(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 December 2014 (11.12.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/197624 A1

(51) International Patent Classification:
C12N 9/88 (2006.01)

(21) International Application Number:
PCT/US2014/040948

(22) International Filing Date:
4 June 2014 (04.06.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/832,759 7 June 2013 (07.06.2013) US

(71) Applicant: **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA** [US/US]; 1111 Franklin Street, 12th Floor, Oakland, CA 94607-5200 (US).

(72) Inventors: **HAMMOND, H., Kirk**; 9500 Gilman Drive, Mail Code 0910, University of California, San Diego, La Jolla, CA 92093-0910 (US). **GAO, Mei, Hua**; 9500 Gilman Drive, Mail Code 0910, University of California, San Diego, La Jolla, CA 92093-0910 (US).

(74) Agents: **EINHORN, Gregory, P.** et al.; Gavrilovich, Dodd & Lindsey LLP, 4660 La Jolla Village Drive, Suite 750, San Diego, CA 92122 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

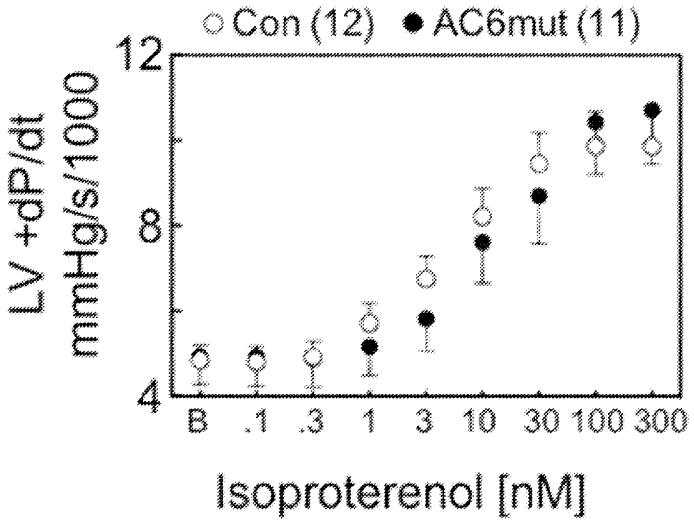
Published:

— *with international search report (Art. 21(3))*

[Continued on next page]

(54) Title: CYCLE ADENOSINE MONOPHOSPHATE-INCOMPETENT ADENYLYL CYCLASE AND COMPOSITIONS AND METHODS FOR TREATING HEART FAILURE AND INCREASING CARDIAC FUNCTION

Figure 3



(57) Abstract: The invention provides methods for treating, ameliorating or protecting (preventing) an individual or a patient having or at risk of having heart disease or heart failure, or decreased cardiac function, comprising: providing a cyclic adenosine monophosphate-incompetent (cAMP-incompetent) adenylyl cyclase type 6 (AC6) protein or polypeptide (also called "an AC6mut"), or an AC6mut -encoding nucleic acid or a gene operatively linked to a transcriptional regulatory sequence.

WO 2014/197624 A1

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

CYCLIC ADENOSINE MONOPHOSPHATE-INCOMPETENT ADENYLYL CYCLASE AND COMPOSITIONS AND METHODS FOR TREATING HEART FAILURE AND INCREASING CARDIAC FUNCTION

RELATED APPLICATIONS

This Patent Convention Treaty (PCT) International Application claims the benefit of priority under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 61/832,759, filed June 07, 2013. The aforementioned application is expressly incorporated herein by reference in its entirety and for all purposes.

TECHNICAL FIELD

This invention relates generally to cellular and molecular biology, gene therapy and medicine; and more specifically, to compositions methods for treating a subject having or at risk of having heart failure or heart disease by administering a cyclic adenosine monophosphate-incompetent (cAMP-incompetent) adenylyl cyclase type 6 (AC6) protein or polypeptide (also called "an AC6mut"), or an AC6mut -encoding nucleic acid sequence.

BACKGROUND

Adenylyl cyclase, a transmembrane protein in cardiac myocytes and other cells, is the key effector molecule that transduces p-adrenergic signaling by generation of intracellular cAMP. Cyclic-AMP is the second messenger for downstream events including protein kinase A activation. Heart failure is associated with impaired cAMP production, which is tightly linked to heart function. It has been shown that increased cardiac AC type 6 (AC6), a dominant AC isoform expressed in mammalian cardiac myocytes, has protean beneficial effects on the failing left ventricle (LV). These include: 1) increased survival in cardiomyopathy and in acute myocardial infarction, 2) reduced action potential duration and facilitation of atrio-ventricular conduction associated with reduction of AV block, 3) reductions in both LV dilation and pathological hypertrophy, 4) beneficial effects on calcium handling through improved SERCA2a activity, increased phospholamban activity, and 5) increased cardiac troponin I phosphorylation.

Consequently, several drugs have been generated which increase intracellular levels of cAMP, and have been tested in patients with heart failure. However, these drugs

typically increase mortality. The current dogma dictates that drugs and proteins that increase levels of intracellular cAMP are deleterious to the failing heart, and therefore, are unsuitable for the treatment of heart failure.

SUMMARY

In alternative embodiments, the invention provides methods for treating, ameliorating or protecting (preventing) an individual or a patient against heart disease or decreased cardiac function, comprising: providing a cyclic adenosine monophosphate-incompetent (cAMP-incompetent) adenylyl cyclase type 6 (AC6) protein or polypeptide (also called "an AC6mut"), or an AC6mut -encoding nucleic acid or a gene operatively linked to a transcriptional regulatory sequence; or an expression vehicle, a vector, a recombinant virus, or equivalent, having contained therein an AC6mut -encoding nucleic acid or gene, and the expression vehicle, vector, recombinant virus, or equivalent can express the an AC6mut -encoding nucleic acid or gene in a cell or *in vivo*; and administering or delivering the AC6mut, or the AC6mut-encoding nucleic acid or gene operatively linked to a transcriptional regulatory sequence, or the expression vehicle, vector, recombinant virus, or equivalent, to an individual or a patient in need thereof, thereby treating, ameliorating or protecting (preventing) the individual or patient against the heart disease or decreased cardiac function. In alternative embodiments, the AC6mut comprises an adenylyl cyclase (AC) polypeptide having a substitution of an uncharged or non-polar amino acid for a charged or an acidic amino acid in the catalytic core of the AC polypeptide.

In alternative embodiments, the invention provides methods, and an *in vivo* method for or method of:

(1) treating a subject having or at risk of having a heart disease or a heart failure;

(2) treating, ameliorating, reversing the effects of, protecting or preventing an individual or a patient against:

a heart disease,

a heart failure,

a decrease in heart function or cardiac output,

a decrease in heart function or cardiac output due to a heart infection or a heart condition,

(3) enhancing calcium handling in intact cardiac myocytes by increasing sarcoplasmic reticulum (SR) Ca^{2+} uptake and/or increased Ca^{2+} transients with reduced time of relaxation in intact cardiac myocytes,

(4) inhibiting the generation of intracellular cAMP levels in cardiac myocytes,

(5) protecting a cardiac myocyte from a programmed cell death (apoptosis) signal, or decreasing the number of cardiac myocytes signaled to programmed cell death (apoptosis) subsequent to an apoptotic signal, or

(6) in heart failure patients or in individuals having a heart infection or a heart condition resulting in a decrease in heart function or cardiac output: increasing heart function or cardiac output, reducing symptom and/or decreasing mortality; or reducing the frequency of hospitalizations for heart failure,

comprising:

(a) providing:

(i) a cyclic adenosine monophosphate-incompetent (cAMP-incompetent) adenylyl cyclase type 6 (AC6) protein or polypeptide (also called "an AC6mut"),

wherein optionally the AC6mut is a recombinant, a synthetic, a peptidomimetic or an isolated AC6mut polypeptide or peptide; or

(ii) a AC6mut-encoding nucleic acid or gene:

wherein optionally the AC6mut-encoding nucleic acid or gene is operatively linked to a transcriptional regulatory sequence, wherein optionally the transcriptional regulatory sequence is a promoter and/or an enhancer, or a cardiac cell-specific promoter or a myocyte-specific promoter; or

wherein optionally the AC6mut-encoding nucleic acid or gene is operatively linked to a transcriptional regulatory sequence, and optionally the AC6mut-encoding nucleic acid or gene is contained in a delivery vehicle, a vector, an expression vector, a recombinant virus, or an equivalent, and the delivery vehicle, expression vehicle, vector, recombinant virus, or equivalent can express the AC6mut-encoding nucleic acid or gene in a cell or *in vivo,*

wherein optionally the cell is a cardiac cell or a myocyte;

wherein the AC6mut does not catalyze the breakdown of ATP to cAMP, or has impaired ability to catalyze the breakdown of ATP to cAMP, and optionally the impaired ability to catalyze the breakdown of ATP to cAMP is defined as the AC6mut having only

about 1%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% of the ATP to cAMP catalytic activity of wild type AC6,

and when the AC6mut is expressed in a cardiac myocyte *in vivo* left ventricular (LV) function is not affected or does not decrease or LV function is substantially not affected or decreased,

and optionally AC6mut expression in a cardiac myocyte increases sarcoplasmic reticulum Ca^{2+} uptake,

and optionally AC6mut expression in a cardiac myocyte reduces the EC50 for SERCA2a activation,

and optionally AC6mut expression in a cardiac myocyte reduces expression of a phospholamban protein, ,

and optionally the substitution inhibits Mg^{2+} binding and alters the efficiency of Gsα-mediated activation of the catalytic core;

(b) delivering or administering the AC6mut, or the AC6mut-encoding nucleic acid or gene, to a cardiac cell or a cardiac myocyte, or expressing the AC6mut in a cardiac cell or a cardiac myocyte, or expressing the AC6mut-encoding nucleic acid or gene in a cardiac cell or a cardiac myocyte,

wherein optionally the AC6mut-encoding nucleic acid is operatively linked to a transcriptional regulatory sequence, or optionally the delivery vehicle, vector, expression vector, recombinant virus, or equivalent, is delivered or administered to a cardiac myocyte cell, or to an individual or a patient in need thereof,

and optionally the delivering or administering of the AC6mut-encoding nucleic acid or gene to the cardiac cell or myocyte *in vivo* is a targeted delivery to a heart muscle or a cardiac myocyte, or comprises direct delivery or administration to a heart, or comprises an intracardiac injection or an infusion,

thereby:

treating the subject having or at risk of having a heart disease or a heart failure,

treating, ameliorating or protecting (preventing) an individual or a patient against a heart disease, a heart failure, a decrease in heart function or cardiac output, a decrease in heart function or cardiac output due to a heart infection or a heart condition,

enhancing calcium handling in intact cardiac myocytes by increasing sarcoplasmic reticulum (SR) Ca^{2+} uptake and/or increased Ca^{2+} transients with reduced time of relaxation in intact cardiac myocytes,

inhibiting the generation of intracellular cAMP levels in cardiac myocytes,

protecting a cardiac myocyte from a programmed cell death (apoptosis) signal, or decreasing the number of cardiac myocytes signaled to programmed cell death (apoptosis) subsequent to an apoptotic signal, or

in heart failure patients or in individuals having a heart infection or a heart condition resulting in a decrease in heart function or cardiac output: increasing heart function or cardiac output, reducing symptom and/or decreasing mortality.

In alternative embodiments, the AC6mut comprises an adenylyl cyclase (AC) polypeptide having a substitution of an uncharged or non-polar amino acid for a charged or an acidic amino acid in the catalytic core of the AC polypeptide,

wherein optionally the uncharged or non-polar amino acid is an alanine (Ala), and optionally the acidic amino acid is an aspartic acid (Asp), or optionally the uncharged or non-polar amino acid is an Ala and the acidic amino acid is an Asp.

In alternative embodiments, the AC6mut comprises:

a murine adenylyl cyclase (AC) polypeptide having a substitution of an Ala for an Asp at position 426 in the catalytic core of the AC polypeptide based on SEQ ID NO:16, where SEQ ID NO:17 is the polypeptide amino acid sequence after the D => A substitution (SEQ ID NO:16 is the amino acid sequence before the D => A substitution); or

a murine AC6mut polypeptide having a substitution of an alanine, or Ala for an Asp at position 436 in the catalytic core of the AC polypeptide based on SEQ ID NO:11, where SEQ ID NO:12 is the polypeptide amino acid sequence after the D => A substitution (SEQ ID NO:11 is the amino acid sequence before the D => A substitution).

In alternative embodiments, the AC6 is a mammalian AC6 polypeptide, or the AC6 is a human AC6 polypeptide. In alternative embodiments, the human AC6 polypeptide comprises a human AC6 polypeptide having a substitution of an Ala for an Asp at position 426 in the catalytic core of the AC polypeptide based on SEQ ID NO:10, where SEQ ID NO:13 is the polypeptide amino acid sequence after the D => A substitution (SEQ ID NO:10 is the amino acid sequence before the D => A substitution).

