for cardiovascular disease patients.

The invention will be more fully understood by reference to the following examples. These examples, however, are merely intended to illustrate the embodiments of the invention and are not to be construed to limit the scope of the invention.

EXAMPLES

Example 1

Experimental Protocols Materials and Methods

Mechanical Strain Device

Experiments of mechanically overloading cardiomyocytes have generally been performed by stretching cells with no control of the cardiac cycle, an approach that does not allow distinction between mechanical overload in contraction versus relaxation. In the present study, we designed and constructed a unique experimental system that allows precisely controlled mechanical strains as well as electrical pacing in

cultured cardiomyocytes, to investigate, inter alia, how cardiomyocyte mechanotransduction is regulated by the cardiac cycle, and identify genes that are involved in such regulation.

The Pacing-Strain Device.

The approach to mechanical stimulation used an apparatus that has multiple platens that contact the underside of silicone elastomer membranes to apply a spatially isotropic biaxial strain profile to the membrane (Schaffer J L, et al., *J Orthop Res,* 1993, 12:709-719; and U.S. Provisional Patent Application Ser. No. 60/144,134 filed on Jul. 16, 1999 entitled "AN APPARATUS FOR STUDYING MYOCARDIAL MECHANICAL OVERLOAD HYPERTROPHY AND USES THREFOR, by Richard T. Lee, and bearing and express mail no. EL110243781US). Six individual 78 mm membranes can be stretched at once with varying amplitudes of strain by controlling displacement of each platen with a stepper motor. Measured Green strains are accurate to ~±0.25% at strains from 1-14% (Cheng G C, et al., *Circ Res,* 1997, 80:28-36; Brown T D, *J Biomechanics,* 2000, 33:3-14). Throughout this study, 8% biaxial strain was used.

To control the timing of mechanical strain relative to the cardiac cycle, the computer paced each dish electrically, and controlled: the phase between the mechanical strain and the electrical impulse, the electrical impulse duration, and the voltage of the impulse. In addition, the electrical impulses had alternating polarity to minimize electrochemical effects such as pH gradients at the electrodes. The two outputs were each connected to a single set of electrodes in each dish. The dishes were paced in parallel with a resistance of approximately 500 ohms per dish.

The positive and negative voltage sources were provided by two power supplies (6545A, Hewlett Packard Company, Palo Alto, Calif.). The control circuit was divided into two parts: a high voltage circuit and a low voltage or digital signal circuit. The high voltage circuit was a gate that switched the output based on the input signal. The low voltage circuit accepted two control signals from the computer and accepted the pulse width from a variable resistor, which controlled both the positive and negative voltage gates. The low voltage circuit allowed a voltage pulse between 0-120V DC amplitude and 2-37 ms duration. Lights provided continuous monitoring of the pulses, and the timing of the circuits and calibration were validated by oscilloscope.

The electrodes for each dish were two arc-shaped $AgCl_2$ wire electrodes at the base of the inner surface of the dish, just above the deformable membrane. The electrodes were premade, ethanol-sterilized, and placed into the dish just prior to each experiment to minimize potential toxicity from silver. Using this method no cellular death or detachment was observed in 24 hr experiments. Each arc was 120 degrees; we performed a two dimensional finite element analysis to estimate the uniformity of the potential field with this configuration. These calculations estimate a spatial variation in the potential field of {root mean square}=29%. Thus, this system provides highly uniform biaxial mechanical strain, with a relatively small variation in the voltage field.

Mechanical Stimulation Protocols.

We imposed strain only during first third of the cardiac cycle by electrical stimulation for strain imposed during the "systolic phase", and only during one third of the cardiac cycle in the relaxation phase for strain imposed during "diastolic phase," respectively. Conditions used in this study were: (1) control; (2) strain, no pacing; (3) pacing, no strain; (4) strain imposed during systolic phase; and (5) strain imposed during diastolic phase.

Neonatal rat ventricular myocytes (NRVM) from 1-day old Sprague-Dawley rats were isolated by previously described methods (Springhorn J P, and Claycomb W C., *Biochem J,* 1989; 258:73-78; Arstall M A, et al., *J Mol Cell Cardiol,* 1998, 30:1019-25). NRVM were plated on the coated membrane dish at a density of 2,000,000 cells/dish in DMEM containing 7% FCS and incubated 24 h. Approximate cell confluence was 85-90%. NRVM were then made quiescent by washing with 10 ml of Hanks' balanced salt solution (HBSS, 138 mM NaCl, 5.3 mM KCl, 4.0 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 5.6 mM glucose; Life Technologies, Inc., Rockville, Md.) twice and incubating with 26 ml of DMEM containing 0.2% FCS for 48-72 hours.

In these cell culture conditions, cells beat at 40-60 beats/minute. At this rate, we have observed negligible competition when pacing at a rate of 70 beats/minute. We performed trial capture experiments; nine locations on each dish were sampled. Capture efficiency was similar at all locations, and maximal capture occurred at 60 V and above with 10 ms of pulse width. Therefore, a voltage of 70 V with 10 ms of impulse duration at a rate of 1.2 Hz (70 beats/minute) was selected. Under these conditions we did not observe partial cell detachment.

Transcriptional Profiling.

The DNA microarray experiment was performed with rat neonatal cardiac myocytes cultured on fibronectin-coated membranes with serum-free medium for 48 hours. Cells were deformed with an 8% deformation imposed only during systole for a period of 30 minutes, and RNA was prepared after 6 hours of subsequent no strain conditions and no pacing conditions. This time point was based upon previous studies demonstrating that the gene tenascin (positive control for cardiomyocytes) is induced at this time period. The DNA microarray hybridization experiment was performed using the Affymatrix GeneChip RGU34A (Affymetrix, Inc., Santa Clara, Calif.). Data were analyzed using Affymatrix software.

Northern Analyses.

The cDNA clones for differentially expressed genes were obtained by PCR using the GenBank sequences. Each clone was sequenced from both 5' and 3' ends to confirm identity. Positive elements in the DNA microarray were confirmed by Northern blot hybridization analysis in at least three independent experiments using three different sources of NRVMs. Total RNA was isolated by the guanidium thiocyanate and phenol chloroform method (Chomcyznski, et al., *Anal. Biochem.,* 1987, 162:156-159). For Northern blotting, 15 μg RNA was loaded on a 1.0% agarose-formaldehyde gel (2.0 mol/l), transferred to a nylon membrane (Amersham Pharmacia Biotech AB, Piscataway, N.J.), and UV cross-linked with a UV Stratalinker (Stratagene, Inc., La Jolla, Calif.). Each probe was hybridized with ExpressHyb solution (Clontech Labs., Inc., Palo Alto, Calif.) at 68° C. for 1 hour. The membrane was washed with 2×SSC, 0.05% SDS solution for 30 to 40 minutes and three times at room temperature and 0.1× SSC, 0.1% SDS solution with continuous shaking at 50° C. for 40 minutes. The membrane was exposed to film at −80° C., and radiographs were scanned and analyzed with Optimas 5.0 software (Optimas Co./Media Cybernetics, Silver Springs, Md.). Densitometric units were normalized to the ethidium-stained 28S ribosomal subunit on the membrane.

Results.

FIG. **1** shows the timecourne (early, left; late, right) of the induction of Fit-1 mRNA expression by 8% cyclic mechanical strain in neonatal cardiac myocytes in culture. Maximal induction occurs at 3 hours and is sustained for 15 hours.

FIG. **2** shows the effects of 8% mechanical strain, angiotensin receptor blockade (ARB, CP-19116, 100 nM), angiotensin II (Ang II, 50 nM), interleukin-1β (IL-1β, 10 ng/ml),

and phorbal ester (Pma, 200 nM) for 3 hours on the induction of Fit-1 mRNA expression in cultured neonatal rat cardiac myocytes. The induction of Fit-1 mRNA expression by strain was not blocked by angiotensin receptor blockade; furthermore, treatment with angiotensin II did not induce Fit-1 mRNA expression. Treatment with both IL-1β and PMA were associated with an induction of Fit-1 mRNA expression in the absence of mechanical strain.

FIG. **3** shows the effects of 8% mechanical strain, hydrogen peroxide (H_2O_2, 100 uM) and the antioxidant, TIRON (10 mN) on the induction of Fit-1 mRNA expression. Unlike the mRNA expression of the mechanically induced Tenascin-C gene which is induced by H_2O_2 in the absence of mechanical strain and blocked by TIRON, H_2O_2 does not induce Fit-1 in the absence of strain and blocks the strain-induced induction of Fit-1. TIRON slightly attenuated the mRNA expression of Fit-1 in the absence and presence of strain.

FIG. **4** shows the effects of actinomycin D (5 μg/ml, left) and cyclohexamide (10 μg/ml, right) on the induction of Fit-1 mRNA expression by 8% mechanical strain. Actinomycin D and cyclohexamide were applied during mechanical strain. Actinomycin D blocked the induction of Fit-1 mRNA expression at both 2 and 4 hours suggesting that the induction of Fit-1 in response to strain is due to increased transcription of Fit-1. The protein synthesis inhibitor, cyclohexamide blocked the induction of Fit-1 mRNA expression in response to strain suggesting that new protein synthesis is required for the induction of Fit-1 mRNA expression.

FIG. **5** shows the effects of 8% mechanical strain alone and in combination with interleukin-1β (IL-1β, 10 ng/ml), and phorbal ester in the absence of strain (PMA, 100 ng/ml) on Fit-1 mRNA expression in cultured neonatal cardiac myocytes. Both IL-1β and mechanical strain alone induced Fit-1 mRNA expression but the induction of Fit-1 by mechanical strain in the presence of IL-1β was not further increased suggesting that mechanical strain and IL-1β do not act in a synergistic or additive manner on the induction of Fit-1. The strongest induction of Fit-1 mRNA expression is seen with PMA. The rank order potency for the induction of Fit-1 mRNA expression is PMA>strain>IL-1β.

FIG. **6** shows neonatal rat cardiac myocytes were exposed to 8% strain for 0, 1, 3, 6, 9, hours. Total RNA was isolated using RNeasy kit. Five μg of total RNA were size-separated on 1% agarose-formaldehyde gel and transferred to nylon membrane. After cross-linking with UV light, membrane was hybridized with ^{32}P-labeled probe specific for V-ATPase B subunit. The membrane was then exposed to x-ray film for 3 hours at −80° C. with an intensifying screen.

Example 2

Introduction

Cytokines and Cardiac Injury.

Stress-activated cytokines participate in many forms of cardiac injury and pathophysiological conditions, the most characterized ones being tumor necrosis factor-α, interleukin-1 and interleukin-6. These molecules are not constitutively expressed in the normal heart but are rapidly induced during ischemia and reperfusion or upon hemodynamic overloading, suggesting that they play an important role in the initial myocardial response to stress, injury or growth stimuli (Mann D L, *Cytokine and Growth Factor Reviews.* 1996; 7:341-354; St. John Sutton M G, et al. *Circulation.* 2000; 101:2981-2988). However, cytokines have also been shown to be stably expressed in pathologic myocardial conditions including ischemic heart disease and heart failure and are associated with a poor prognosis (Pulkki K J, et al. *Annals of Medicine.* 1997; 29:339-343; Kubota T, et al *Proc Natl Acad Sci.* 1998; 95:6930-6935; Aukrust P, et al. *Am J Cardiol* 1999; 83:376-382; MacGowan G A, et al. *Am J Cardiol* 1997; 79:1128-1132; Roig E, et al. *Am J Cardiol* 1998; 688-690; Tsutamoto T, et al. *J Am Coll Cardiol* 1998; 31:391-398; Prabhu S D, et al. *Circulation.* 2000; 101:2103-2109; Murray D R, et al. *Annu Rev Immunol.* 2000; 18:451-494).

Interleukin-1 signaling through the interleukin-1 receptor is an early event in inflammatory cytokine signaling in many different systems (Trehu E G., *Clin Cancer Res.* 1996; 8:1341-51). In cardiac injury, interleukin-6 is produced by cardiac myocytes secondary to stimulation with interleukin-1, tumor necrosis factor-α, or lipopolysaccharide and has been detected in the post-ischemic lymph during reperfusion of ischemic myocardium (Gwechenberger M, et al. *Circulation* 1999; 99:546-551). Recently recognized is the potential expression of counteracting anti-inflammatory cytokines in cardiac disease secondary to interleukin-1 signaling. Interleukin-4 and interleukin-10 can suppress the synthesis of tumor necrosis factor-α and enhance the release of soluble tumor necrosis factor receptors, which are ligand sinks for tumor necrosis factor (Joyce D A., 1994*; Eur. J. Immunol.* 11:2699-705). Interleukin-10 is increased in patients with heart failure (Yamaoka M, et al. *Jpn Circ J.* 1999; 63:951-956) and interleukin-10 serum levels are increased when tumor necrosis factor-α serum levels are increased in patients with dilated cardiomyopathy (Ohtsuka T, et al. *J Am Coll Cardiol.* 2001; 37:412-417).

T1/ST2 (Fit-1): A Novel Mechanically Induced Receptor.

We have identified a novel potential stress-activated signaling pathway in the heart: regulation of the induction of an interleukin-1 family member gene, T1/ST2. Little is known of the induction, signaling and function of T1/ST2 in any cell type and T1/ST2 was shown in separate areas of investigation to have two seemingly unrelated functions. One of these is growth regulation and the other is immune modulation. Both compensatory hypertrophic growth and immune/inflammatory modulation are involved in the pathophysiology of cardiovascular diseases.

Growth.

The T1/ST2 gene was first identified by its induction following serum stimulation of resting mouse 3T3 fibroblasts, suggesting that the T1/ST2 gene participates in growth regulation (Tominaga S., *FEBS Letters* 1989; 258:301-304). The same group later identified a longer transcript consisting of transmembrane and cytoplasmic domains homologous to the full-length interleukin-1 receptor (Yanagisawa K, et al. *FEBS Letters.* 1993; 318:83-87).

Immunity.

T1/ST2 is expressed on T helper-2, but not T helper-1, cells of the adaptive immune system, which produce interleukin-4, interleukin-5 and interleukin-10 (Yanagisawa K I, et al. *J Biochem.* 1997; 121:95-103; Coyle A J, et al. *J Exp Med.* 1999; 190:895-902). T helper-2 cells mediate beneficial responses to infection, but are detrimental in the development of allergy and asthma. There is a strong correlation between expression of T1/ST2 and interleukin-4 production on T helper-2 cells (Coyle A J, et al. *J Exp Med.* 1999; 190:895-902). T1/ST2 plays a critical role in differentiation to and activation of T helper-2 but not T helper-1 cells (O'Neill L A J, et al. *Immunology Today.* 2000; 21:206-209).

Inhibition of T1/ST2 signaling attenuated T helper 2-mediated induction of eosinophil inflammatory responses in lung and inhibited cytokine secretion from T helper-2 cells without modifying interferon-gamma secretion from T helper-1 cells (Coyle A J, et al. *J Exp Med.* 1999; 190:895-902). These studies indicate that expression of T1/ST2 can alter the cytokine profile in favor of expression of interleukin-4, interleukin-5 and interleukin-10. Interleukin-10 has recently been shown to have anti-inflammatory effects in the setting of cardiac injury (Ohtsuka T, et al. *J Am Coll Cardiol.* 2001; 37:412-417). Similarly, the absence of T1/ST2 expression could result in a shift towards interferon-gamma expression, which may be deleterious following myocardial injury.

Taken together, the involvement of T1/ST2 in growth responses and immune function coupled with the clinical recognition of the role of cytokines in the inflammatory response to ischemia/reperfusion are suggestive that T1/ST2 activation is a growth- or stress-activated signaling pathway that contributes to myocardial growth and remodeling.

Phenotype of T1/ST2 Null Mice.

(Townsend M J, et al. *J Exp Med.* 2000; 191:1069-1075). The absence of T1/ST2 in T1/ST2 null mice does not compromise their basal immune function in the absence of immune challenge. However, T1/ST2 null mice have an impaired ability to generate IL-4, IL-5, and IL-10, but not IFN-γ (a Th1 cytokine) and to generate a T helper-2 inflammatory response during eosinophilic infiltration in the lung (a Th2 response).

We have begun to study the induction of T1/ST2 in cardiac myocytes and its involvement in survival/death signaling within the context of the myocyte signaling pathways. Preliminary studies presented below show that T1/ST2 is induced in cardiac myocytes in response to interleukin-1 and mechanical strain and that the induction of T1/ST2 by interleukin-1 may be dependent on NF-κB activation. T1/ST2 mRNA is also induced in human adult vascular smooth muscle cells in response to interleukin-1. T1/ST2 protein is expressed in the mouse heart early after myocardial ischemia in vivo as well as in human aorta tissue from patients with unstable plaque.

Results:

In Vitro Studies.

The following studies demonstrate the induction of T1/ST2 by mechanical strain and interleukin-1, possibly through activation of NF-κB. Both transcripts of T1/ST2 (that is, Fit-1S and Fit-1M) are induced by strain in cardiac myocytes. T1/ST2 mRNA is induced by mechanical strain in cultured neonatal cardiac myocytes (FIG. **8**).

T1/ST2 mRNA is induced by mechanical strain in cultured neonatal cardiac myocytes. Neonatal rat ventricular myocytes were isolated by collagenase digestion, plated on fibronectin-coated silicone membrane dishes at a density of 3.5 million cells/dish in 13 ml media as previously described (Yamamoto K, et al. *J Biol Chem.* 1999; 274:21840-21846). This technique yields cultures with ≥95% myocytes. Mechanical deformation was applied using a device that provides uniform biaxial cyclic strain as previously described (Yamamoto K, et al. *J Biol Chem.* 1999; 274:21840-21846). RNA was extracted (Qiagen) and Northern blotting was performed using as a probe a ^{32}P-labelled 600 bp PCR fragment specific to rat T1/ST2. Maximal induction occurs at 3 hours, is sustained for 9 hours and declines by 15 hours.