In alternative embodiments of the methods:

(a) the AC6mut-encoding nucleic acid or gene is stably inserted into a chromosome of a cell;

(b) the delivery vehicle, vector, expression vector, recombinant virus, or equivalent, is or comprises: an adeno-associated virus (AAV); a recombinant AAV virus or vector; an AAV virion, or an adenovirus vector, or any pseudotype, hybrid or derivative thereof;

(c) the method of (b), wherein the adeno-associated virus (AAV), recombinant AAV virus or vector, AAV virion, or adenovirus vector, is or comprises: an AAV serotype AAV5, AAV6, AAV7, AAV8 or AAV9; a rhesus macaque AAV (AAVrh), or an AAVrh10; or any hybrid or derivative thereof;

(d) the AC6mut -encoding nucleic acid or gene is operatively linked to a regulated or inducible transcriptional regulatory sequence;

(e) the method of (d), wherein the regulated or inducible transcriptional regulatory sequence is a regulated or inducible promoter;

(f) the method of any of (a) to (e), wherein administering the AC6mut -encoding nucleic acid or gene operatively linked to a transcriptional regulatory sequence, or the delivery vehicle, vector, expression vector, recombinant virus, or equivalent, to an individual or a patient in need thereof results in: targeted delivery and expression of the AC6mut in a cardiac myocyte, or a AC6mut being released into the bloodstream or general circulation; or

(g) the method of any of (a) to (f), wherein a disease, infection or condition responsive to an increased AC6mut level *in vivo* is a cardiac contractile dysfunction; a congestive heart failure (CHF); a cardiac fibrosis; a cardiac myocyte disease; a cardiac myocyte dysfunction or a cardiac myocyte apoptosis.

In alternative embodiments of the methods:

(a) the AC6mut -encoding nucleic acid or gene operatively linked to the transcriptional regulatory sequence; or the delivery vehicle, vector, expression vector, recombinant virus, or equivalent, is administered or delivered to the individual or a patient in need thereof, by oral administration, by intramuscular (IM) injection, by intravenous (IV) injection, by subcutaneous (SC) injection, by intradermal injection, by intrathecal injection, by intra-arterial (IA) injection, by intracoronary or intracardiac injection, by intraocular injection or application, by inhalation, or by a biolistic particle delivery system, or by using a "gene gun", air pistol or a HELIOS™ gene gun (Bio-Rad Laboratories, Hercules, CA),

wherein optionally the AC6mut -encoding nucleic acid or gene is delivered by intravenous (IV) injection of an AAV vector, or AAV-9 vector; or

(b) the AC6mut -encoding nucleic acid or gene operatively linked to the transcriptional regulatory sequence; or the expression vehicle, vector, recombinant virus, or equivalent, is administered or delivered to the individual or a patient in need thereof, by introduction into any cell, organ, tissue or fluid space within the body that is adjacent to or is drained by the bloodstream, such that the encoded AC6mut protein may be secreted from cells in the tissue and released into the bloodstream.

In alternative embodiments of the methods:

(a) the individual, patient or subject is administered a stimulus or signal that induces expression of the AC6mut -expressing nucleic acid or gene, or induces or activates a promoter (e.g., a promoter operably linked to the AC6mut -expressing nucleic acid or gene) that induces expression of or up-regulates expression of the AC6mut -expressing nucleic acid or gene;

(b) the individual, patient or subject is administered a stimulus or signal that induces synthesis of an activator of a promoter, wherein optionally the promoter is an AC gene promoter, or a myocyte cell-specific promoter;

(c) the individual, patient or subject is administered a stimulus or signal that induces synthesis of a natural or a synthetic activator of the AC6mut -expressing nucleic acid or gene or the AC6mut -expressing nucleic acid or gene-specific promoter,

wherein optionally the natural activator is an endogenous transcription factor;

(d) the method of (c), wherein the synthetic activator is a zinc-finger DNA binding protein designed to specifically and selectively turn on an endogenous or exogenous target gene, wherein optionally the endogenous target is an AC6mut -expressing nucleic acid or gene or an activator of an AC6mut, or a AC6mut -expressing nucleic acid or gene, or an activator of a promoter operatively linked to a AC6mut -expressing nucleic acid or gene;

(e) the method of any of (a) to (c), wherein the stimulus or signal comprises a biologic, a light, a chemical or a pharmaceutical stimulus or signal;

(f) the individual, patient or subject is administered a stimulus or signal that stimulates or induces expression of a post-transcriptional activator of an AC6mut, or a AC6mut -expressing nucleic acid or gene, or an activator of a promoter operatively linked to a AC6mut -expressing nucleic acid or gene, or

(g) the individual, patient or subject is administered a stimulus or signal that inhibits or induces inhibition of a transcriptional repressor or a post-transcriptional repressor of a AC6-expressing nucleic acid or gene.

In alternative embodiments: the chemical or pharmaceutical that induces expression of the AC6mut, or the AC6mut -expressing nucleic acid or gene, or induces expression of the regulated or inducible promoter operatively linked to the AC6mut -expressing nucleic acid or gene, is or comprises an oral antibiotic, a doxycycline or a rapamycin; or a tet-regulation system using doxycycline is used to induce expression of the AC6mut, or the AC6mut -expressing nucleic acid or gene, or an equivalent thereof.

In alternative embodiments: the AC6mut, or the AC6mut -expressing nucleic acid or gene, or the delivery vehicle, vector, expression vector, recombinant virus, or equivalent, is formulated in or as a lyophilate, a liquid, a gel, a hydrogel, a powder, a spray, an ointment, or an aqueous or a saline formulation.

In alternative embodiments: the AC6mut, or the AC6mut -expressing nucleic acid or gene or the delivery vehicle, vector, expression vector, recombinant virus, or equivalent, comprises, or is formulated in, a vesicle, a hydrogel, a gel, a liposome, a nanoliposome, a nanoparticle or a nanolipid particle (NLP).

In alternative embodiments: the AC6mut, or the AC6mut -expressing nucleic acid or gene or the delivery vehicle, vector, expression vector, recombinant virus, or equivalent, is formulated in an isolated or cultured cell, and optionally the cell is a mammalian cell, a cardiac cell, or a human cell, a non-human primate cell, a monkey cell, a mouse cell, a rat cell, a guinea pig cell, a rabbit cell, a hamster cell, a goat cell, a bovine cell, an equine cell, an ovine cell, a canine cell or a feline cell.

In alternative embodiments: the AC6mut, or the AC6mut -expressing nucleic acid or gene, or the delivery vehicle, vector, expression vector, recombinant virus, or equivalent, is formulated as a pharmaceutical or sterile.

In alternative embodiments: the AC6mut, or the AC6mut -expressing nucleic acid or gene or the delivery vehicle, vector, expression vector, recombinant virus, or equivalent, is formulated or delivered with, on, or in conjunction with a product of manufacture, an artificial organ or an implant.

In alternative embodiments: the AC6mut, or the AC6mut -expressing nucleic acid or gene or the delivery vehicle, vector, expression vector, recombinant virus, or equivalent expresses a AC6mut polypeptide *in vitro* or *ex vivo*.

In alternative embodiments, the invention provides methods for treating, ameliorating, reversing, protecting or preventing an individual or a patient against a AC6mut -responsive pathology, infection, disease, illness, or condition, comprising practicing a method of the invention.

In alternative embodiments, the invention provides methods for treating, ameliorating, reversing, protecting or preventing a cardiopathy or a cardiovascular disease in an individual or a patient in need thereof, comprising practicing a method of the invention. In alternative embodiments, the cardiopathy or cardiovascular disease comprises: a coronary artery disease (CAD); an atherosclerosis; a thrombosis; a restenosis; a vasculitis, an autoimmune or a viral vasculitis; a polyarteritis nodosa; a Churg-Strass syndrome; a Takayasu's arteritis; a Kawasaki Disease; a Rickettsial vasculitis; an atherosclerotic aneurism; a myocardial hypertrophy; a congenital heart disease (CHD); an ischemic heart disease; an angina; an acquired valvular or an endocardial disease; a primary myocardial disease; a myocarditis; an arrhythmia; a transplant rejection; a metabolic myocardial disease; a myocardiomyopathy; a congestive, a hypertrophic or a restrictive cardiomyopathy; and/or, a heart transplant.

In alternative embodiments, the invention provides uses comprising:

an AC6mut; an AC6mut -expressing nucleic acid or gene; a delivery vehicle, a vector, an expression vector, a recombinant virus, or equivalent; an adeno-associated virus (AAV); a recombinant AAV virus or vector; or an adenovirus vector, or any pseudotype, hybrid or derivative thereof, as set forth in any of claims 1 to 16,

wherein optionally the AAV or recombinant AAV virus or vector comprises an AAV serotype AAV5, AAV6, AAV7, AAV8 or AAV9; a rhesus macaque AAV (AAVrh), or an AAVrh10; or any hybrid or derivative thereof, or an AC6mut-expressing cell or cardiac myocyte,

in the preparation of a medicament for:

(1) treating a subject having or at risk of having a heart disease or a heart failure;

(2) treating, ameliorating, reversing the effects of, protecting or preventing an individual or a patient against:

a heart disease,

a heart failure,

a decrease in heart function or cardiac output,

a decrease in heart function or cardiac output due to a heart infection or a heart condition,

(3) enhancing calcium handling in intact cardiac myocytes by increasing sarcoplasmic reticulum (SR) Ca^{2+} uptake and/or increased Ca^{2+} transients with reduced time of relaxation in intact cardiac myocytes,

(4) inhibiting the generation of intracellular cAMP levels in cardiac myocytes,

(5) protecting a cardiac myocyte from a programmed cell death (apoptosis) signal, or decreasing the number of cardiac myocytes signaled to programmed cell death (apoptosis) subsequent to an apoptotic signal,

(6) in heart failure patients or in individuals having a heart infection or a heart condition resulting in a decrease in heart function or cardiac output: increasing heart function or cardiac output, reducing symptom and/or decreasing mortality; or reducing the frequency of hospitalizations for heart failure;

(7) a cardiopathy or a cardiovascular disease; or

(8) a coronary artery disease (CAD); an atherosclerosis; a thrombosis; a restenosis; a vasculitis, an autoimmune or a viral vasculitis; a polyarteritis nodosa; a Churg-Strass syndrome; a Takayasu's arteritis; a Kawasaki Disease; a Rickettsial vasculitis; an atherosclerotic aneurism; a myocardial hypertrophy; a congenital heart disease (CHD); an ischemic heart disease; an angina; an acquired valvular or an endocardial disease; a primary myocardial disease; a myocarditis; an arrhythmia; a transplant rejection; a metabolic myocardial disease; a myocardiomyopathy; a congestive, a hypertrophic or a restrictive cardiomyopathy; and/or, a heart transplant.

In alternative embodiments, therapeutic formulations as used or as set forth herein, or as in any methods of the invention, for use in the treatment of or for:

(1) a heart disease, a heart failure, a decrease in heart function or cardiac output, a decrease in heart function or cardiac output due to a heart infection or a heart condition,

(2) enhancing calcium handling in intact cardiac myocytes by increasing sarcoplasmic reticulum (SR) Ca^{2+} uptake and/or increased Ca^{2+} transients with reduced time of relaxation in intact cardiac myocytes,

(3) inhibiting the generation of intracellular cAMP levels in cardiac myocytes,

(4) protecting a cardiac myocyte from a programmed cell death (apoptosis) signal, or decreasing the number of cardiac myocytes signaled to programmed cell death (apoptosis) subsequent to an apoptotic signal,

(5) in heart failure patients or in individuals having a heart infection or a heart condition resulting in a decrease in heart function or cardiac output: increasing heart function or cardiac output, reducing symptom and/or decreasing mortality; or reducing the frequency of hospitalizations for heart failure;

(6) a cardiopathy or a cardiovascular disease; or

(7) a coronary artery disease (CAD); an atherosclerosis; a thrombosis; a restenosis; a vasculitis, an autoimmune or a viral vasculitis; a polyarteritis nodosa; a Churg-Strass syndrome; a Takayasu's arteritis; a Kawasaki Disease; a Rickettsial vasculitis; an atherosclerotic aneurism; a myocardial hypertrophy; a congenital heart disease (CHD); an ischemic heart disease; an angina; an acquired valvular or an endocardial disease; a primary myocardial disease; a myocarditis; an arrhythmia; a transplant rejection; a metabolic myocardial disease; a myocardiomyopathy; a congestive, a hypertrophic or a restrictive cardiomyopathy; and/or, a heart transplant.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

All publications, patents, patent applications cited herein are hereby expressly incorporated by reference for all purposes.

DESCRIPTION OF DRAWINGS

Figure 1 illustrates the design, expression, activity and cellular distribution of the exemplary AC6mut of the invention:

Fig. 1A schematically illustrates a diagram depicting the site of substitution of alanine (ala) for aspartic acid (asp) (a D => A substitution) at position 426 (position number based on SEQ ID NO:17, where SEQ ID NO:16 is the sequence before the D => A substitution) in the C1 domain (intracellular loop) in the construction of an exemplary murine AC6mut of the invention;

Fig. 1B graphically illustrates AC6mut mRNA expression as assessed by qRT-PCR using primers common to endogenous AC6 and transgene AC6mut;

Fig. 1C illustrates an immunoblot detecting AC6mut protein using anti-AC5/6 antibody and confirmed using anti-AU1 tag antibody;

Fig. 1D graphically illustrates cyclic AMP production in isolated cardiac myocytes from AC6mut and control mice, before (Basal) and after stimulation with isoproterenol, as measured by cAMP Enzyme immunoassay;

Fig. 1E illustrates a double immunofluorescence staining of AC6mut protein in cardiac myocytes isolated from AC6mut vs control mice using anti-AU1 antibody (red); anti-caveolin 3 (Cav-3) antibody (green, for caveolae); anti-protein disulphide-isomerase (PDI) antibody (green, for sarcoplasmic reticulum); anti-lamin A antibody (green, for nuclear envelope), and anti-voltage dependent anion selective channel protein (VDAC) antibody (green, for mitochondria); Nucleus is blue;

as discussed in detail in Example 1, below.