Both interleukin-1β and mechanical strain each induce T1/ST2 RNA in cardiac myocytes (FIG. **9**). Shown is the induction of T1/ST2 by interleukin-1 and strain. We also found that the induction of T1/ST2 by mechanical strain in the presence of interleukin-1β was not further increased suggesting that interleukin-1 does not sensitize myocytes to the effects of mechanical strain (or vice versa) on the induction of T1/ST2. The 1 hour time point was included in the event that induction by strain is saturated at 3 hours and therefore masks an additive effect of interleukin-1β. Shown in the two right lanes are the effects of phorbol ester (PMA) at 1 and 3 hours. The rank order potency for the induction of T1/ST2 mRNA expression is PMA>strain>interleukin-1β. Since interleukin-1β signals through NF-κB and PMA through PKC these results suggest that NF-κB and PKC activation both participate in the induction of T1/ST2.

T1/ST2 may be a NF-κB target gene in cardiac myocytes through interleukin-1/interleukin-1 receptor signaling (FIG. **10**). Previously reported by us (Yamamoto K, et al. *J Biol Chem.* 1999; 274:21840-21846), mechanical strain of cardiac myocytes activates NF-κB. To investigate the role of NF-κB in interleukin-1β and strain induction of T1/ST2 RNA, we overexpressed IκBα, which decreases NF-κB DNA binding activity. Cultured cardiac myocytes were infected with IκBα overexpression adenovirus vector or with β-galactosidase control vector and exposed for 4 hours to 8% cyclic mechanical strain or interleukin-1 (10 ng/ml). RNA was analyzed by Northern blotting with ^{32}P-labeled Fit-1 cDNA probe. Ectopic expression of IκBα blocked interleukin-1β induction of T1/ST2-1 mRNA and partially blocked strain induction of T1/ST2 mRNA expression when compared with T1/ST2 induction in cells treated with the β-galactosidase control vector. These results suggest that T1/ST2 is an early, NF-κB target gene through interleukin-1/interleukin-1 receptor signaling. In contrast, pathways in addition to NF-κB activation may be involved in the induction of T1/ST2 RNA by mechanical strain. T1/ST2 mRNA is also induced by interleukin-1 but not PMA or tumor necrosis factor (TNF) in human adult vascular smooth muscle cells.

In addition to the above-noted results, we have shown that T1/ST2 is induced secondary to NF-κB activation by interleukin-1 and NF-κB is linked to cardiac myocyte survival. Further in vitro studies are performed to confirm that T1/ST2 activation is linked to cell growth and survival.

In Vivo Studies. In Vivo Expression of T1/ST2 Protein in Myocardial Infarction in Mice.

FIG. **11** shows protein expression of T1/ST2 using immunohistochemistry in paraffin sections of mouse hearts 1 and 3 days post-infarction. T1/ST2 protein was visualized by DAB staining. This antibody (Morwell Diagnostics) does not distinguish between the two isoforms of T1/ST2. Positive staining (brownish color) is seen 1 day post-infarction (post-MI) in the normal, infarct and border zones but not at 3 days post-MI. These results suggest that ST2 protein is rapidly expressed in response to myocardial injury during the early phase of post-infarction remodeling before the migration of macrophages into the infarct and border zones (see 3 days post-MI). Magnification: 40×.

In addition to the above, we are generating an operational colony of T1/ST2 null mice. Our in vivo studies indicate that T1/ST2 is expressed in the mouse heart following myocardial infarction. The in vivo studies confirm the hypothesis that local cardiac expression of T1/ST2 favorably modifies the process of LV remodeling following ischemia and left ventricular pressure overload. We have also generated adeno-associated viruses to express isoforms of these genes and their effects on null mice are determined.

More recently, we have obtained results which support the utility of the gene/protein called fit-1, or ST-2, as a diagnostic indicator of a cardiovascular condition in humans. We assayed serum levels on 69 patients who participated in the

HEART study, a clinical trial of acute myocardial infarction patients. The assay employed a monoclonal assay for the human ST2 protein. The results show that the levels of ST2 correlated with serum creatine phosphokinase levels, which is a standard way of looking at size of heart attack. Also, such levels rapidly decline after the infarct. The levels were: Day 1: 3.8+/−3.3 ng/ml; Day 14: 0.9+/−0.5; and Day 90: 0.8+/−0.5 and are highly statistically significant. These results also establish that the protein is secreted during heart attacks and can be easily measured, thereby supporting the asserted utility of the invention.

EQUIVALENTS

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

All references disclosed herein are incorporated by reference in their entirety.

What is claimed is presented below and is followed by a Sequence Listing.

SEQUENCE LISTING

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Ile Asn Pro Val Glu Trp Tyr Tyr Ser Asn Thr Asn Glu Arg Ile Pro
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Thr Gln Lys Arg Asn Arg Ile Phe Val Ser Arg Asp Arg Leu Lys Phe
65                  70                  75                  80

Leu Pro Ala Lys Val Glu Asp Ser Gly Ile Tyr Thr Cys Val Ile Arg
                85                  90                  95

Ser Pro Glu Ser Ile Lys Thr Gly Ser Leu Asn Val Thr Ile Tyr Lys
            100                 105                 110

Arg Pro Pro Asn Cys Lys Ile Pro Asp Tyr Met Met Tyr Ser Thr Val
        115                 120                 125

Asp Gly Ser Asp Lys Asn Ser Lys Ile Thr Cys Pro Thr Ile Ala Leu
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Tyr Asn Trp Thr Ala Pro Val Gln Trp Phe Lys Asn Cys Lys Ala Leu
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Glu Glu Lys Gly Phe Ser Thr Phe Pro Val Ile Thr Asn Pro Pro His
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Ser Ala Cys Phe Gly Thr Ala Ser Gln Phe Val Ala Val Leu Trp Gln
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Ile Asn Lys Thr Arg Ile Gly Ser Phe Gly Lys Ala Arg Ile Gln Glu
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Glu Lys Gly Pro Asn Lys Ser Ser Ser Asn Gly Met Ile Cys Leu Thr
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Ser Leu Leu Arg Ile Thr Gly Val Thr Asp Lys Asp Phe Ser Leu Lys
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Tyr Asp Cys Val Ala Met Asn His His Gly Val Ile Arg His Pro Val
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Thr Gln Lys Arg Asn Arg Ile Phe Val Ser Arg Asp Arg Leu Lys Phe
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Leu Pro Ala Lys Val Glu Asp Ser Gly Ile Tyr Thr Cys Val Ile Arg
                85   90   95

Ser Pro Glu Ser Ile Lys Thr Gly Ser Leu Asn Val Thr Ile Tyr Lys
            100   105   110

Arg Pro Pro Asn Cys Lys Ile Pro Asp Tyr Met Met Tyr Ser Thr Val
        115   120   125

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Tyr Asn Trp Thr Ala Pro Val Gln Trp Phe Lys Asn Cys Lys Ala Leu
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Glu Glu Lys Gly Phe Ser Thr Phe Pro Val Ile Thr Asn Pro Pro His
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Asn Tyr Thr Val Glu Val Glu Ile Gly Lys Thr Ala Asn Ile Ala Cys
225                 230                 235                 240

Ser Ala Cys Phe Gly Thr Ala Ser Gln Phe Val Ala Val Leu Trp Gln
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    50                  55                  60

Phe Pro Arg Tyr Ala Glu Ile Val His Leu Thr Leu Pro Asp Gly Thr
65                  70                  75                  80

Lys Arg Ser Gly Gln Val Leu Glu Val Ser Gly Ser Lys Ala Val Val
                85                  90                  95

Gln Val Phe Glu Gly Thr Ser Gly Ile Asp Ala Lys Lys Thr Ser Cys
            100                 105                 110

Glu Phe Thr Gly Asp Ile Leu Arg Thr Pro Val Ser Glu Asp Met Leu
        115                 120                 125

Gly Arg Val Phe Asn Gly Ser Gly Lys Pro Ile Asp Arg Gly Pro Val
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Val Leu Ala Glu Asp Phe Leu Asp Ile Met Gly Gln Pro Ile Asn Pro
145                 150                 155                 160

Gln Cys Arg Ile Tyr Pro Glu Glu Met Ile Gln Thr Gly Ile Ser Ala
                165                 170                 175

Ile Asp Gly Met Asn Ser Ile Ala Arg Gly Gln Lys Ile Pro Ile Phe
            180                 185                 190

Ser Ala Ala Gly Leu Pro His Asn Glu Ile Ala Ala Gln Ile Cys Arg
        195                 200                 205

Gln Ala Gly Leu Val Lys Lys Ser Lys Asp Val Val Asp Tyr Ser Glu
    210                 215                 220

Glu Asn Phe Ala Ile Val Phe Ala Ala Met Gly Val Asn Met Glu Thr
225                 230                 235                 240

Ala Arg Phe Phe Lys Ser Asp Phe Glu Glu Asn Gly Ser Met Asp Asn
                245                 250                 255