Figure 2 illustrates the activities and expression of PKA, PKS and PDE:

Fig. 2A *Upper Graph* graphically illustrates levels of PKA activity in isolated cardiac myocytes without stimulation (Basal) or stimulated with isoproterenol or NKH477; and Fig. 2A *Lower illustration* illustrates a gel immunoblot showing PKA protein in left ventricle (LV) homogenates;

Fig. 2B illustrates immunoblots showing the phosphorylation of key signaling proteins using left ventricular homogenates from AC6mut and control mice; shown are phospho (P) and Total (T) PKA regulatory subunits II-α and II-β, PKCα, phosphor-diesterase type 3A (PDE3A), phospho-troponin I (P22/23-TnI), and total TnI;

Fig. 2C illustrates immunoblots showing the phosphorylation of RyR2, PLB and TnI before and after isoproterenol stimulation was assessed in cultured cardiac myocytes isolated from each group;

Fig. 2D graphically illustrates the data from Fig. 2**C** indicating that isoproterenol stimulation in AC6mut mice was associated with increased phosphorylation of RyR2, PLB, and TnI in cardiac myocytes; data is normalized for loading (GAPDH);

as discussed in detail in Example 1, below.

Figure 3 graphically illustrates Left Ventricular Contractile Function: isolated hearts from AC6mut TG mice (closed circles) showed preserved LV dP/dt in response to isoproterenol stimulation through a wide range of isoproterenol doses; open circles represent transgene negative control mice; as discussed in detail in Example 1, below.

Figure 4 illustrates SR Ca^{2+} uptake, Ca^{2+} signaling proteins, and transcriptional factors:

Fig. 4A *Upper graph,* graphically illustrates Ca^{2+} uptake activity in pooled LV samples from AC6mut and TG negative sibling control mice; and Fig. 4A *Lower graph,* graphically illustrates expression of AC6mut decreased SERCA2a affinity for Ca^{2+};

Fig. 4B *Upper graph* graphically illustrates AC6mut expression was associated with decreased LV phospholamban (PLB) expression; and Fig. 4B *Lower graph* graphically illustrates AC6mut expression was associated with increased LV CREM-1 protein expression; and Fig. 4B *lower illustration* illustrates immunoblots of the gels showing protein levels; data is normalized for loading (GAPDH);

Fig. 4C *Upper graph* graphically illustrates AC6mut expression was associated with increased LV S100A1 protein expression; and Fig. 4C *Lower graph* graphically illustrates AC6mut expression was associated with increased LV P133-CREB protein expression; and Fig. 4C *lower illustration* illustrates immunoblots of the gels showing protein levels; data is normalized for loading (GAPDH);

Fig. 4D illustrates immunoblots of the gels showing AC6mut expression did not affect LV expression of SERCA2a, calreticulin, calsequestrin or phospho-S16-PLB proteins;

Fig. 4E illustrates a double immunofluorescence staining of AC6mut protein in isolated cardiac myocytes from AC6mut and control mice using anti-AU1 antibody (red) and anti–CREM-1 antibody (green) or anti-AU1 and anti-phospho-CREB (S133, green); nucleus was showing in blue;

as discussed in detail in Example 1, below.

Figure 5 illustrates cytosolic Ca^{2+} transients in isolated cardiac myocytes from AC6mut and control mice:

Fig. 5A graphically illustrates data showing that basal Ca^{2+} released (systolic-diastolic Ca^{2+}) showed no group difference between AC6mut and control;

Fig. 5B graphically illustrates data showing that representative Indo-1 Ca^{2+} transient recordings in cardiac myocytes stimulated with isoproterenol were higher in cardiac myocytes from AC6mut mice; summary data are displayed in Fig. 5C;

Fig. 5C graphically illustrates data showing that Ca^{2+} released in the presence of isoproterenol was increased in cardiac myocytes from AC6mut mice;

Fig. 5D graphically illustrates data showing that time-to-peak Ca^{2+} transient in the presence of isoproterenol was decreased in cardiac myocytes from AC6mut mice;

Fig. 5E graphically illustrates data showing that time to 50% relaxation (tau) in the presence of isoproterenol was decreased in cardiac myocytes from AC6mut mice; as discussed in detail in Example 1, below.

Like reference symbols in the various drawings indicate like elements.

DETAILED DESCRIPTION

The invention provides compositions and *in vivo* and *ex vivo* methods comprising administration of a cyclic adenosine monophosphate-incompetent (cAMP-incompetent) adenylyl cyclase type 6 (AC6) protein or polypeptide (also called "an AC6mut"), or an AC6mut-encoding nucleic acid or a gene to treat, ameliorate or protect (as a preventative or a prophylaxis) individuals with a heart disease, a decreased cardiac function or output, or a heart infection or a condition responsive to decreased cAMP, increased sarcoplasmic reticulum (SR) Ca^{2+} uptake and/or increased Ca^{2+} transients with reduced time of relaxation in intact cardiac myocytes *in vivo*.

In alternative embodiments, the invention provides an AC6mut that inhibits or substantially reduces amounts of, or does not catalyze generation of, intracellular cAMP. In alternative embodiments, the AC6mut of the invention alters intracellular signaling in a manner that 1) enhances calcium handling in intact cardiac myocytes, 2) inhibits generation of intracellular cAMP levels in cardiac myocytes, and 3) protects cardiac myocytes from programmed cell death (apoptosis). In alternative embodiments, when the AC6mut is expressed in or delivered to the failing hearts of patients, heart function increases, symptoms are reduced, and mortality decreases. Therefore, delivery of the AC6mut of the invention to the heart increases cardiac function with no deleterious effects due to cAMP generation. Thus, in alternative embodiments, the invention provides an ideal therapy for heart failure and decrease heart function.

In alternative embodiments, the invention provides compositions and methods for the delivery and expression (e.g., a controlled expression) of an AC6mut-encoding nucleic acid or gene, or an expression vehicle (e.g., vector, recombinant virus, and the like) comprising (having contained therein) an AC6mut-encoding nucleic acid or gene, that results in an AC6mut protein selectively expressed in a cardiac myocyte, or only delivered to cardiac myocytes, or alternatively, released into the bloodstream or general circulation where it can have a beneficial effect on in the body, e.g., such as the heart in the case of treating cardiovascular disease.

In alternative embodiments, the invention provides delivery vehicles, vectors, expression vectors, recombinant viruses and the like for *in vivo* expression of an AC6mut -encoding nucleic acid or gene to practice the methods of this invention. In alternative embodiments, the delivery vehicles, vectors, expression vectors, recombinant viruses and the like expressing the AC6mut or the AC6mut nucleic acid or gene can be delivered by intramuscular (IM) injection, direct injection into the heart, by intravenous (IV) injection, by subcutaneous injection, by inhalation, by a biolistic particle delivery system (e.g., a so-called "gene gun"), and the like, e.g., as an outpatient, e.g., during an office visit.

In alternative embodiments, AC6mut -encoding nucleic acids or genes (for example, including delivery vehicles (such as e.g., liposomes), vectors, expression vectors, recombinant viruses and the like carrying them as a "payload") are targeted to myocytes, cardiac myocytes or delivered directly to cardiac myocytes for directed cAMP-incompetent AC expression, or expression directly in the target heart organ.

In alternative embodiments, this "peripheral" mode of delivery, e.g., delivery vehicles, vectors, expression vectors, recombinant viruses and the like, are injected IM or IV, can circumvent problems encountered when genes or nucleic acids are expressed directly in an organ (e.g., the heart, lung or kidney) itself. Sustained secretion of a desired AC6mut protein(s), or delivery vehicles, vectors, expression vectors, recombinant viruses and the like, in the bloodstream or general circulation also circumvents the difficulties and expense of administering proteins, delivery vehicles, vectors, expression vectors, recombinant viruses and the like, by infusion, which can be particularly problematic for many proteins, delivery vehicles, vectors, expression vectors, recombinant viruses and the like, which exhibit very short half lives in the body.

In alternative embodiments, the invention provides methods for being able to turn on and turn off AC6mut -expressing nucleic acid or gene expression easily and efficiently for tailored treatments and insurance of optimal safety.

In alternative embodiments, the AC6mut protein or proteins expressed by the AC6mut-expressing nucleic acid(s) or gene(s) have a beneficial or favorable effects (e.g., therapeutic or prophylactic) on a tissue or an organ, e.g., the heart, blood vessels, lungs, kidneys, or other targets, even though secreted into the blood or general circulation at a distance (e.g., anatomically remote) from their site or sites of action.

In an exemplary embodiment of the invention AC6mut -expressing nucleic acids or genes encoding cAMP-incompetent AC are used to practice methods of this invention,

including but not limited to, e.g., treating a heart disease, a heart failure, a congestive heart failure (CHF), any decrease in cardiac output or function, or any combination thereof.

For example, in alternative embodiments, delivery vehicles, vectors, expression vectors, recombinant viruses and the like, e.g., a long-term virus or viral vector, can be injected, e.g., in a systemic vein (e.g., IV), or by intramuscular (IM) injection, by inhalation, or by a biolistic particle delivery system (e.g., a so-called "gene gun"), e.g., as an outpatient, e.g., in a physician's office. In alternative embodiments, days or weeks later (e.g., four weeks later), the individual, patient or subject is administered (e.g., inhales, is injected or swallows), a chemical or pharmaceutical that induces expression of the AC6mut -expressing nucleic acids or genes; for example, an oral antibiotic (e.g., doxycycline or rapamycin) is administered once daily (or more or less often), which will activate the expression of the gene. In alternative embodiments, after the "activation", or inducement of expression (e.g., by an inducible promoter) of the nucleic acid or gene, an AC6mut protein is synthesized and released into the subject's circulation (e.g., into the blood), and subsequently has favorable physiological effects, e.g., therapeutic or prophylactic, that benefit the individual or patient (e.g., benefit heart function). When the physician or subject desires discontinuation of the treatment, the subject simply stops taking the activating chemical or pharmaceutical, e.g., antibiotic.

In alternative embodiments, applications of the present invention include: the treatment of severe, low ejection fraction heart failure; the treatment of pulmonary hypertension; the treatment of heart failure with preserved ejection fraction; replacement of current therapies that require hospitalization and sustained intravenous infusions of vasoactive peptides for the treatment of a pulmonary hypertension and heart failure; and, the treatment of other conditions in which controlled expression of an AC6mut or an AC6mut nucleic acid or gene to promote favorable effects in the body.

Generating and Manipulating Nucleic Acids

In alternative embodiments, to practice the methods of the invention, the invention provides isolated, synthetic and/or recombinant nucleic acids or genes encoding AC6mut polypeptides. In alternative embodiments, to practice the methods of the invention, the invention provides AC6mut -expressing nucleic acids or genes in recombinant form in an

(e.g., spliced into) an expression vehicle for *in vivo* expression, e.g., in a vector, e.g., an AAV, or any pseudotype, hybrid or derivative thereof, or a recombinant virus.

In alternative embodiments, a mammalian, e.g., human or murine, AC6mut can be used to practice this invention, wherein the AC6mut comprises an adenylyl cyclase (AC) polypeptide having a substitution of an uncharged or non-polar amino acid for a charged or an acidic amino acid in the catalytic core of the AC polypeptide. The catalytic core (also called the catalytic region 1 (C1)) of human AC6 polypeptide (SEQ ID NO:10) is from amino acid residue 307 to 675. The catalytic core of murine AC6 polypeptide (SEQ ID NO:11) is from amino acid residue 315 to 683.

In alternative embodiments, the uncharged or non-polar amino acid is an alanine (Ala), and optionally the acidic amino acid is an aspartic acid (Asp), or optionally the uncharged or non-polar amino acid is an Ala and the acidic amino acid is an Asp.

In alternative embodiments, the invention provides a (murine) AC6mut polypeptide (SEQ ID NO:12) comprising a murine adenylyl cyclase (AC) polypeptide having a substitution of an alanine, or Ala (or "A") for an aspartic acid, or Asp (or "D") at position 436 in the catalytic core of the AC polypeptide; i.e., in this embodiment, the murine adenylyl cyclase (AC) polypeptide has a substitution D => A, or of an Ala for an Asp, at position 436 in the catalytic core of the murine AC polypeptide (SEQ ID NO:11 is the amino acid sequence before the D => A substitution).

In alternative embodiments, the invention provides a (murine) AC6mut polypeptide (SEQ ID NO:17) comprising a murine adenylyl cyclase (AC) polypeptide having a substitution of an alanine, or Ala (or "A") for an aspartic acid, or Asp (or "D") (i.e., a D => A substitution) at position 426 in the catalytic core of the AC polypeptide. The SEQ ID NO:17 polypeptide differs from the SEQ ID NO:12 polypeptide in that the SEQ ID NO:17 polypeptide is missing the first ten amino acids of the SEQ ID NO:12 polypeptide; otherwise the polypeptides are identical. SEQ ID NO:16 is the murine amino acid sequence before the D => A substitution. The isoform lacking the amino terminal is believed to be the wild type murine polypeptide, with the first ten amino acids of SEQ ID NO:11 and SEQ ID NO:12 being untranslated.

In alternative embodiments, the invention provides a (human) AC6mut polypeptide (SEQ ID NO:13) comprising a human adenylyl cyclase (AC) polypeptide having a substitution of an alanine, or Ala (or "A") for an aspartic acid, or Asp (or "D") at position 428 in the catalytic core of the AC polypeptide; i.e., in this embodiment, the

murine adenylyl cyclase (AC) polypeptide has a substitution D => A, or of an Ala for an Asp, at position 428 in the catalytic core of the murine AC polypeptide.

Human AC6 nucleic acid coding sequence (SEQ ID NO:14) vs murine coding sequence: 86% homology (SEQ ID NO:15). Human AC6 polypeptide (SEQ ID NO:10) vs murine AC6 polypeptide (SEQ ID NO:11) at amino acid levels: 94% homology.

The AC6mut D => A substitution is in the exact same relative structural position in the catalytic core of the human AC6mut as the murine AC6mut, as illustrated below (showing the wild type still having the aspartic acid, or "D" residue, as underlined below:

```
Human   1    MSWFSGLLVPKVDERKTAWGERNGQKRSRRRGTRAGGFCTPRYMSCLRDAEPPSPTPAGP  60
Murine  11   MSWFSGLLVPKVDERKTAWGERNGQKRPRH-ANRASGFCAPRYMSCLKNAEPPSPTPAAH  69
Human   61   PRCPWQDDAFIRRGGPGKGKELGLRAVALGFEDTEVTTTAGGTAEVAPDAVPRSGRSCWR  120
Murine  70   TRCPWQDEAFIRRAGPGRGVELGLRSVALGFDDTEVTTPMG-TAEVAPDTSPRSGPSCWH  128
Human   121  RLVQVFQSKQFRSAKLERLYQRYFFQMNQSSLTLLMAVLVLLTAVLLAFHAAPARPQPAY  180
Murine  129  RLVQVFQSKQFRSAKLERLYQRYFFQMNQSSLTLLMAVLVLLMAVLLTFHAAPAQPQPAY  188
Human   181  VALLACAAALFVGLMVVCNRHSFRQDSMWVVSYVVLGILAAVQVGGALAADPRSPSAGLW  240
Murine  189  VALLTCASVLFVVLMVVCNRHSFRQDSMWVVSYVVLGILAAVQVGGALAANPHSPSAGLW  248
Human   241  CPVFFVYIAYTLLPIRMRAAVLSGLGLSTLHLILAWQLNRGDAFLWRQLGANVLLFLCTN  300
Murine  249  CPVFFVYITYTLLPIRMRAAVLSGLGLSTLHLILAWQLNSSDPFLWKQLGANVVLFLCTN  308
Human   301  VIGICT HYPAEVSQRQAFQETRGYIQARLHLQHENRQQERLLLSVLPQHVAMEMKEDINT  360
Murine  309  AIGVCT HYPAEVSQRQAFQETRGYIQARLHLQHENRQQERLLLSVLPQHVAMEMKEDINT  368
Human   361  KKEDMMFHKIYIQKHDNVSILFADIEGFTSLASQCTAQELVMTLNELFARFDKLAAENHC  420
Murine  369  KKEDMMFHKIYIQKHDNVSILFADIEGFTSLASQCTAQELVMTLNELFARFDKLAAENHC  428
Human   421  LRIKILGDCYYCVSGLPEARADHAHCCVEMGVDMIEAISLVREVTGVNVNMRVGIHSGRV  480
Murine  429  LRIKILGDCYYCVSGLPEARADHAHCCVEMGVDMIEAISLVREVTGVNVNMRVGIHSGRV  488
Human   481  HCGVLGLRKWQFDVWSNDVTLANHMEAGGRAGRIHITRATLQYLNGDYEVEPGRGGERNA  540
Murine  489  HCGVLGLRKWQFDVWSNDVTLANHMEAGGRAGRIHITRATLQYLNGDYEVEPGRGGERNA  548
Human   541  YLKEQHIETFLILGASQKRKEEKAMLAKLQRTRANSMEGLMPRWVPDRAFSRTKDSKAFR  600
Murine  549  YLKEQCIETFLILGASQKRKEEKAMLAKLQRTRANSMEGLMPRWVPDRAFSRTKDSKAFR  608
Human   601  QMGIDDSSKDNRGTQDALNPEDEVDEFLSRAIDARSIDQLRKDHVRRFLLTFQREDLEKK  660
Murine  609  QMGIDDSSKDNRGAQDALNPEDEVDEFLGRAIDARSIDQLRKDHVRRFLLTFQREDLEKK  668
Human   661  YSRKVDPRFGAYVACALLVFCFICFIQLLIFPHSTLMLGIYASIFLLLLITVLICAVYSC  720
Murine  669  YSRKVDPRFGAYVACALLVFCFICFIQLLVFPYSTLILGIYAAIFLLLLVTVLICAVCSC  728
```

```
Human  721   GSLFPKALQRLSRSIVRSRAHSTAVGIFSVLLVFTSAIANMFTCNHTPIRSCAARMLNLT  780
Murine  729  GSFFPKALQRLSRNIVRSRVHSTAVGIFSVLLVFISAIANMFTCNHTPIRTCAARMLNLT  788
Human  781   PADITACHLQQLNYSLGLDAPLCEGTMPTCSFPEYFIGNMLLSLLASSVFLHISSIGKLA  840
Murine  789  PADVTACHLQQLNYSLGLDAPLCEGTAPTCSFPEYFVGNVLLSLLASSVFLHISSIGKLA  848
Human  841   MIFVLGLIYLVLLLLGPPATIFDNYDLLLGVHGLASSNETFDGLDCPAAGRVALKYMTPV  900
Murine  849  MTFILGFTYLVLLLLGPPAAIFDNYDLLLGVHGLASSNETFDGLDCPAVGRVALKYMTPV  908
Human  901   ILLVFALALYLHAQQVESTARLDFLWKLQATGEKEEMEELQAYNRRLLHNILPKDVAAHF  960
Murine  909  ILLVFALALYLHAQQVESTARLDFLWKLQATGEKEEMEELQAYNRRLLHNILPKDVAAHF  968
Human  961   LARERRNDELYYQSCECVAVMFASIANFSEFYVELEANNEGVECLRLLNEIIADFDEIIS  1020
Murine  969  LARERRNDELYYQSCECVAVMFASIANFSEFYVELEANNEGVECLRLLNEIIADFDEIIS  1028
Human  1021  EERFRQLEKIKTIGSTYMAASGLNASTYDQVGRSHITALADYAMRLMEQMKHINEHSFNN  1080
Murine  1029 EERFRQLEKIKTIGSTYMAASGLNASTYDQVGRSHITALADYAMRLMEQMKHINEHSFNN  1088
Human  1081  FQMKIGLNMGPVVAGVIGARKPQYDIWGNTVNVSSRMDSTGVPDRIQVTTDLYQVLAAKG  1140
Murine  1089 FQMKIGLNMGPVVAGVIGARKPQYDIWGNTVNVSSRMDSTGVPDRIQVTTDLYQVLAAKG  1148
Human  1141  YQLECRGVVKVKGKGEMTTYFLNGGPSS  1168  (SEQ ID NO:10)
Murine  1149 YQLECRGVVKVKGKGEMTTYFLNGGPSS  1176  (SEQ ID NO:11)
```

++++++++++++

In alternative embodiments, both the human ACmut nucleic acid coding sequence (SEQ ID NO:13) and the murine ACmut nucleic acid coding sequence (SEQ ID NO:12) were made by changing an adenosine (or "A") to a cytosine (or "C"), as indicated below, where the "A" residue before its change to "C" is underlined, below; i.e., illustrated below is the wild type human AC6 (SEQ ID NO:10) and wild type murine AC6 (SEQ ID NO:11):

```
Murine  90   CCTCCCAGCAGC▒▒▒TCATGGTTTAGTGGCCTCCTGGTTCCCAAAGTGGATGAACGGAAA  149
             ||| |||||| |||||||||||||||||||||||||| || ||||||||||||||||||
Human  649   CCTACCAGCAAC▒▒▒TCATGGTTTAGTGGCCTCCTGGTCCCTAAAGTGGATGAACGGAAA  708

Murine  150  ACAGCTTGGGGGGAACGCAATGGGCAGAAGCG--C-CCACGCCACGCGAATCGAGCCAGT  206
             ||||| ||||| ||||||||||||||||||  | |  ||||  |  | ||| ||  ||
Human  709   ACAGCCTGGGGTGAACGCAATGGGCAGAAGCGTTCGCGGCGCCGTGGCACTCGGGCAGGT  768

Murine  207  GGCTTCTGCGCACCTCGCTACATGAGCTGCCTCAAGAATGCGGAGCCACCCAGCCCCACT  266
             ||||||||| | || ||||| ||||||||||  | |||| ||||||||||||||||||
Human  769   GGCTTCTGCACGCCCCGCTATATGAGCTGCCTCCGGGATGCAGAGCCACCCAGCCCCACC  828

Murine  267  CCTGCAGCTCACACTCGGTGCCCCTGGCAGGATGAAGCCTTCATCAGGAGGGCGGGCCCG  326
             ||||| |  | | |||||||||||||||||||||| ||||||||| ||||||  |||||
Human  829   CCTGCGGGCCCCCCTCGGTGCCCCTGGCAGGATGACGCCTTCATCCGGAGGGGCGGCCCA  888

Murine  327  GGCAGGGGTGTGGAGCTGGGGCTGCGGTCAGTGGCCTTGGGGTTTGACGACACTGAGGTG  386
             |||| |||   |||||||||||||||| |||||||| |||| || || || || ||||||
Human  889   GGCAAGGGCAAGGAGCTGGGGCTGCGGGCAGTGGCCCTGGGCTTCGAGGATACCGAGGTG  948
```