Val Cys Leu Phe Leu Asn Leu Ala Asn Asp Pro Thr Ile Glu Arg Ile
            260                 265                 270

Ile Thr Pro Arg Leu Ala Leu Thr Thr Ala Glu Phe Leu Ala Tyr Gln
        275                 280                 285

Cys Glu Lys His Val Leu Val Ile Leu Thr Asp Met Ser Ser Tyr Ala
    290                 295                 300

Glu Ala Leu Arg Glu Val Ser Ala Ala Arg Glu Glu Val Pro Gly Arg
305                 310                 315                 320

Arg Gly Phe Pro Gly Tyr Met Tyr Thr Asp Leu Ala Thr Ile Tyr Glu
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Arg Ala Gly Arg Val Glu Gly Arg Asn Gly Ser Ile Thr Gln Ile Pro
            340                 345                 350

Ile Leu Thr Met Pro Asn Asp Asp Ile Thr His Pro Ile Pro Asp Leu
        355                 360                 365

Thr Gly Tyr Ile Thr Glu Gly Gln Ile Tyr Val Asp Arg Gln Leu His
    370                 375                 380

Asn Arg Gln Ile Tyr Pro Pro Ile Asn Val Leu Pro Ser Leu Ser Arg
385                 390                 395                 400

Leu Met Lys Ser Ala Ile Gly Glu Gly Met Thr Arg Lys Asp His Ala
                405                 410                 415

Asp Val Ser Asn Gln Leu Tyr Ala Cys Tyr Ala Ile Gly Lys Asp Val
            420                 425                 430
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```
Gln Ala Met Lys Ala Val Val Gly Glu Glu Ala Leu Thr Ser Asp Asp
        435                 440                 445

Leu Leu Tyr Leu Glu Phe Leu Gln Lys Phe Glu Lys Asn Phe Ile Thr
    450                 455                 460

Gln Gly Pro Tyr Glu Asn Arg Thr Val Tyr Glu Thr Leu Asp Ile Gly
465                 470                 475                 480

Trp Gln Leu Leu Arg Ile Phe Pro Lys Glu Met Leu Lys Arg Ile Pro
                485                 490                 495

Gln Ser Thr Leu Ser Glu Phe Tyr Pro Arg Asp Ser Ala Lys His
            500                 505                 510


<210> SEQ ID NO 7
<211> LENGTH: 2747
<212> TYPE: DNA
<213> ORGANISM: Rattus norvegicus
<220> FEATURE:
<221> NAME/KEY: mRNA
<222> LOCATION: (1)...(2747)
<223> OTHER INFORMATION: glycoprotein CD44

<400> SEQUENCE: 7

ctcattgccc agcagccccc agccagtgac aggttccatt caccctcttt gcccccttccc     60

ccgcgaccct tttccagagg ctactagatc ctttggtttc atcctgcaca tcatggacaa    120

ggtttggtgg cacacagctt ggggactact ttgcctctta cagttgagcc tggcacagca    180

gcagatcgat ttgaatataa cctgccgtta cgcaggtgta ttccatgtgg agaaaaatgg    240

ccgctacagt atctccagga ctgaagcagc tgacctctgc gaggctttca acaccacctt    300

gcccaccatg gctcagatgg agttagccct gagaaagggg tttgaaacat gcaggtatgg    360

gttcatagaa ggacacgtgg taatcccgag gatccacccc aacgctatct gtgcagccaa    420

caacacagga gtgtatatcc tcctcgcatc caacacctcc cactatgaca catattgctt    480

caatgcctca gctcctcttg aagaagactg tacatcagtc acagacctac ccaattcctt    540

cgatggacca gttaccataa ctattgtcaa ccgtgatggc acccgctaca gcaagaaggg    600

cgagtataga acacaccaag aagacatcga tgcctcaaac attatagatg aggatgtcag    660

cagtggatcc accattgaga agagcacccc agaaggctac attttgcaca ccgaccttcc    720

cacttcacag cctactggag accgggatga cgccttcttt attgggagca ccctggccac    780

cagtgatgga gactcatcca tggaccccag gggtggtttc gacactgtga ctcatggatc    840

cgaattagct ggacactcaa gtgggaatca agacagtgga gtgaccacaa cttctggtcc    900

tgcgaggaga cctcagattc cagagtggct tatcatcttg gcatccctcc tggcgctggc    960

tctgattctt gccgtctgca ttgctgtcaa cagtaggaga aggtgtgggc agaagaagaa   1020

gctggtgatc aacagtggca atggaacagt ggaagacagg aaaccaagtg aactcaacgg   1080

ggaggccagc aagtctcagg aaatggtgca tttggtgaac aaggaaccaa cagagactcc   1140

ggaccagttt atgacagctg atgagacccg gaatctgcag agtgtggata tgaagattgg   1200

ggtgtagtgc ctatgccact aacttgaaaa gacacaacaa ttggagacat gtcattactg   1260

ggagctggga cccttaacag atgcaatgtg ctactgatta ttttttattg ggattatttt   1320

gggcataaaa tttccctttt tttgtttttt aaaagtttgt tttccaattt atgaaaatag   1380

cattgctttc tgaaatgagg gtctcttcca gttcctcctt agaggccttg cattaccagg   1440

gtatgctacc ataggcttct accaaatgaa tactcttggt cccgattgaa cccaaagtcc   1500

caggtaacat ccaccagcta aggatttccc cagaacttag agagattggt ctctgggagg   1560
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aaatttgaat gggtccatat tgcctcccag cagtccaatc tgtaggcatt gctttgcagt   1620

ggatgggaga tcaggtgtac tggttacaca ctctctttat agactccctt ctgctggaaa   1680

atttccacat gcttctgaga gattccccaa aggtgacgct atttatcttt agtaagctat   1740

ttatctttgt ttttgaaata tcaaaccctg gaggtccttt tttcagtatg actttttta   1800

ttttgttttt ttttattttg ttttttaggt tactttgtca gaagcataac agggtataag   1860

ttgattcata ataaatacct gtccatcttc catcttgacc tgttgtgctg tgatccttca   1920

gtttctaaat cagcaaggtc tgagtctttg tagcacatca atgtgacctt agtatggtcc   1980

tctgaaactc atgttagagc atccgtgccc tgcttgggtt tacccagctg aatctcagaa   2040

gatcaaggac aggagcactg ttttcattct aggactatca aaggggtttc tctcctgttc   2100

aagaatctga attgggagta ggagagcttc tgtccctttt atgtttcgat aaccacccat   2160

ttctctttct taaagggcac attaagtttt tatatcttac aacattcgcg gtcctgtttc   2220

atagacactg atcttattgg cactttcaca aaacagtgtg gaggggactt ctgacacctt   2280

atagtaaaag gagaagccaa cagaaatgaa agtgtggaca gagagcagta gattggcatg   2340

aggaggcatg atgtacaacc cccagaccac tctttccatc accacatttg ttgatgcttt   2400

cgcaagccag ttggtactta gaatcagttc cccagggaat ccttcaaaaa gccataagaa   2460

tgcccacccc tggaatctta ccaccaccag atgagcaggt ttatggttta gcaaaaggag   2520

aatgctgtca ccctctgacc tcatagtttt cacatactgg gcaagtgttc atctgccagg   2580

atgccccatt gctcctaggt cttcccaggt accttgtaga agaacttaaa tctataaaat   2640

aaggctttct ctaaaatgga acttcctttc taaggctccc atttttactg ttgactaaat   2700

ttatatgttt aatagttttt tttcaaataa aaacaaacac aaaaagg                 2747
```

<210> SEQ ID NO 8
<211> LENGTH: 364
<212> TYPE: PRT
<213> ORGANISM: Rattus norvegicus
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)...(364)
<223> OTHER INFORMATION: glycoprotein CD44

<400> SEQUENCE: 8

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Met Asp Lys Val Trp Trp His Thr Ala Trp Gly Leu Leu Cys Leu Leu
 1               5                  10                  15

Gln Leu Ser Leu Ala Gln Gln Gln Ile Asp Leu Asn Ile Thr Cys Arg
            20                  25                  30

Tyr Ala Gly Val Phe His Val Glu Lys Asn Gly Arg Tyr Ser Ile Ser
        35                  40                  45

Arg Thr Glu Ala Ala Asp Leu Cys Glu Ala Phe Asn Thr Thr Leu Pro
    50                  55                  60

Thr Met Ala Gln Met Glu Leu Ala Leu Arg Lys Gly Phe Glu Thr Cys
65                  70                  75                  80

Arg Tyr Gly Phe Ile Glu Gly His Val Val Ile Pro Arg Ile His Pro
                85                  90                  95

Asn Ala Ile Cys Ala Ala Asn Asn Thr Gly Val Tyr Ile Leu Leu Ala
            100                 105                 110

Ser Asn Thr Ser His Tyr Asp Thr Tyr Cys Phe Asn Ala Ser Ala Pro
        115                 120                 125

Leu Glu Glu Asp Cys Thr Ser Val Thr Asp Leu Pro Asn Ser Phe Asp
    130                 135                 140
```