Murine 387 AC--C-ACACCGATGGGCACAGCTGAAGTGGCACCGGATACATCGCCTCGGAGCGGTCCG 443
Human 949 ACAACGACAGCGGGCGGGACGGCTGAGGTGGCGCCCGACGCGGTGCCCAGGAGTGGGCGA 1008
Murine 444 TCCTGCTGGCACCGGCTTGTGCAGGTGTTCCAGTCTAAGCAGTTCCGCTCTGCCAAGCTG 503
Human 1009 TCCTGCTGGCGCCGTCTGGTGCAGGTGTTCCAGTCGAAGCAGTTCCGTTCGGCCAAGCTG 1068
Murine 504 GAGCGGCTGTACCAGCGGTACTTCTTCCAGATGAACCAGAGCAGCCTCACGCTGCTCATG 563
Human 1069 GAGCGCCTGTACCAGCGGTACTTCTTCCAGATGAACCAGAGCAGCCTGACGCTGCTGATG 1128
Murine 564 GCGGTGCTGGTGCTGCTCATGGCTGTACTGTTGACTTTCCACGCTGCGCCTGCCCAGCCT 623
Human 1129 GCGGTGCTGGTGCTGCTCACAGCGGTGCTGCTGGCTTTCCACGCCGCACCCGCCCGCCCT 1188
Murine 624 CAGCCTGCTTACGTGGCCCTGCTGACCTGTGCCTCTGTCCTTTTTGTGGTACTCATGGTG 683
Human 1189 CAGCCTGCCTATGTGGCACTGTTGGCCTGTGCCGCCGCCCTGTTCGTGGGGCTCATGGTG 1248
Murine 684 GTGTGTAACCGACACAGCTTCCGCCAGGACTCCATGTGGGTGGTGAGCTATGTGGTCCTG 743
Human 1249 GTGTGTAACCGGCATAGCTTCCGCCAGGACTCCATGTGGGTGGTGAGCTACGTGGTGCTG 1308
Murine 744 GGCATCCTAGCAGCCGTGCAAGTCGGGGGTGCCCTGGCAGCCAATCCACACAGCCCCTCG 803
Human 1309 GGCATCCTGGCGGCAGTGCAGGTCGGGGGCGCTCTCGCAGCAGACCCGCGCAGCCCCTCT 1368
Murine 804 GCGGGCCTTTGGTGCCCCGTGTTCTTCGTCTACATCACCTACACTCTTCTTCCCATTCGC 863
Human 1369 GCGGGCCTCTGGTGCCCTGTGTTCTTTGTCTACATCGCCTACACGCTCCTCCCCATCCGC 1428
Murine 864 ATGCGAGCCGCAGTACTCAGCGGCCTGGGCCTCTCTACTCTGCATTTGATTTTGGCCTGG 923
Human 1429 ATGCGGGCTGCCGTCCTCAGCGGCCTGGGCCTCTCCACCTTGCATTTGATCTTGGCCTGG 1488
Murine 924 CAGCTCAACAGCAGCGACCCCTTCCTTTGGAAGCAGCTCGGTGCTAACGTGGTGCTCTTC 983
Human 1489 CAACTTAACCGTGGTGATGCCTTCCTCTGGAAGCAGCTCGGTGCCAATGTGCTGCTGTTC 1548
Murine 984 CTCTGCACCAATGCCATCGGTGTCTGCACACACTACCCTGCTGAAGTGTCTCAGCGCCAA 1043
Human 1549 CTCTGCACCAACGTCATTGGCATCTGCACACACTATCCAGCAGAGGTGTCTCAGCGCCAG 1608
Murine 1044 GCTTTTCAGGAGACCCGAGGTTACATCCAGGCGCGGCTGCACCTGCAGCATGAGAACCGT 1103
Human 1609 GCCTTTCAGGAGACCCGCGGTTACATCCAGGCCCGGCTCCACCTGCAGCATGAGAATCGG 1668
Murine 1104 CAGCAGGAACGGCTGCTGCTATCGGTGTTGCCCCAGCACGTTGCCATGGAGATGAAAGAA 1163
Human 1669 CAGCAGGAGCGGCTGCTGCTGTCGGTATTGCCCCAGCACGTTGCCATGGAGATGAAAGAA 1728
Murine 1164 GACATCAACAC[illegible]GAGGACATGATGTTCCATAAGATCTACATCCAGAAGCATGAT 1223
Human 1729 GACATCAACACAAAAAAAGAAGACATGATGTTCCACAAGATCTACATACAGAAGCATGAC 1788
Murine 1224 AATGTCAGCATCCTGTTTGCGGACATTGAGGGCTTCACCAGCCTGGCCTCCCAGTGCACT 1283
Human 1789 AATGTCAGCATCCTGTTTGCAGACATTGAGGGCTTCACCAGCCTGGCATCCCAGTGCACT 1848
Murine 1284 GCACAGGAACTGGTCATGACCTTGAATGAGCTCTTTGCCCGGTTTGACAAGCTGGCTGCG 1343
Human 1849 GCGCAGGAGCTGGTCATGACCCTGAATGAGCTCTTTGCCCGGTTTGACAAGCTGGCTGCG 1908
Murine 1344 GAGAATCACTGTCTGAGGATCAAGATCTTAGGAGACTGTTACTACTGCGTGTCAGGGCTG 1403

```
             ||||||||||| ||||||||||||||||| || |||||||||||||| ||||||||||||
Human   1909 GAGAATCACTGCCTGAGGATCAAGATCTTGGGGGACTGTTACTACTGTGTGTCAGGGCTG  1968

Murine  1404 CCCGAGGCCCGGGCAGATCACGCCCACTGCTGTGTGGAGATGGGGGTAGACATGATCGAA  1463
             || ||||||||||| || || ||||||||||||||||||||||||||||||||||| ||
Human   1969 CCGGAGGCCCGGGCCGACCATGCCCACTGCTGTGTGGAGATGGGGGTAGACATGATTGAG  2028

Murine  1464 GCCATCTCGCTGGTGCGTGAGGTAACAGGTGTGAACGTGAACATGCGTGTGGGCATCCAC  1523
             |||||||||||||| |||||||| ||||||||||| ||||||||||| ||||||||||||
Human   2029 GCCATCTCGCTGGTACGTGAGGTGACAGGTGTGAATGTGAACATGCGCGTGGGCATCCAC  2088

Murine  1524 AGCGGACGTGTGCATTGCGGCGTCCTTGGCCTACGGAAATGGCAGTTTGATGTCTGGTCA  1583
             ||||| || ||||| ||||||||||||||| | |||||||||||||| ||||| |||||
Human   2089 AGCGGGCGCGTGCACTGCGGCGTCCTTGGCTTGCGGAAATGGCAGTTCGATGTGTGGTCC  2148

Murine  1584  AACGATGTGACCCTGGCTAACCACATGGAGGCCGG-GGGC-GGCCGG-CGCATCCACATC  1640
             || |||||||||||||| |||||||||||||| || || | ||| || ||||||||||||
Human   2149 AATGATGTGACCCTGGCCAACCACATGGAGGCAGGAGGCCGGGCTGGCCGCATCCACATC  2208

Murine  1641 ACTCGGGCTACACTGCAGTACTTGAACGGGGACTATGAGGTGGAGCCAGGCCGTGGTGGT  1700
             |||||||| |||||||||||| ||||||||||||| |||||||||||||||||||||||
Human   2209 ACTCGGGCAACACTGCAGTACCTGAACGGGGACTACGAGGTGGAGCCAGGCCGTGGTGGC  2268

Murine  1701  GAACGCAATGCGTACCTCAAGGAGCAGTGCATTGAGACCTTCCTCATACTTGGCGCCAGC  1760
             || ||||| ||||||||||||||||||  ||||||||| |||||||| || |||||||||
Human   2269 GAGCGCAACGCGTACCTCAAGGAGCAGCACATTGAGACTTTCCTCATCCTGGGCGCCAGC  2328

Murine  1761 CAAAAACGGAAAGAGGAGAAAGCCATGCTGGCCAAGCTTCAGCGGACACGGGCCAACTCC  1820
             || ||||||||||||||||| ||||||||||||||||| |||||||| ||||||||||||
Human   2329 CAGAAACGGAAAGAGGAGAAGGCCATGCTGGCCAAGCTGCAGCGGACTCGGGCCAACTCC  2388

Murine  1821 ATGGAAGGACTGATGCCCCGCTGGGTTCCTGACCGTGCCTTCTCCCGGACCAAGGACTCT  1880
             |||||||| |||||||| |||||||||||||| ||||||||||||||||||||||||||
Human   2389 ATGGAAGGGCTGATGCCGCGCTGGGTTCCTGATCGTGCCTTCTCCCGGACCAAGGACTCC  2448

Murine  1881 AAGGCATTCCGCCAGATGGGCATTGATGATTCTAGCAAAGACAACCGGGGTGCCCAAGAT  1940
             ||||| |||||||||||||||||||||||||| |||||||||||||||||  ||||||||
Human   2449 AAGGCCTTCCGCCAGATGGGCATTGATGATTCCAGCAAAGACAACCGGGGCACCCAAGAT  2508

Murine  1941 GCTCTGAACCCTGAAGATGAGGTGGATGAGTTCCTGGGCCGAGCCATCGATGCCCGAAGC  2000
             || ||||||||||| ||||||||||||||||||||| |||| |||||||||||||| |||
Human   2509 GCCCTGAACCCTGAGGATGAGGTGGATGAGTTCCTGAGCCGTGCCATCGATGCCCGCAGC  2568

Murine  2001 ATCGACCAACTGCGTAAGGACCATGTGCGCCGGTTCCTGCTCACCTTCCAGAGAGAGGAT  2060
             || || || ||||| |||||||||||||||||||| ||||||||||||||||||||||||
Human   2569 ATTGATCAGCTGCGGAAGGACCATGTGCGCCGGTTTCTGCTCACCTTCCAGAGAGAGGAT  2628

Murine  2061 CTTGAGAAGAAGTATTCACGGAAAGTAGATCCTCGCTTCGGAGCCTACGTCGCCTGTGCC  2120
             |||||||||||||| || ||||| || ||||| ||||||||||||||||| |||||||||
Human   2629 CTTGAGAAGAAGTACTCCCGGAAGGTGGATCCCCGCTTCGGAGCCTACGTTGCCTGTGCC  2688

Murine  2121 CTCCTGGTTTTTTGCTTCATCTGTTTTATCCAGCTCCTTGTGTTCCCATACTCCACCCTG  2180
             ||  |||| || ||||||||||| || |||||||| ||  | |||||| |||||||||||
Human   2689 CTGTTGGTCTTCTGCTTCATCTGCTTCATCCAGCTTCTCATCTTCCCACACTCCACCCTG  2748

Murine  2181 ATACTCGGGATTTATGCC-GCTATCTTCCTGCTGTTGCTGGTCACTGTGCTGATCTGTGC  2239
             || || ||||| |||||| || |||||||||||| ||||  |||| ||||||||||||||
Human   2749 ATGCTTGGGATCTATGCCAGC-ATCTTCCTGCTGCTGCTAATCACCGTGCTGATCTGTGC  2807

Murine  2240 CGTGTGCTCCTGCGGTTCTTTCTTCCCCAAGGCCCTGCAACGCCTGTCCCGCAATATTGT  2299
              |||| |||||| |||||| | ||||| |||||||||||||| ||||||||||  |||||
Human   2808 TGTGTACTCCTGTGGTTCTCTGTTCCCTAAGGCCCTGCAACGTCTGTCCCGCAGCATTGT  2867

Murine  2300 CCGCTCACGGGTGCACAGCACCGCGGTTGGAATCTTCTCGGTTCTGCTTGTGTTCATCTC  2359
             |||||||||||  || |||||||| ||||| ||||| || || ||||||||||| |  ||
Human   2868 CCGCTCACGGGCACATAGCACCGCAGTTGGCATCTTTTCCGTCCTGCTTGTGTTTACTTC  2927
```

```
Murine  2360 TGCCATCGCCAACATGTTTACCTGTAATCACACCCCAATAAGGACCTGCGCGGCCCGGAT  2419
             |||||| |||||||||| |||||||| |||||||| ||| ||| ||| || ||||||||
Human   2928 TGCCATTGCCAACATGTTCACCTGTAACCACACCCCCATACGGAGCTGTGCAGCCCGGAT  2987

Murine  2420 GCTGAACTTAACACCAGCGGATGTCACCGCCTGCCACCTACAACAGCTCAATTACTCTCT  2479
             |||||| |||||||| || ||  |||| |||||||||| || ||||||||||||||||
Human   2988 GCTGAATTTAACACCTGCTGACATCACTGCCTGCCACCTGCAGCAGCTCAATTACTCTCT  3047

Murine  2480 GGGACTGGATGCTCCCCTGTGTGAGGGCACCGCACCCACCTGCAGCTTCCCTGAGTACTT  2539
             ||| ||||||||||||||||||||||||||   |||||||||||||| ||||||||||
Human   3048 GGGCCTGGATGCTCCCCTGTGTGAGGGCACCATGCCCACCTGCAGCTTTCCTGAGTACTT  3107

Murine  2540 CGTCGGGAACGTGCTGCTGAGTCTTCTAGCCAGCTCTGTCTTCCTACACATCAGCAGCAT  2599
             | |||||||| ||||||||||||  | ||||||||||||||||| ||||||||||||||
Human   3108 CATCGGGAACATGCTGCTGAGTCTCTTGGCCAGCTCTGTCTTCCTGCACATCAGCAGCAT  3167

Murine  2600 CGGCAAGCTGGCCATGACCTTCATCTTGGGGTTCACCTACTTGGTGCTGCTTTTGCTGGG  2659
             ||| ||| ||||||||| |||  |||||||| ||| ||| |||||||||||| |||||||
Human   3168 CGGGAAGTTGGCCATGATCTTTGTCTTGGGGCTCATCTATTTGGTGCTGCTTCTGCTGGG  3227

Murine  2660 TCCCCCGGCCGCCATCTTTGACAACTATGATCTACTGCTTGGCGTCCATGGCTTGGCTTC  2719
             |||||| ||| |||||||||||||||||| |||||||||||||||||||||||||||||
Human   3228 TCCCCCAGCCACCATCTTTGACAACTATGACCTACTGCTTGGCGTCCATGGCTTGGCTTC  3287

Murine  2720 CTCCAATGAGACCTTTGATGGGCTGGACTGCCCAGCTGTGGGGAGGGTAGCGCTCAAATA  2779
              |||||||||||||||||||||||||||||||| |||||||  |||||||| || ||||||||
Human   3288 TTCCAATGAGACCTTTGATGGGCTGGACTGTCCAGCTGCAGGGAGGGTGGCCCTCAAATA  3347

Murine  2780 TATGACCCCCGTGATTCTGCTGGTGTTTGCCCTGGCACTGTATCTGCATGCACAACAGGT  2839
             ||||||||| |||||||||||||||||||| ||||| |||||||||||||| || |||||
Human   3348 TATGACCCCTGTGATTCTGCTGGTGTTTGCGCTGGCGCTGTATCTGCATGCTCAGCAGGT  3407

Murine  2840 GGAATCGACTGCCCGCCTGGACTTCCTGTGGAAGTTACAGGCAACAGGGGAGAAGGAGGA  2899
             ||| |||||||||||||| |||||||| |||||  |||||||||||||||||||||||||
Human   3408 GGAGTCGACTGCCCGCCTAGACTTCCTCTGGAAACTACAGGCAACAGGGGAGAAGGAGGA  3467

Murine  2900 GATGGAGGAGCTACAGGCATACAACCGGAGGTTGCTGCATAACATTCTTCCCAAGGACGT  2959
             |||||||||||||||||||||||||||||||| ||||||||||||||| ||||||||||
Human   3468 GATGGAGGAGCTACAGGCATACAACCGGAGGCTGCTGCATAACATTCTGCCCAAGGACGT  3527

Murine  2960 GGCCGCCCACTTCCTGGCCCGGGAACGCCGCAACGATGAGCTGTACTACCAGTCGTGTGA  3019
             ||| |||||||||||||||||| |||||||| ||||| || ||||| ||||||||||
Human   3528 GGCGGCCCACTTCCTGGCCCGGGAGCGCCGCAATGATGAACTCTACTATCAGTCGTGTGA  3587

Murine  3020 ATGTGTGGCTGTCATGTTTGCCTCCATCGCCAATTTCTCGGAGTTCTACGTGGAGCTCGA  3079
              |||||||||| |||||||||||||| ||||| ||||| |||||||| |||||||| ||
Human   3588 GTGTGTGGCTGTTATGTTTGCCTCCATTGCCAACTTCTCTGAGTTCTATGTGGAGCTGGA  3647

Murine  3080 GGCAAACAACGAGGGCGTGGAGTGCCTGCGGCTGCTCAATGAGATCATCGCAGACTTTGA  3139
             |||||||| ||||| || |||||||||||||||||||| |||||||||| ||||||||
Human   3648 GGCAAACAATGAGGGTGTCGAGTGCCTGCGGCTGCTCAACGAGATCATCGCTGACTTTGA  3707

Murine  3140 CGAGATCATCAGTGAGGAGAGATTCCGGCAGTTGGAGAAGATCAAGACCATCGGTAGCAC  3199
              ||||| ||||| |||||| | ||||||||| |||| |||||||||| || ||||||||
Human   3708 TGAGATTATCAGCGAGGAGCGGTTCCGGCAGCTGGAAAAGATCAAGACGATTGGTAGCAC  3767

Murine  3200 CTACATGGCCGCCTCTGGGCTAAATGCCAGCACCTATGACCAGGTCGGCCGCTCACACAT  3259
             |||||||| ||||| ||||| || |||||||||| || ||||| |||||||| |||||
Human   3768 CTACATGGCTGCCTCAGGGCTGAACGCCAGCACCTACGATCAGGTGGGCCGCTCCCACAT  3827

Murine  3260 CACGGCGCTGGCTGACTATGCCATGCGGCTCATGGAGCAGATGAAACACATCAATGAACA  3319
             ||| || |||||||||| |||||||||||||||||||||||| |||||||||| ||
Human   3828 CACTGCCCTGGCTGACTACGCCATGCGGCTCATGGAGCAGATGAAGCACATCAATGAGCA  3887

Murine  3320 CTCTTTCAACAATTTCCAGATGAAGATCGGGTTGAACATGGGTCCGGTTGTAGCAGGCGT  3379
```

```
                 ||| ||||||||||||||||||||||| ||| |||||||||| || || || ||||| ||
Human   3888  CTCCTTCAACAATTTCCAGATGAAGATTGGGCTGAACATGGGCCCAGTCGTGGCAGGTGT  3947

Murine  3380 CATTGGGGCCCGAAAGCCACAGTATGACATCTGGGGAAATACCGTGAATGTTTCCAGTCG  3439
                 ||| ||||| || |||||||||||||||||||||| || || |||||||| || |||||
Human   3948  CATCGGGGCTCGGAAGCCACAGTATGACATCTGGGGGAACACAGTGAATGTCTCTAGTCG  4007

Murine  3440 TATGGACAGCACTGGAGTTCCTGACCGAATACAGGTGACTACGGACCTATACCAGGTTCT  3499
                 |||||||||||| || || || |||||||| |||||||| |||||||| ||||||||||
Human   4008  TATGGACAGCACGGGGGTCCCCGACCGAATCCAGGTGACCACGGACCTGTACCAGGTTCT  4067

Murine  3500 AGCTGCCAAGGGCTACCAGCTGGAGTGTCGAGGGGTGGTCAAGGTGAAGGGAAAGGGGGA  3559
                 |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Human   4068  AGCTGCCAAGGGCTACCAGCTGGAGTGTCGAGGGGTGGTCAAGGTGAAGGGCAAGGGGGA  4127

Murine  3560 GATGACCACCTACTTCCTCAACGGGGGCCCCAGCAGT  3596  (from SEQ ID NO:10)
                 |||||||||||||||||||| ||||||||||||||
Human   4128  GATGACCACCTACTTCCTCAATGGGGGCCCCAGCAGT  4164  (from SEQ ID NO:11)
```