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Gly Pro Val Thr Ile Thr Ile Val Asn Arg Asp Gly Thr Arg Tyr Ser
145                 150                 155                 160

Lys Lys Gly Glu Tyr Arg Thr His Gln Glu Asp Ile Asp Ala Ser Asn
                165                 170                 175

Ile Ile Asp Glu Asp Val Ser Ser Gly Ser Thr Ile Glu Lys Ser Thr
            180                 185                 190

Pro Glu Gly Tyr Ile Leu His Thr Asp Leu Pro Thr Ser Gln Pro Thr
        195                 200                 205

Gly Asp Arg Asp Asp Ala Phe Phe Ile Gly Ser Thr Leu Ala Thr Ser
    210                 215                 220

Asp Gly Asp Ser Ser Met Asp Pro Arg Gly Gly Phe Asp Thr Val Thr
225                 230                 235                 240

His Gly Ser Glu Leu Ala Gly His Ser Ser Gly Asn Gln Asp Ser Gly
                245                 250                 255

Val Thr Thr Thr Ser Gly Pro Ala Arg Arg Pro Gln Ile Pro Glu Trp
            260                 265                 270

Leu Ile Ile Leu Ala Ser Leu Leu Ala Leu Ala Leu Ile Leu Ala Val
        275                 280                 285

Cys Ile Ala Val Asn Ser Arg Arg Arg Cys Gly Gln Lys Lys Lys Leu
    290                 295                 300

Val Ile Asn Ser Gly Asn Gly Thr Val Glu Asp Arg Lys Pro Ser Glu
305                 310                 315                 320

Leu Asn Gly Glu Ala Ser Lys Ser Gln Glu Met Val His Leu Val Asn
                325                 330                 335

Lys Glu Pro Thr Glu Thr Pro Asp Gln Phe Met Thr Ala Asp Glu Thr
            340                 345                 350

Arg Asn Leu Gln Ser Val Asp Met Lys Ile Gly Val
        355                 360


<210> SEQ ID NO 9
<211> LENGTH: 5028
<212> TYPE: DNA
<213> ORGANISM: Rattus norvegicus
<220> FEATURE:
<221> NAME/KEY: mRNA
<222> LOCATION: (1)...(5028)
<223> OTHER INFORMATION: Lot-1

<400> SEQUENCE: 9

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ggagcggaga gcgggcatct cctgagcgcc gtcatggctg cttaggctgc gcctgccagc    120

ggaccgacgg tgtcgcccga atccggctcg gataggtctg gttggagtct gtgcctgctt    180

gcttggcgtg tggttgttcc tgcttgattg gcacggtgcc attggcttcg tatttgggaa    240

tcggaggagt taatcttgtc tcttctcaca ggttcgagtc ctcagacctt ctgcaggact    300

ccatccatat ctgcctcgca gctgactctc ctgctcacac agaagacggc catcctagat    360

ccccagctat tgtgctgacc atccccttcc tgctccggat ctcgcctggc tgctaggctg    420

tggtgctgcc ttttcagagt caggctgtag cgactccccg ccttcgtccc ggctgggctt    480

aggtggaaca gtggttcatc tcatctcatc agcacttctg aagaagaaag tgtgagaagc    540

agaggccatg gctccttttc gctgtcaaaa atgcggcaag tccttcctca ccctggagaa    600

gttcaccatc cacaattatt cccacaccag ggagcgccca ttcaagtgct ccaagactga    660

gtgtggcaaa gccttcgtct ccaagtataa gctgatgaga cacatggcta cgcactctcc    720

ccagaagacg caccagtgca ctcattgtga aaagactttc aaccggaagg atcatctgaa    780
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gaatcacctc cagacccacg atcccaacaa gatgatctac gcctgcgaag attgtggcaa 840

gaaataccac accatgctgg gctacaagag gcacatggcc ctgcattcgg ccagcagcgg 900

cgatctcacc tgcggcgtct gcaccctgga gctggggagc accgaggtcc tgctggacca 960

cctcaagtct cacgcggaag aaaaggccca ccacgcgccc agggagaaga aacaccagtg 1020

cgaccactgc gagagatgct tctacacccg gaaggatgtg cgtcgccacc tggtggtcca 1080

cacaggatgc aaggacttcc tgtgtcagtt ctgcgcccag agatttgggc gcaaagacca 1140

cctcactcgt cacaccaaga agacccactc ccaggagctg atgcaagaga gcctgcaagc 1200

aggagaatac cagggcggtt accaacccat tgcgcctccg ttccagatca aggctgatcc 1260

catgcctcct ttccagttag aaatgccccc cgagagcggg cttgatgggg gcttgcctcc 1320

tgagattcat ggtctagtgc ttgcttcccc agaggaggtt ccccagccta tgctgtctat 1380

gccgccaatg cagccaatgc cagagcagcc tttcactctg caccctgggg tagttccctc 1440

ctctcctccc ccgatcattc ttcaggagca taagtacagc ccagttccta cctcttttgc 1500

cccgttcgta agcatgccga tgaaagcaga tctcaagggc ttttgcaaca tgggtctctt 1560

tgaggaattt cctctgcaag agtgtcagtc gcctgtcaag ttcagtcagt gctttgagat 1620

ggctaaggaa gggtttggga aagtcaccct gcccaaagag ctgctggtag atgctgtaaa 1680

tatagccatt cctggctctc tggagatttc ctctctcttg gggttctggc agctgccccc 1740

tcctcctccc cagaatggct tcatgaatgg caccatccct gtgggggccg gggagccgct 1800

gccccatagg ataacttgtc tggcacagca gcagccacca cctctgctac ctccgccgcc 1860

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gctccagccc cagccccaga tgcagcccca gatgcagctg cagcctctac agctgcagct 1980

gccccagctg ctgccccagc tgcagcccga gcctgagcca gagccagagc cagaggaaga 2040

agaggaagaa gaagaagaga tagaagaaga agaagagatc gaagaagaag aagaagccga 2100

accagaagca gaagaagaag aggaggcaga agacgaagag gaggcagagg aagaggaaga 2160

agagccacag ccagaagaag cccaaatagc aatgagcgct gtgaatatgg gccagccccc 2220

gctacccccg acccctcatg ttttcacagc tggcaccaac actgctatcc tgccccattt 2280

ccaccacgcg ttcagataaa ttggtttttt aagagggtgc ttctcttctg gaagatgttt 2340

caaacaccag ttccagttcc agacatcagt tacagtttga agagaagcgt tggaaaaaca 2400

ggaatggggt ttctagctta ttgccatgag tagattgaga aaaagaactc tcttaactgc 2460

atgcactgtg ccaatacata tatatatata tatatatata tatatatgta tatatatata 2520

tatatatatc atccttagta ttcatgcttt gtaccaaact tagtgagtgc gggcgttctc 2580

cgtaatcgaa ctgcaagtag tatcatatta ttaccctgat attgttagtc tcatattatt 2640

agccttgtat tattctcata taatcaaaac caagatccaa aacatgagct gctaatttgt 2700

aaatatcgtg ttgagtgtta gccgtcgtag tgatgttagc tgcgtagttg cgtgttagca 2760

ctgcctagga agggcacgag ggccaagttg ggcttctccc acttggaaga tgttttgaag 2820

agaaggggt gatctccgta gggcgtccgt aactaggccg tgtgttcttt tcagggaccc 2880

gtctaccttc aggattggat gtagtttagt cgctcttctt cttagctcgc tttgtagttt 2940

gtccttctgg tagcctactg tgtgtgtctg tgtgtagctt tataggaaag ttccgtgtga 3000

agctgtcggt gtcttcgttt tcaaaagtga attttaaatg tatttttcaa tatttttcat 3060

gtgatgttgt accaatgtga attatgactt cgtttatctt aaagacaaaa ctggttgtca 3120

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gtcatatctg acaggaagaa agaaatccct gtgggtaggc aagtcaagtg gccaactaat 3180

gagaagaagc atcaatcgaa agtgttggct gactgggaca ctcatgattc tcacaggact 3240

ttgagaaacg tactggaatt aaaaaaaaaa aagcttaagt acattagata agaattttct 3300

ttgcctagct taacctacta cttaagcctc ttaagttctg aagtattgtg atcaaccaat 3360

aggaaaatgt atctgtagtt gatgaatttc agtccttgtt actttgtatc ccaagaggtt 3420

tgtgttttgg gaatgtaacc gtacttgtaa tctcagttgg tatcttgcta atcgatttga 3480

aagtgtaaaa cctaaccctt gaagactctg tatttccttt tttgagactg tatttcccag 3540

catgtatacc ctaacctttg gagactctgt attctgtttt tgagactttc cccccgcccc 3600

ccagcatatg taccccgacc cttgaagact gtatttcgtt tttgagagcg tatttcccag 3660

catatataca ctaacccttg aagactctgt atttcctttt ttgagactgt atttcccagc 3720

atatatacac taacccttga agactctgta tttccttttt tgagactgta tttcccagca 3780

tatatacact aacctttgaa gactctgtat tctgtttttg agacccccccc ccagcatatg 3840

taccctaacc cttgaagact gtatttcgtt tttgagaacg tatttcccag catatataca 3900

ctaacctttg gaagactctg tatttcattt ttgagactgt gtttcttagt atacataccc 3960

taacctttga aagactccat ttttgagact tccccccccc cagcatttgt gccctaaccc 4020

ttggaggctt tgtatttttt ttttgagact tttccgccag catatataca ctaacccttg 4080

aagactctgt atttcatttt tgagactttt ttccccagca tatataccgt aacccttgaa 4140

gactctgtat tccgtttttg agattttttt ccctcagcat atatacccca acctttgaag 4200

actctgtatt tcatttttga gacttttttcc cagcatatat accctaacct ttgaagactc 4260

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ctgtattcca ttttttgaga cttttttccc cagcatatat accctaacct ttgaagactc 4560