In alternative embodiments, nucleic acids of the invention are made, isolated and/or manipulated by, e.g., cloning and expression of cDNA libraries, amplification of message or genomic DNA by PCR, and the like. The nucleic acids and genes used to practice this invention, including DNA, RNA, iRNA, antisense nucleic acid, cDNA, genomic DNA, vectors, viruses or hybrids thereof, can be isolated from a variety of sources, genetically engineered, amplified, and/or expressed/ generated recombinantly. Recombinant polypeptides (e.g., cAMP-incompetent AC chimeric proteins used to practice this invention) generated from these nucleic acids can be individually isolated or cloned and tested for a desired activity. Any recombinant expression system can be used, including e.g. bacterial, fungal, mammalian, yeast, insect or plant cell expression systems or expression vehicles.

Alternatively, nucleic acids used to practice this invention can be synthesized *in vitro* by well-known chemical synthesis techniques, as described in, e.g., Adams (1983) J. Am. Chem. Soc. 105:661; Belousov (1997) Nucleic Acids Res. 25:3440-3444; Frenkel (1995) Free Radic. Biol. Med. 19:373-380; Blommers (1994) Biochemistry 33:7886-7896; Narang (1979) Meth. Enzymol. 68:90; Brown (1979) Meth. Enzymol. 68:109; Beaucage (1981) Tetra. Lett. 22:1859; U.S. Patent No. 4,458,066.

Techniques for the manipulation of nucleic acids used to practice this invention, such as, e.g., subcloning, labeling probes (e.g., random-primer labeling using Klenow polymerase, nick translation, amplification), sequencing, hybridization and the like are well described in the scientific and patent literature, see, e.g., Sambrook, ed., MOLECULAR CLONING: A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold

Spring Harbor Laboratory, (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel, ed. John Wiley & Sons, Inc., New York (1997); LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY: HYBRIDIZATION WITH NUCLEIC ACID PROBES, Part I. Theory and Nucleic Acid Preparation, Tijssen, ed. Elsevier, N.Y. (1993).

Another useful means of obtaining and manipulating nucleic acids used to practice the methods of the invention is to clone from genomic samples, and, if desired, screen and re-clone inserts isolated or amplified from, e.g., genomic clones or cDNA clones. Sources of nucleic acid used in the methods of the invention include genomic or cDNA libraries contained in, e.g., mammalian artificial chromosomes (MACs), see, e.g., U.S. Patent Nos. 5,721,118; 6,025,155; human artificial chromosomes, see, e.g., Rosenfeld (1997) Nat. Genet. 15:333-335; yeast artificial chromosomes (YAC); bacterial artificial chromosomes (BAC); P1 artificial chromosomes, see, e.g., Woon (1998) Genomics 50:306-316; P1-derived vectors (PACs), see, e.g., Kern (1997) Biotechniques 23:120-124; cosmids, recombinant viruses, phages or plasmids.

In alternative embodiments, to practice the methods of the invention, AC6mut fusion proteins and nucleic acids encoding them are used. Any AC6mut polypeptide can be used to practice this invention. In alternative embodiments, the AC6mut protein can be fused to a heterologous peptide or polypeptide, such as a peptide for targeting the polypeptide to a desired cell type, such a cardiac myocyte.

In alternative embodiments, a heterologous peptide or polypeptide joined or fused to a protein used to practice this invention can be an N-terminal identification peptide which imparts a desired characteristic, such as fluorescent detection, increased stability and/or simplified purification. Peptides and polypeptides used to practice this invention can also be synthesized and expressed as fusion proteins with one or more additional domains linked thereto for, e.g., producing a more immunogenic peptide, to more readily isolate a recombinantly synthesized peptide, to identify and isolate antibodies and antibody-expressing B cells, and the like. Detection and purification facilitating domains include, e.g., metal chelating peptides such as polyhistidine tracts and histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle WA). The inclusion of a cleavable linker sequences such as Factor Xa or enterokinase (Invitrogen,

San Diego CA) between a purification domain and the motif-comprising peptide or polypeptide to facilitate purification. For example, an expression vector can include an epitope-encoding nucleic acid sequence linked to six histidine residues followed by a thioredoxin and an enterokinase cleavage site (see e.g., Williams (1995) Biochemistry 34:1787-1797; Dobeli (1998) Protein Expr. Purif. 12:404-414). The histidine residues facilitate detection and purification while the enterokinase cleavage site provides a means for purifying the epitope from the remainder of the fusion protein. Technology pertaining to vectors encoding fusion proteins and application of fusion proteins are well described in the scientific and patent literature, see e.g., Kroll (1993) DNA Cell. Biol., 12:441-53.

Nucleic acids or nucleic acid sequences used to practice this invention, e.g., AC6mut-encoding nucleic acids, can be an oligonucleotide, nucleotide, polynucleotide, or to a fragment of any of these, to DNA or RNA of genomic or synthetic origin which may be single-stranded or double-stranded and may represent a sense or antisense strand, to peptide nucleic acid (PNA), or to any DNA-like or RNA-like material, natural or synthetic in origin. Compounds use to practice this invention include "nucleic acids" or "nucleic acid sequences" including oligonucleotide, nucleotide, polynucleotide, or any fragment of any of these; and include DNA or RNA (e.g., mRNA, rRNA, tRNA, iRNA) of genomic or synthetic origin which may be single-stranded or double-stranded; and can be a sense or antisense strand, or a peptide nucleic acid (PNA), or any DNA-like or RNA-like material, natural or synthetic in origin, including, e.g., iRNA, ribonucleoproteins (e.g., e.g., double stranded iRNAs, e.g., iRNPs). Compounds use to practice this invention include nucleic acids, i.e., oligonucleotides, containing known analogues of natural nucleotides. Compounds use to practice this invention include nucleic-acid-like structures with synthetic backbones, see e.g., Mata (1997) Toxicol. Appl. Pharmacol. 144:189-197; Strauss-Soukup (1997) Biochemistry 36:8692-8698; Samstag (1996) Antisense Nucleic Acid Drug Dev 6:153-156. Compounds use to practice this invention include "oligonucleotides" including a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands that may be chemically synthesized. Compounds use to practice this invention include synthetic oligonucleotides having no 5' phosphate, and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide can ligate to a fragment that has not been dephosphorylated.

In alternative aspects, compounds used to practice this invention include genes or any segment of DNA involved in producing an AC6mut polypeptide; it can include regions preceding and following the coding region (leader and trailer) as well as, where applicable, intervening sequences (introns) between individual coding segments (exons). "Operably linked" can refer to a functional relationship between two or more nucleic acid (e.g., DNA) segments. In alternative aspects, it can refer to the functional relationship of transcriptional regulatory sequence to a transcribed sequence. For example, a promoter can be operably linked to a coding sequence, such as a nucleic acid used to practice this invention, if it stimulates or modulates the transcription of the coding sequence in an appropriate host cell or other expression system. In alternative aspects, promoter transcriptional regulatory sequences can be operably linked to a transcribed sequence where they can be physically contiguous to the transcribed sequence, i.e., they can be *cis*-acting. In alternative aspects, transcriptional regulatory sequences, such as enhancers, need not be physically contiguous or located in close proximity to the coding sequences whose transcription they enhance.

In alternative aspects, the invention comprises use of "expression cassettes" comprising a nucleotide sequences used to practice this invention, which can be capable of affecting expression of the nucleic acid, e.g., a structural gene or a transcript (e.g., encoding AC6mut protein) in a host compatible with such sequences. Expression cassettes can include at least a promoter operably linked with the polypeptide coding sequence or inhibitory sequence; and, in one aspect, with other sequences, e.g., transcription termination signals. Additional factors necessary or helpful in effecting expression may also be used, e.g., enhancers.

In alternative aspects, expression cassettes used to practice this invention also include plasmids, expression vectors, recombinant viruses, any form of recombinant "naked DNA" vector, and the like. In alternative aspects, a "vector" used to practice this invention can comprise a nucleic acid that can infect, transfect, transiently or permanently transduce a cell. In alternative aspects, a vector used to practice this invention can be a naked nucleic acid, or a nucleic acid complexed with protein or lipid. In alternative aspects, vectors used to practice this invention can comprise viral or bacterial nucleic acids and/or proteins, and/or membranes (e.g., a cell membrane, a viral lipid envelope, etc.). In alternative aspects, vectors used to practice this invention can include, but are not limited to replicons (e.g., RNA replicons, bacteriophages) to which fragments of

DNA may be attached and become replicated. Vectors thus include, but are not limited to RNA, autonomous self-replicating circular or linear DNA or RNA (e.g., plasmids, viruses, and the like, see, e.g., U.S. Patent No. 5,217,879), and can include both the expression and non-expression plasmids. In alternative aspects, the vector used to practice this invention can be stably replicated by the cells during mitosis as an autonomous structure, or can be incorporated within the host's genome.

In alternative aspects, "promoters" used to practice this invention include all sequences capable of driving transcription of a coding sequence in a cell, e.g., a mammalian cell such as a heart, lung, muscle, nerve or brain cell. Thus, promoters used in the constructs of the invention include *cis*-acting transcriptional control elements and regulatory sequences that are involved in regulating or modulating the timing and/or rate of transcription of a gene. For example, a promoter used to practice this invention can be a *cis*-acting transcriptional control element, including an enhancer, a promoter, a transcription terminator, an origin of replication, a chromosomal integration sequence, 5' and 3' untranslated regions, or an intronic sequence, which are involved in transcriptional regulation. These cis-acting sequences typically interact with proteins or other biomolecules to carry out (turn on/off, regulate, modulate, etc.) transcription.

In alternative embodiments, "constitutive" promoters used to practice this invention can be those that drive expression continuously under most environmental conditions and states of development or cell differentiation. In alternative embodiments, "Inducible" or "regulatable" promoters used to practice this invention can direct expression of the nucleic acid of the invention under the influence of environmental conditions, administered chemical agents, or developmental conditions.

Adenovirus Vector and Adeno-associated virus (AAV) delivery

In alternative embodiments, delivery vehicles, vectors, expression vectors, recombinant viruses, or equivalent are or comprise: an adeno-associated virus (AAV); a recombinant AAV virus, vector or virion; or, an adenovirus vector. In alternative embodiments, the AAV, recombinant AAV virus or vector, or adenovirus vector, is or comprises: an AAV serotype AAV5, AAV6, AAV7, AAV8 or AAV9; a rhesus macaque AAV (AAVrh), or an AAVrh10; or any pseudotype, hybrid or derivative thereof.

In alternative embodiments, any of these vectors (or any delivery vehicle of the invention) is tropic for, or is designed for specific delivery to, a particular cell, tissue or

organ. For example, in alternative embodiments, an AAV used to practice the invention (or any vector or delivery vehicle used to practice the invention) is tropic (or has tropism) for the heart. In other embodiments, an AAV used to practice the invention (or any vector or delivery vehicle) is tropic for, or is designed for specific delivery another tissue or organ, for example, the liver. In alternative embodiments, this "peripheral" mode of delivery, e.g., delivery vehicles, vectors, recombinant viruses and the like, are injected IM or IV, can circumvent problems encountered when genes or nucleic acids are expressed directly in an organ (e.g., the heart, lung or kidney) itself. For example, AAV5, AAV6, and AAV9 have been found to be tropic for the heart, see e.g., Fang et al., Hum Gene Ther Methods 2012 Oct 17; Zincarelli, et al., Clin Transl Sci. 2010 Jun;3(3):81-9.

Adeno-associated virus (AAV) used to practice the invention can be any non-pathogenic member of the *Parvoviridae* family of small, non-enveloped, single-stranded DNA animal viruses. AAV require helper virus (e.g., adenovirus) for replication and, thus, AAVs used to practice the invention do not replicate upon administration to a subject. AAV can infect a relatively wide range of cell types and stimulate only a mild immune response, particularly as compared to a number of other viruses, such as adenovirus. AAV serotypes used to practice this invention include, e.g., AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, and AAV12. AAV used to practice the invention can be from other animals, including: e.g., birds (e.g., avian AAV, or AAAV), bovines (e.g., bovine AAV, or BAAV), canines, equines, ovines, and porcines.

In alternative embodiments, AAV vectors used to practice the invention are recombinant nucleic acid molecules in which at least a portion of the AAV genome is replaced by a heterologous nucleic acid molecule; one can replace about 4.7 kilobases (kb) of AAV genome DNA, e.g., by removing the viral replication and capsid genes. In alternative embodiments, the heterologous nucleic acid molecule is simply flanked by AAV inverted terminal repeats (ITRs) on each terminus. The ITRs serve as origins of replication and contain cis acting elements required for rescue, integration, excision from cloning vectors, and packaging. In alternative embodiments AAVs used to practice the invention comprise a promoter operatively linked to the heterologous nucleic acid molecule to control expression.

An AAV vector can be packaged into an AAV capsid *in vitro* with the assistance of a helper virus or helper functions expressed in cells to yield an AAV virion. The

serotype and cell tropism of an AAV virion are conferred by the nature of the viral capsid proteins. AAV vectors and AAV virions can transduce cells efficiently, including both dividing and non-dividing cells. AAV vectors and virions have been shown to be safe and to lead to long term in vivo persistence and expression in a variety of cell types.

In alternative embodiments, an ITR joined to 5' terminus of the AC6mut-encoding nucleic acid molecule, and an ITR joined to the 3' terminus of the AC6mut-encoding nucleic acid molecule. Examples of ITRs include, but are not limited, to AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAAV, BAAV, and other AAV ITRs known to those skilled in the art. In one embodiment, an AAV ITR is selected from an AAV2 ITR, an AAV5 ITR, an AAV6 ITR, and a BAAV ITR. In one embodiment, an AAV ITR is an AAV2 ITR. In one embodiment, an AAV ITR is an AAV5 ITR. In one embodiment, an AAV ITR is an AAV6 ITR. In one embodiment, an AAV ITR is a BAAV ITR.

In alternative embodiments, AAV vectors (and other vectors, recombinant viruses and the like) used to practice the invention comprise other sequences, such as expression control sequences, e.g., a promoter, an enhancer, a repressor, a ribosome binding site, an RNA splice site, a polyadenylation site, a transcriptional terminator sequence, and a microRNA binding site. Examples of promoters include, but are not limited to, an AAV promoter, such as a p5, p19 or p40 promoter, an adenovirus promoter, such as an adenoviral major later promoter, a cytomegalovirus (CMV) promoter, a papilloma virus promoter, a polyoma virus promoter, a respiratory syncytial virus (RSV) promoter, a sarcoma virus promoter, an SV40 promoter, other viral promoters, an actin promoter, an amylase promoter, an immunoglobulin promoter, a kallikrein promoter, a metallothionein promoter, a heat shock promoter, an endogenous promoter, a promoter regulated by rapamycin or other small molecules, other cellular promoters, and other promoters known to those skilled in the art. In one embodiment, the promoter is an AAV promoter. In one embodiment, the promoter is a CMV promoter. Selection of expression control sequences to include can be accomplished by one skilled in the art.

In alternative embodiments, AAV vectors of different serotypes (as determined by the serotype of the ITRs within such vector) are used, e.g.,: an AAV1 vector, an AAV2 vector, an AAV3 vector, an AAV4 vector, an AAV5 vector, an AAV6 vector, an AAV7 vector, an AAV8 vector, an AAV9 vector, an AAV10 vector, an AAV11 vector, an AAV

12 vector, an AAAV vector, and a BAAV vector. In alternative embodiments the AAV vector is an AAV2 vector, an AAV5 vector, an AAV6 vector or a BAAV vector.

In alternative embodiments, chimeric, shuffled or capsid-modified AAV derivatives are used to provide one or more desired functionalities for the viral vector. alternative embodiments, these derivatives may display increased efficiency of gene delivery, decreased immunogenicity (humoral or cellular), an altered tropism range and/or improved targeting of a particular cell type compared to an AAV viral vector comprising a naturally occurring AAV genome. In alternative embodiments increased efficiency of gene delivery is achieved by improved receptor or co-receptor binding at the cell surface, improved internalization, improved trafficking within the cell and into the nucleus, improved uncoating of the viral particle and/or improved conversion of a single-stranded genome to double-stranded form. In alternative embodiments an altered tropism range or targeting of a specific cell population results in increased efficiency, such that the vector dose is not diluted by administration to tissues where it is not needed.

In alternative embodiments, capsid-free AAV vectors are used as described e.g., in U.S. patent app. No. 20140107186. In alternative embodiments, AAV9 vectors that are heart- or liver-tropic are used as described e.g., in U.S. patent app. No. 20140056854. In alternative embodiments, AAV vectors are described in e.g., in U.S. patent app. Nos. 20130310443; 20130136729, are used to practice the invention.

In alternative embodiments, AAV vectors are pseudotyped for e.g., improved or altered performance, e.g., to improve or alter the tropism or other features of the virus, as described e.g., in U.S. patent app. No 20120220492. For example, specific or improved targeting allows the delivery vehicle (e.g., the AAV viral particle) to infect and deliver the therapeutic nucleic acid (e.g., an AC6mut) only to those cells intended to be infected, thus decreasing the risk of unwanted side effects from gene therapy and increasing the efficacy of the gene therapy.

In alternative embodiments, dosages of the viral vector are determined by factors such as the condition being treated, the age, weight and health of the patient, and may thus vary among patients. For example, a therapeutically effective human dosage of a viral vector is generally in the range of from about 0.1 ml to about 100 ml of solution containing concentrations of from about 1 $x10^{9}$ to 1 $x10^{16}$ genomes virus vector. An exemplary human dosage for delivery to large organs (e.g., liver, muscle, heart and lung) may be about 5 x 10^{10}, to 5 x 10^{13} AAV genomes per 1 kg, at a volume of about 1 to 100

mL. The dosages are adjusted to balance the therapeutic benefit against any side effects and such dosages may vary depending upon the therapeutic application for which the recombinant vector is employed. The levels of expression of the transgene can be monitored to determine the frequency of dosage resulting in viral vectors, e.g., AAV vectors.

Formulations

In alternative embodiments, the invention provides compositions and methods for delivering and expressing AC6mut *in vivo* in a cardiac myocyte cell. In alternative embodiments, these compositions comprise AC6mut -encoding nucleic acids formulated for these purposes, e.g., expression vehicles or AC6mut -encoding nucleic acids formulated in a buffer, in a saline solution, in a powder, an emulsion, in a vesicle, in a liposome, in a nanoparticle, in a nanolipoparticle and the like.

In alternative embodiments, the compositions can be formulated in any way and can be applied in a variety of concentrations and forms depending on the desired *in vivo* or *ex vivo* conditions, including a desired *in vivo* or *ex vivo* method of administration and the like. Details on techniques for *in vivo* or *ex vivo* formulations and administrations are well described in the scientific and patent literature.

Formulations and/or carriers of the AC6mut -encoding nucleic acids used to practice this invention are well known in the art. Formulations and/or carriers used to practice this invention can be in forms such as tablets, pills, powders, capsules, liquids, gels, syrups, slurries, suspensions, etc., suitable for *in vivo* or *ex vivo* applications.

In alternative embodiments, AC6mut -encoding nucleic acids used to practice this invention can be in admixture with an aqueous and/or buffer solution or as an aqueous and/or buffered suspension, e.g., including a suspending agent, such as sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia, and dispersing or wetting agents such as a naturally occurring phosphatide (e.g., lecithin), a condensation product of an alkylene oxide with a fatty acid (e.g., polyoxyethylene stearate), a condensation product of ethylene oxide with a long chain aliphatic alcohol (e.g., heptadecaethylene oxycetanol), a condensation product of ethylene oxide with a partial ester derived from a fatty acid and a hexitol (e.g., polyoxyethylene sorbitol mono-oleate), or a condensation product of ethylene oxide with a partial ester derived from fatty acid and a hexitol

anhydride (e.g., polyoxyethylene sorbitan mono-oleate). The aqueous suspension can also contain one or more preservatives such as ethyl or n-propyl p-hydroxybenzoate. Formulations can be adjusted for osmolarity, e.g., by use of an appropriate buffer.

In practicing this invention, the compounds (e.g., formulations) of the invention can comprise a solution of AC6mut -encoding nucleic acids or genes dissolved in a pharmaceutically acceptable carrier, e.g., acceptable vehicles and solvents that can be employed include water and Ringer's solution, an isotonic sodium chloride. In addition, sterile fixed oils can be employed as a solvent or suspending medium. For this purpose any fixed oil can be employed including synthetic mono- or diglycerides, or fatty acids such as oleic acid. In one embodiment, solutions and formulations used to practice the invention are sterile and can be manufactured to be generally free of undesirable matter. In one embodiment, these solutions and formulations are sterilized by conventional, well known sterilization techniques.

The solutions and formulations used to practice the invention can comprise auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents, e.g., sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of active agent (e.g., AC6mut -encoding nucleic acids or genes) in these formulations can vary widely, and can be selected primarily based on fluid volumes, viscosities and the like, in accordance with the particular mode of *in vivo* or *ex vivo* administration selected and the desired results, e.g., increasing *in vivo* AC6mut expression.

The solutions and formulations used to practice the invention can be lyophilized; for example, the invention provides a stable lyophilized formulation comprising AC6mut -encoding nucleic acids or genes. In one aspect, this formulation is made by lyophilizing a solution comprising AC6mut -encoding nucleic acid or gene and a bulking agent, e.g., mannitol, trehalose, raffinose, and sucrose or mixtures thereof. A process for preparing a stable lyophilized formulation can include lyophilizing a solution about 2.5 mg/mL protein, about 15 mg/mL sucrose, about 19 mg/mL NaCl, and a sodium citrate buffer having a pH greater than 5.5 but less than 6.5. See, e.g., U.S. patent app. no. 20040028670.

The compositions and formulations of the invention can be delivered by the use of liposomes (see also discussion, below). By using liposomes, particularly where the

liposome surface carries ligands specific for target cells, e.g., cardiac myocytes, or are otherwise preferentially directed to a specific tissue or organ type, e.g., a heart, one can focus the delivery of the active agent into a target cell, e.g., a cardiac myocyte, in an *in vivo* or *ex vivo* application.