tgtatttcat ttttgagact ttttccccag catatatacc ctaacctttg aagactctgt 4620

attccgtttt tgagaccccc cccccggcat gaatacccta atctttgaag actctggtat 4680

ttcatttttg agatttttttt cccctcagca tatatacact aacctttgta gactctgtat 4740

tccgtttttg agactttccc cccccagcat gtatacccta acctttgaag actctgtatt 4800

tccagcattt gtaccctacc cttgaagact ctgtatttcc cagcatttgt accctaaccc 4860

ttgaagaccc tgtatttcgt ttgtaagact tttccccagc atatatatcc tacatataat 4920

aaacgctaag catctagcaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 4980

aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaa 5028

<210> SEQ ID NO 10
<211> LENGTH: 583
<212> TYPE: PRT
<213> ORGANISM: Rattus norvegicus
<220> FEATURE:
<221> NAME/KEY: PEPTIDE
<222> LOCATION: (1)...(583)
<223> OTHER INFORMATION: Lot-1

<400> SEQUENCE: 10

Met Ala Pro Phe Arg Cys Gln Lys Cys Gly Lys Ser Phe Leu Thr Leu
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```
Glu Lys Phe Thr Ile His Asn Tyr Ser His Thr Arg Glu Arg Pro Phe
            20                  25                  30

Lys Cys Ser Lys Thr Glu Cys Gly Lys Ala Phe Val Ser Lys Tyr Lys
        35                  40                  45

Leu Met Arg His Met Ala Thr His Ser Pro Gln Lys Thr His Gln Cys
    50                  55                  60

Thr His Cys Glu Lys Thr Phe Asn Arg Lys Asp His Leu Lys Asn His
65                  70                  75                  80

Leu Gln Thr His Asp Pro Asn Lys Met Ile Tyr Ala Cys Glu Asp Cys
                85                  90                  95

Gly Lys Lys Tyr His Thr Met Leu Gly Tyr Lys Arg His Met Ala Leu
            100                 105                 110

His Ser Ala Ser Ser Gly Asp Leu Thr Cys Gly Val Cys Thr Leu Glu
        115                 120                 125

Leu Gly Ser Thr Glu Val Leu Leu Asp His Leu Lys Ser His Ala Glu
    130                 135                 140

Glu Lys Ala His His Ala Pro Arg Glu Lys Lys His Gln Cys Asp His
145                 150                 155                 160

Cys Glu Arg Cys Phe Tyr Thr Arg Lys Asp Val Arg Arg His Leu Val
                165                 170                 175

Val His Thr Gly Cys Lys Asp Phe Leu Cys Gln Phe Cys Ala Gln Arg
            180                 185                 190

Phe Gly Arg Lys Asp His Leu Thr Arg His Thr Lys Lys Thr His Ser
        195                 200                 205

Gln Glu Leu Met Gln Glu Ser Leu Gln Ala Gly Glu Tyr Gln Gly Gly
    210                 215                 220

Tyr Gln Pro Ile Ala Pro Pro Phe Gln Ile Lys Ala Asp Pro Met Pro
225                 230                 235                 240

Pro Phe Gln Leu Glu Met Pro Pro Glu Ser Gly Leu Asp Gly Gly Leu
                245                 250                 255

Pro Pro Glu Ile His Gly Leu Val Leu Ala Ser Pro Glu Glu Val Pro
            260                 265                 270

Gln Pro Met Leu Ser Met Pro Pro Met Gln Pro Met Pro Glu Gln Pro
        275                 280                 285

Phe Thr Leu His Pro Gly Val Val Pro Ser Ser Pro Pro Pro Ile Ile
    290                 295                 300

Leu Gln Glu His Lys Tyr Ser Pro Val Pro Thr Ser Phe Ala Pro Phe
305                 310                 315                 320

Val Ser Met Pro Met Lys Ala Asp Leu Lys Gly Phe Cys Asn Met Gly
                325                 330                 335

Leu Phe Glu Glu Phe Pro Leu Gln Glu Cys Gln Ser Pro Val Lys Phe
            340                 345                 350

Ser Gln Cys Phe Glu Met Ala Lys Glu Gly Phe Gly Lys Val Thr Leu
        355                 360                 365

Pro Lys Glu Leu Leu Val Asp Ala Val Asn Ile Ala Ile Pro Gly Ser
    370                 375                 380

Leu Glu Ile Ser Ser Leu Leu Gly Phe Trp Gln Leu Pro Pro Pro Pro
385                 390                 395                 400

Pro Gln Asn Gly Phe Met Asn Gly Thr Ile Pro Val Gly Ala Gly Glu
                405                 410                 415

Pro Leu Pro His Arg Ile Thr Cys Leu Ala Gln Gln Gln Pro Pro Pro
            420                 425                 430

Leu Leu Pro Pro Pro Pro Pro Leu Pro Leu Pro Glu Pro Leu Pro Gln
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        435                 440                 445

Pro Gln Leu Pro Pro Gln Phe Gln Leu Gln Leu Gln Pro Gln Pro Gln
    450                 455                 460

Met Gln Pro Gln Met Gln Leu Gln Pro Leu Gln Leu Gln Leu Pro Gln
465                 470                 475                 480

Leu Leu Pro Gln Leu Gln Pro Glu Pro Glu Pro Glu Pro Glu Pro Glu
                485                 490                 495

Glu Glu Glu Glu Glu Glu Glu Glu Ile Glu Glu Glu Glu Glu Ile Glu
            500                 505                 510

Glu Glu Glu Glu Ala Glu Pro Glu Ala Glu Glu Glu Glu Glu Ala Glu
        515                 520                 525

Asp Glu Glu Glu Ala Glu Glu Glu Glu Glu Glu Pro Gln Pro Glu Glu
    530                 535                 540

Ala Gln Ile Ala Met Ser Ala Val Asn Met Gly Gln Pro Pro Leu Pro
545                 550                 555                 560

Pro Thr Pro His Val Phe Thr Ala Gly Thr Asn Thr Ala Ile Leu Pro
                565                 570                 575

His Phe His His Ala Phe Arg
            580
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<210> SEQ ID NO 11
<211> LENGTH: 658
<212> TYPE: DNA
<213> ORGANISM: Rattus norvegicus
<220> FEATURE:
<221> NAME/KEY: mRNA
<222> LOCATION: (1)...(658)
<223> OTHER INFORMATION: AA892598 (EST196401)

<400> SEQUENCE: 11

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tttatatcat accaaagcaa tctagaaaca ctgtacagag cacacttgaa catttagaag    120

gctatatata atctgtggta aagtcatagg catcgtcttc ttcactcatt ttatccaaga    180

taaaggatct gtcagatggt ttacttgctg ttgattgccc aggtgacatc tccctggtct    240

cttctacagg agtcacatct gagatctctg catttttttc accagtaaca tgttcttgat    300

catcaccatc ctgttggtct tctgtctgtt ttggtgactc ttcggggatg tccttttctt    360

ctagtattcc atttgtcagg cccgaagacc ggaaaaggat tttattagtt aaatgagggc    420

ccttgaggac ttgtatgctg tgtgcattat tcttttctag ttcttctaga ttaaagcccc    480

tcttcatgat tgctgtaata ttctcattaa aatgaggaga atgattccag gatgcagggg    540

gatggcagta gtaacctaat gaggcacctg tccactcaga ccatagcagc ttagcagcac    600

tttcgacatt tgggcttcca ccttttttggt gcagacctct tctctgagca agtttagt      658
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<210> SEQ ID NO 12
<211> LENGTH: 480
<212> TYPE: DNA
<213> ORGANISM: Rattus norvegicus
<220> FEATURE:
<221> NAME/KEY: mRNA
<222> LOCATION: (1)...(480)
<223> OTHER INFORMATION: AA900476 (Mrg-1)

<400> SEQUENCE: 12

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gtgggggcag ggagggtgat ttctttcagc cgcgaggtta accgagtcaa cagctgactc    120
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tgctgggctg ctgtttgcac acgaagtccg tcataaaatc aaactcattt tggcccagcc 180

agagttctgg cagctccttg atgcggtcca aacccatctc tatcactaag gacataagca 240

cttcctcgtc gatgaaatca gtgtctatga cattgggcgg cagcattgcc gcggggacgt 300

gagccaccga ggcgggcatg gtgctgccac cgccactgcc gcccgcgctg ctgccacccg 360

cgccgcccga ggtgccgccg gagccgccgg gggtgccgct gccacccgcg ccgccagggg 420

tgctgctgcc tccactgtgc ttggggttgc aatctcggaa gtgctggttt gtcccgttca 480

<210> SEQ ID NO 13
<211> LENGTH: 2646
<212> TYPE: DNA
<213> ORGANISM: Mus musculus
<220> FEATURE:
<221> NAME/KEY: mRNA
<222> LOCATION: (1)...(2646)
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<223> OTHER INFORMATION: ST2L

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<210> SEQ ID NO 15
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<212> TYPE: DNA
<213> ORGANISM: Bos taurus
<220> FEATURE:
<221> NAME/KEY: mRNA
<222> LOCATION: (1)...(2681)
<223> OTHER INFORMATION: Bovine vacuolar H+-ATPase

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<223> OTHER INFORMATION: human vacuolar H+-ATPase

<400> SEQUENCE: 16

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<212> TYPE: DNA
<213> ORGANISM: Mus musculus
<220> FEATURE:
<221> NAME/KEY: mRNA
<222> LOCATION: (1)...(1536)
<223> OTHER INFORMATION: mouse vacuolar H+-ATPase

<400> SEQUENCE: 17

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tcccagcctc gtctcaccta caagactgtc tctggggtga atggtccact agtgatccta 180

gatcatgtga agtttcccag atacgctgag attgtccact tgacattacc agatggcaca 240

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gggacatctg gtatagacgc caagaaaaca tcctgtgagt ttactggaga tattctccga 360

acaccagtgt ctgaggatat gcttggtcgg gtattcaacg gatcaggaaa acccattgac 420

cgaggccctg tggtgctggc tgaagacttc cttgacatca tgggtcagcc aatcaaccct 480

cagtgtcgga tctatccaga agagatgatt cagacgggca tttccgccat cgatggcatg 540

aacagtattg ctaggggaca gaaaatcccg atcttttctg ctgctggatt accccataac 600

gagattgcag ctcagatctg tcgccaggct ggtttggtaa agaaatccaa agatgtagtg 660

gactatagtg aagaaaattt tgccattgtg tttgctgcta tgggagtaaa catggaaaca 720

gcccggttct ttaaatctga ctttgaagaa aatggctcaa tggacaatgt ctgcctgttt 780

ttgatcttgc ctaatgaccc aaccattgag cggatcatca ctccctgcct ggctctgacc 840

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<210> SEQ ID NO 18
<211> LENGTH: 2820
<212> TYPE: DNA
<213> ORGANISM: Homo Sapiens
<220> FEATURE:
<221> NAME/KEY: mRNA
<222> LOCATION: (1)...(2820)
<223> OTHER INFORMATION: human vacuolar H+-ATPase (56,000 subunit -HO57)

<400> SEQUENCE: 18

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ctctcccagc ctcgcctcac atacaagaca gtatctggag tcaatggtcc actagtgatc 180

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gaagggactt caggtataga tgctaagaaa acgtcctgtg agtttactgg ggatattctc 360

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gacagaggtc ctgttgtact ggccgaagac ttcttggata tcatgggtca gccaatcaac 480

cctcaatgtc gaatctaccc agaggaaatg attcagactg gcatttcggc catcgatggg 540

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tgccagtgtt gcaacgtttt aaactgctaa cagaccttaa aatatccccc tacctgggtc 1740

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<210> SEQ ID NO 19
<211> LENGTH: 2457
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: mRNA
<222> LOCATION: (1)...(2457)
<223> OTHER INFORMATION: human vacuolar H+-ATPase B subunit

<400> SEQUENCE: 19

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ctggctgaag acttccttga tatcatgggt cagccaatca accctcaatg tcgaatctac 180

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atctgtcgcc aggctggttt ggtaaagaaa tccaaagatg tagtagacta cagtgaggaa 360

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tctgactttg aagaaaatgg ctcaatggac aatgtctgcc tctttttgaa cttggcgaat 480

gacccaacca ttgagcgaat tatcactcct cgcctggctc taaccacagc tgaatttctg 540

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gcacttcgag aggtttcagc agccagggaa gaggttcctg gtcgacgagg cttcccaggt 660

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cgagactctg cgaacgatta gctgccgctt ctgcactgct ccacactctt gtgaaatact 1260

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cctgagcgat cttctatgca gttctgccat gtgtcctgtt tggtctctct gtgttctttg 2340

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<210> SEQ ID NO 20
<211> LENGTH: 2676
<212> TYPE: DNA
<213> ORGANISM: Bos taurus
<220> FEATURE:
<221> NAME/KEY: mRNA
<222> LOCATION: (1)...(2676)
<223> OTHER INFORMATION: bovine vacuolar H+-ATPase B subunit

<400> SEQUENCE: 20

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<210> SEQ ID NO 21
<211> LENGTH: 3035
<212> TYPE: DNA
<213> ORGANISM: Gallus gallus
<220> FEATURE:
<221> NAME/KEY: mRNA

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<222> LOCATION: (1)...(3035)
<223> OTHER INFORMATION: gallus vacuolar H+-ATPase

<400> SEQUENCE: 21

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aaaggtccaa attgtacctt gaagcttgca aagaaagatt cctacttaac ttttcttttt 2220

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<210> SEQ ID NO 22
<211> LENGTH: 1737
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: mRNA
<222> LOCATION: (1)...(1737)
<223> OTHER INFORMATION: human CD44R

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<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
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<222> LOCATION: (1)...(1297)
<223> OTHER INFORMATION: human CD44

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tggtcgctac agcatctctc ggacggaggc cgctgacctc tgcaaggctt tcaatagcac 240

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tgatggacca attaccataa ctattgttaa ccgtgatggc acccgctatg tccagaaagg 540

agaatacaga acgaatcctg aagacatcta ccccagcaac cctactgatg atgacgtgag 600

cagcggctcc tccagtgaaa ggagcagcac ttcaggaggt tacatctttt acacctttc 660

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<211> LENGTH: 1177
<212> TYPE: DNA

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<213> ORGANISM: Mus musculus
<220> FEATURE:
<221> NAME/KEY: mRNA
<222> LOCATION: (1)...(1177)
<223> OTHER INFORMATION: mouse CD44

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gccgctacgc aggtgtattc catgtggaga aaaatggccg ctacagtatc tcccggactg 180

aggcagctga cctctgccag gctttcaaca gtaccttacc caccatggac caaatgaagt 240

tggccctgag caagggtttt gaaacatgca ggtatgggtt catagaagga aatgtggtaa 300

ttccgaggat tcatcccaac gctatctgtg cagccaacca cacaggagta tatatcctcg 360

tcacgtccaa cacctcccac tatgacacat attgcttcaa tgcctcagcc cctcctgaag 420

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<210> SEQ ID NO 25
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<212> TYPE: DNA
<213> ORGANISM: Cricetulus sp.
<220> FEATURE:
<221> NAME/KEY: mRNA
<222> LOCATION: (1)...(1089)
<223> OTHER INFORMATION: hamster CD44

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aatggccgct acagcatctc acggactgag gcagctgacc tctgccaagc tttcaacagc 180

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gccaaccaca ctggggtgta tatcctcaca tccaacacat ctcactacga tacatattgc 360

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tccaaattaa ctgaacactc aagtgggaat caagacagtg ggcttaactc aacttctcgt 780

cctggaggaa aacctcgagt tccagaatgg ctcatcgtct tggcatctct cctggcgctg 840

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<210> SEQ ID NO 26
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<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: mRNA
<222> LOCATION: (1)...(4632)
<223> OTHER INFORMATION: human LOT1

<400> SEQUENCE: 26

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aaaaaaaaaa aa 4632

<210> SEQ ID NO 27
<211> LENGTH: 2828
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: mRNA
<222> LOCATION: (1)...(2828)
<223> OTHER INFORMATION: human ZAC zinc finger protein

<400> SEQUENCE: 27

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<210> SEQ ID NO 28
<211> LENGTH: 3975
<212> TYPE: DNA
<213> ORGANISM: Mus musculus
<220> FEATURE:
<221> NAME/KEY: mRNA
<222> LOCATION: (1)...(3975)
<223> OTHER INFORMATION: mouse ZAC1 zinc finger protein

<400> SEQUENCE: 28

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<211> LENGTH: 536
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

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<210> SEQ ID NO 30
<211> LENGTH: 2059
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
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<221> NAME/KEY: mRNA

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<222> LOCATION: (1)...(2059)
<223> OTHER INFORMATION: putative nucleotide binding protein, estradiol-induced (E2IG3)

<400> SEQUENCE: 30

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agcagactgc taaactgttc tctgtataag ttatggtatg catgagctgt gtaaattttg 1920

tgaatatgta ttatattaaa accaggcaac ttggaatccc taaattctgt aaaaagacaa 1980

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aaaaaaaaaa aaaaaaaaa 2059

<210> SEQ ID NO 31
<211> LENGTH: 1943

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<212> TYPE: DNA
<213> ORGANISM: Mus musculus
<220> FEATURE:
<221> NAME/KEY: mRNA
<222> LOCATION: (1)...(1943)
<223> OTHER INFORMATION: mouse mrg-1

<400> SEQUENCE: 31

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ccatcggctg tccctctatg tgctgctgag ccggtcctgg actcgacgag cccgccttcg 180

gtgttccgag cagaaatcgc aaagacggaa ggactggaaa tggcagacca tatgatggcc 240

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<210> SEQ ID NO 32
<211> LENGTH: 6324
<212> TYPE: DNA

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<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: mRNA
<222> LOCATION: (1)...(6324)
<223> OTHER INFORMATION: human p35srj

<400> SEQUENCE: 32

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acatgaagat gaaggtgggg atcagtgtga tgcatctaca agtcaaggga caccaaagat 180

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I claim:

1. A method of treating a subject having a cardiovascular condition, said method comprising:

performing an assay to determine a level of soluble Interleukin-1 Receptor-Like-1 (ST2) in a biological sample from a subject having a cardiovascular condition;

comparing the level of soluble ST2 in the biological sample to a predetermined value;

identifying a subject having an elevated level of soluble ST2 as compared to the predetermined value; and

administering to the identified subject a treatment comprising a therapeutically effective amount of a renin-angiotensin system inhibitor and a therapeutically effective amount of a beta-adrenergic receptor blocker.

2. The method of claim **1**, wherein the cardiovascular condition is selected from the group consisting of cardiac hypertrophy, myocardial infarction, stroke, and heart failure.

3. The method of claim **1**, wherein the sample comprises a biological fluid.

4. The method of claim **3**, wherein the biological fluid is blood or serum.

5. The method of claim **1**, wherein the assay comprises contacting the biological sample with an antibody that specifically binds to soluble ST2.

6. The method of claim **1**, wherein the subject is a human.

7. The method of claim **1**, wherein the renin-angiotensin system inhibitor is selected from the group consisting of: an angiotensin-converting enzyme (ACE) inhibitor, an angiotensin II receptor blocker (ARB), an angiotensin II receptor antagonist, an agent that activates the catabolism of angiotensin II, and an agent that prevents the synthesis of angiotensin I.

8. The method of claim **1**, wherein the beta-adrenergic receptor blocker is selected from the group consisting of:

atenolol, acebutolol, alprenolol, befunolol, betaxolol, bunitrolol, carteolol, celiprolol, hedroxalol, indenolol, labetalol, levobunolol, mepindolol, methypranol, metindol, metoprolol, metrizoranolol, oxprenolol, pindolol, propranolol, practolol, sotalol, nadolol, tiprenolol, tomalolol, timolol, bupranolol, penbutolol, and trimepranol.

9. The method of claim **1**, wherein the predetermined value is a level of secreted ST2 in a healthy subject.

10. The method of claim **1**, wherein the treatment is administered orally, nasally, intradermally, by infusion, intravenously, or intramuscularly.

11. A method of treating a subject having a cardiovascular condition, said method comprising:

performing an assay to determine a level of soluble Interleukin-1 Receptor-Like-1 (ST2) in a biological sample from a subject having a cardiovascular condition;

comparing the level of soluble ST2 in the biological sample to a predetermined value;

identifying a subject having an elevated level of soluble ST2 as compared to the predetermined value; and

administering to the identified subject a treatment comprising a therapeutically effective amount of a renin-angiotensin system inhibitor.

12. The method of claim **11**, wherein the cardiovascular condition is selected from the group consisting of cardiac hypertrophy, myocardial infarction, stroke, and heart failure.

13. The method of claim **11**, wherein the sample comprises a biological fluid.

14. The method of claim **13**, wherein the biological fluid is blood or serum.

15. The method of claim **11**, wherein the assay comprises contacting the biological sample with an antibody that specifically binds to soluble ST2.

16. The method of claim **11**, wherein the subject is a human.

17. The method of claim **11**, wherein the renin-angiotensin system inhibitor is selected from the group consisting of: an angiotensin-converting enzyme (ACE) inhibitor, an angiotensin II receptor blocker (ARB), an angiotensin II receptor antagonist, an agent that activates the catabolism of angiotensin II, and an agent that prevents the synthesis of angiotensin I.

18. The method of claim **11**, wherein the predetermined value is a level of secreted ST2 in a healthy subject.

19. The method of claim **11**, wherein the treatment is administered orally, nasally, intradermally, by infusion, intravenously, or intramuscularly.

20. A method of treating a subject having a cardiovascular condition, said method comprising:

performing an assay to determine a level of soluble Interleukin-1 Receptor-Like-1 (ST2) in a biological sample from a subject having a cardiovascular condition;

comparing the level of soluble ST2 in the biological sample to a predetermined value;

identifying a subject having an elevated level of soluble ST2 as compared to the predetermined value; and

administering to the identified subject a treatment comprising a therapeutically effective amount of a beta-adrenergic receptor blocker.

21. The method of claim **20**, wherein the cardiovascular condition is selected from the group consisting of cardiac hypertrophy, myocardial infarction, stroke, and heart failure.

22. The method of claim **20**, wherein the sample comprises a biological fluid.

23. The method of claim **22**, wherein the biological fluid is blood or serum.

24. The method of claim **20**, wherein the assay comprises contacting the biological sample with an antibody that specifically binds to soluble ST2.

25. The method of claim **20**, wherein the subject is a human.

26. The method of claim **20**, wherein the beta-adrenergic receptor blocker is selected from the group consisting of: atenolol, acebutolol, alprenolol, befunolol, betaxolol, bunitrolol, carteolol, celiprolol, hedroxalol, indenolol, labetalol, levobunolol, mepindolol, methypranol, metindol, metoprolol, metrizoranolol, oxprenolol, pindolol, propranolol, practolol, sotalol, nadolol, tiprenolol, tomalolol, timolol, bupranolol, penbutolol, and trimepranol.

27. The method of claim **20**, wherein the predetermined value is a level of secreted ST2 in a healthy subject.

28. The method of claim **20**, wherein the treatment is administered orally, nasally, intradermally, by infusion, intravenously, or intramuscularly.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 8,871,452 B2
APPLICATION NO. : 13/788922
DATED : October 28, 2014
INVENTOR(S) : Richard T. Lee

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

IN THE CLAIMS:

Col. 133, line 2, Claim 8, delete "hedroxalol," and insert -- hydroxalol, --, therefor.

Col. 134, line 32, Claim 26, delete "hedroxalol," and insert -- hydroxalol, --, therefor.

Signed and Sealed this
Seventeenth Day of February, 2015

Michelle K. Lee

Michelle K. Lee
Deputy Director of the United States Patent and Trademark Office

专利名称(译)	治疗心血管疾病的方法		
公开(公告)号	US8871452	公开(公告)日	2014-10-28
申请号	US13/788922	申请日	2013-03-07
[标]申请(专利权)人(译)	布赖汉姆妇女医院		
申请(专利权)人(译)	杨百翰妇女医院有限公司，		
当前申请(专利权)人(译)	布里格姆妇女医院，INC.		
[标]发明人	LEE RICHARD T		
发明人	LEE, RICHARD, T.		
IPC分类号	G01N33/50 A61P9/00 C12Q1/68 G01N33/68 G01N33/00 G01N33/53 A61K45/06 G01N33/566 C12P21/08 C07K16/28 C12Q1/6883		
CPC分类号	A61K45/06 G01N2800/32 G01N2800/323 G01N33/6887 G01N33/5008 G01N2800/324 G01N33/5061 G01N33/5091 C12Q2600/158 G01N33/6893 G01N33/5041 C12Q2600/136 G01N33/5023 C12Q1/6883 G01N33/6869 G01N2333/7155 G01N2800/2871 G01N2800/325 G01N2800/52		
代理机构(译)	FISH & RICHARDSON P.C.		
审查员(译)	同人，ROBERT		
优先权	60/247457 2000-11-09 US		
其他公开文献	US20130317030A1		
外部链接	Espacenet USPTO		

摘要(译)

本发明涉及用于诊断和治疗心血管疾病的方法和组合物。更具体地，本发明涉及可用于诊断和/或治疗心血管疾病的分离的分子，包括心脏肥大，心肌梗塞，中风，动脉硬化和心力衰竭。

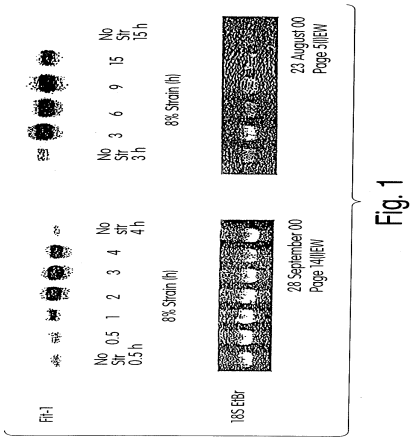

Fig. 1