Nanoparticles, Nanolipoparticles and Liposomes

The invention also provides nanoparticles, nanolipoparticles, vesicles and liposomal membranes comprising compounds (e.g., AC6mut or AC6mut -encoding nucleic acids or genes) used to practice the methods of this invention, e.g., to deliver AC6mut or AC6mut -encoding nucleic acids or genes to cardiac myocyte cells *in vivo* or *ex vivo*. In alternative embodiments, these compositions are designed to target specific molecules, including biologic molecules, such as polypeptides, including cell surface polypeptides, e.g., for targeting a desired cell type, e.g., a mammalian cardiac cell, a cardiac myocyte and the like.

The invention provides multilayered liposomes comprising compounds used to practice this invention, e.g., as described in Park, et al., U.S. Pat. Pub. No. 20070082042. The multilayered liposomes can be prepared using a mixture of oil-phase components comprising squalane, sterols, ceramides, neutral lipids or oils, fatty acids and lecithins, to about 200 to 5000 nm in particle size, e.g., to entrap a cAMP-incompetent AC-encoding nucleic acid or gene.

Liposomes can be made using any method, e.g., as described in Park, et al., U.S. Pat. Pub. No. 20070042031, including method of producing a liposome by encapsulating an active agent (e.g., AC6mut -encoding nucleic acids or genes), the method comprising providing an aqueous solution in a first reservoir; providing an organic lipid solution in a second reservoir, and then mixing the aqueous solution with the organic lipid solution in a first mixing region to produce a liposome solution, where the organic lipid solution mixes with the aqueous solution to substantially instantaneously produce a liposome encapsulating the active agent; and immediately then mixing the liposome solution with a buffer solution to produce a diluted liposome solution.

In one embodiment, liposome compositions used to practice this invention comprise a substituted ammonium and/or polyanions, e.g., for targeting delivery of a compound (e.g., AC6mut -encoding nucleic acids or genes) used to practice this invention to a desired cell type, as described e.g., in U.S. Pat. Pub. No. 20070110798.

The invention also provides nanoparticles comprising compounds (e.g., AC6mut -encoding nucleic acids or genes) used to practice this invention in the form of active agent-containing nanoparticles (e.g., a secondary nanoparticle), as described, e.g., in U.S. Pat. Pub. No. 20070077286. In one embodiment, the invention provides nanoparticles comprising a fat-soluble active agent of this invention or a fat-solubilized water-soluble active agent to act with a bivalent or trivalent metal salt.

In one embodiment, solid lipid suspensions can be used to formulate and to deliver AC6mut -encoding nucleic acids or genes used to practice the invention to a mammalian cell *in vivo* or *ex vivo*, as described, e.g., in U.S. Pat. Pub. No. 20050136121.

Delivery vehicles

In alternative embodiments, any delivery vehicle can be used to practice the methods or compositions of this invention, e.g., to deliver AC6mut or AC6mut -encoding nucleic acids or genes to practice the methods of the invention *in vivo* or *ex vivo*. For example, delivery vehicles comprising polycations, cationic polymers and/or cationic peptides, such as polyethyleneimine derivatives, can be used e.g. as described, e.g., in U.S. Pat. Pub. No. 20060083737.

In one embodiment, a dried polypeptide-surfactant complex is used to formulate a composition of the invention, wherein a surfactant is associated with a nucleic acid via a non-covalent bond e.g. as described, e.g., in U.S. Pat. Pub. No. 20040151766.

In one embodiment, a nucleic acid used to practice this invention can be applied to cells as polymeric hydrogels or water-soluble copolymers, e.g., as described in U.S. Patent No. 7,413,739; for example, a nucleic acid can be polymerized through a reaction between a strong nucleophile and a conjugated unsaturated bond or a conjugated unsaturated group, by nucleophilic addition, wherein each precursor component comprises at least two strong nucleophiles or at least two conjugated unsaturated bonds or conjugated unsaturated groups.

In one embodiment, a nucleic acid is applied to cells using vehicles with cell membrane-permeant peptide conjugates, e.g., as described in U.S. Patent Nos. 7,306,783; 6,589,503. In one aspect, the nucleic acid itself is conjugated to a cell membrane-permeant peptide. In one embodiment, a nucleic acid and/or the delivery vehicle are conjugated to a transport-mediating peptide, e.g., as described in U.S. Patent No.

5,846,743, describing transport-mediating peptides that are highly basic and bind to poly-phosphoinositides.

In one embodiment, electro-permeabilization is used as a primary or adjunctive means to deliver AC6mut -encoding nucleic acids or genes to a cell, e.g., using any electroporation system as described e.g. in U.S. Patent Nos. 7,109,034; 6,261,815; 5,874,268.

Implanting cells *in vivo*

In alternative embodiments, the methods of the invention also comprise implanting or engrafting cells, e.g., cardiac or cardiac myocyte cells, comprising or expressing AC6mut -encoding nucleic acids or genes used to practice the invention; and in one aspect, methods of the invention comprise implanting or engrafting the AC6mut -encoding nucleic acids or genes (or cells expressing them) in a vessel, tissue or organ *ex vivo* or *in vivo*, e.g., a heart or a cardiac myocyte, or implanting or engrafting the re-programmed differentiated cell in an individual in need thereof.

Cells can be removed from an individual, treated using the compositions and/or methods of this invention, and reinserted (e.g., injected or engrafted) into a tissue, organ or into the individual, using any known technique or protocol. For example, de-differentiated re-programmed cells, stem cells, or re-programmed differentiated cells, can be re-implanted (e.g., injected or engrafted) using e.g., microspheres e.g., as described in U.S. Pat. No. 7,442,389; e.g., in one aspect, the cell carrier comprises a bulking agent comprising round and smooth polymethylmethacrylate microparticles preloaded within a mixing and delivery system and an autologous carrier comprising these cells. In another embodiment, the cells are re-administered to a tissue, an organ, e.g., a heart, and/or to an individual in need thereof in a biocompatible crosslinked matrix, as described e.g., in U.S. Pat. App. Pub. No. 20050027070.

In another embodiment, the cells of the invention (e.g., cells made by practicing the methods of this invention) are re-administered (e.g., injected or engrafted) to a tissue, an organ and/or an individual in need thereof within, or protected by, a biocompatible, nonimmunogenic coating, e.g., as on the surface of a synthetic implant, e.g., as described in U.S. Pat. No. 6,969,400, describing e.g., a protocol where AC6mut can be conjugated to a polyethylene glycol that has been modified to contain multiple nucleophilic groups, such as primary amino or thiol group.

In one embodiment, the cells of the invention (e.g., cells made by practicing the methods of this invention) are re-administered (e.g., injected or engrafted) to a tissue, an organ and/or an individual in need thereof using grafting methods as described e.g. by U.S. Pat. Nos. 7,442,390; 5,733,542.

Any method for delivering polypeptides, nucleic acids and/or cells to a tissue or organ (e.g., a cardiac myocyte, heart) can be used, and these protocols are well known in the art, e.g., as described in U.S. Patent No. (USPN) 7,514,401, describing e.g., using intracoronary (IC), intravenous (IV), and/or local delivery (direct myocardial injection) of polypeptides, nucleic acids and/or cells to a heart *in situ*. For example, in alternative embodiments, aerosol drug particles into the lungs and into the bloodstream, gene therapy, continuous infusions, repeated injections and/or sustained release polymers can be used for delivering polypeptides, nucleic acids and/or cells to a tissue or organ (e.g., a lung, kidney, heart). In alternative embodiments, nucleic acids and/or cells can be given through a catheter into the coronary arteries or by direct injection into the left atrium or ventricular myocardium via a limited thoracotomy; or delivered into the myocardium via a catheter passed during cardiac catheterization; or delivered into the pericardial space.

In alternative embodiments, nucleic acids used to practice this invention, or a vector comprising a nucleic acid used to practice the invention (e.g., an adenovirus-associated virus or vector (AAV), or an adenoviral gene therapy vector), or a vesicle, liposome, nanoparticle or nanolipid particle (NLP) of the invention, and the like, to a tissue or organ (e.g., a lung, kidney, heart); e.g. as described in USPN 7,501,486.

Compositions used to practice this invention can be used in combination with other therapeutic agents, e.g. angiogenic agents, anti-thrombotic agents, anti-inflammatory agents, immunosuppressive agents, anti-arrhythmic agents, tumor necrosis factor inhibitors, endothelin inhibitors, angiotensin-converting enzyme inhibitors, calcium antagonists, antibiotic agents, antiviral agents and viral vectors.

Compositions used to practice this invention can be used for ameliorating or treating any of a variety of cardiopathies and cardiovascular diseases, e.g., cardiopathies and cardiovascular diseases, e.g., coronary artery disease (CAD); atherosclerosis; thrombosis; restenosis; vasculitis including autoimmune and viral vasculitis such as polyarteritis nodosa, Churg-Strass syndrome, Takayasu's arteritis, Kawasaki Disease and Rickettsial vasculitis; atherosclerotic aneurisms; myocardial hypertrophy; congenital heart diseases (CHD); ischemic heart disease and anginas; acquired valvular/endocardial

diseases; primary myocardial diseases including myocarditis; arrhythmias; and transplant rejections; metabolic myocardial diseases and myocardiomyopathies such as congestive, hypertrophic and restrictive cardiomyopathies, and/or heart transplants.

Kits and Instructions

The invention provides kits comprising compositions and methods of the invention, including instructions for use thereof. As such, cells, delivery vehicles, vectors, expression vectors, recombinant viruses and the like, of the invention, can also be provided.

For example, in alternative embodiments, the invention provides kits comprising compositions comprising (a) AC6mut -encoding nucleic acid, (b) delivery vehicles, vectors, expression vectors, recombinant viruses and the like, of the invention, (c) a liquid or aqueous formulation of the invention, or (d) the vesicle, liposome, nanoparticle or nanolipid particle of the invention. In one aspect, the kit further comprising instructions for practicing any methods of the invention, e.g., *in vitro* or *ex vivo* methods for delivering a desired AC6mut or AC6mut-expressing nucleic acid, vector, and the like, to a cardiac myocyte cell.

The invention will be further described with reference to the following examples; however, it is to be understood that the invention is not limited to such examples.

EXAMPLES

EXAMPLE 1: Delivery of cAMP-incompetent AC increases cardiac function

This example demonstrates the effectiveness of an exemplary embodiment of the invention: delivery of cAMP-incompetent AC to cardiac myocytes for the treatment of heart failure. In this study, we asked whether an AC mutant molecule that *reduces* LV cAMP production would have favorable effects on left ventricle (LV) function through its effects on Ca^{2+} handling alone.

So many clinical trials of positive inotropes have failed, that it is now axiomatic that agents that increase cAMP are deleterious to the failing heart. An alternative strategy is to alter myocardial Ca^{2+} handling or myofilament response to Ca^{2+} using agents that do not affect cAMP. Although left ventricular (LV) function is tightly linked to adenylyl

cyclase (AC) activity, the beneficial effects of AC may be independent of cAMP and instead stem from effects on Ca^{2+} handling.

In this study, we generated transgenic mice with cardiac-directed expression of a cyclic adenosine monophosphate-incompetent (cAMP-incompetent) adenylyl cyclase type 6 (AC6) polypeptide, the so-called "AC6 mutant", or "AC6mut". Cardiac myocytes of these AC6mut transgenic mice showed impaired cAMP production in response to isoproterenol (74% reduction; $p<0.001$), but LV size and function were normal. Isolated hearts showed preserved LV function in response to isoproterenol stimulation. AC6mut expression was associated with increased sarcoplasmic reticulum Ca^{2+} uptake and the EC50 for SERCA2a activation was reduced. Cardiac myocytes isolated from AC6mut mice showed increased amplitude of Ca^{2+} transients in response to isoproterenol ($p=0.0001$). AC6mut expression also was associated with increased expression of LV S100A1 ($p=0.03$) and reduced expression of phospholamban protein ($p=0.01$). This study determined that LV AC mutant expression is associated with normal cardiac function despite impaired cAMP generation. The mechanism appears to be through effects on Ca^{2+} handling — effects that occur despite diminished cAMP.

Data from previous studies indicated that increased cardiac AC type 6 (AC6), a dominant AC isoform expressed in mammalian cardiac myocytes [6], has protean beneficial effects on the failing left ventricle (LV) [7],[8],[9],[10],[11],[12]. These unexpected beneficial effects must be reconciled with the dire consequences on the heart of beta (ß) adrenergic receptor (ßAR) stimulation and elevations in intracellular cAMP [13],[14],[15],[16],[17],[18]. Indeed, the apparent benefits of AC6 expression in the failing heart is paradoxical. Using pharmacological inhibitors, data from previous studies suggest that some of the beneficial effects of increased cardiac AC6 expression do not depend upon increased cAMP generation [2],[3]. Because of the inherent limitations of studies using pharmacological inhibition in cultured cardiac myocytes, we generated a catalytically inactive murine AC6 mutant (AC6mut) molecule by substitution of Ala for Asp at position 426 in the catalytic core (position 426: position number based on SEQ ID NO:16), a change predicted to alter Mg^{2+} binding but not influence G-protein dynamics [4]. This murine AC6mut molecule, when studied *in vitro,* markedly impairs cAMP generation, but retains the cellular distribution pattern associated with AC6 [4]. Such *in vitro* studies fall far short of establishing how such a molecule might influence cardiac function *in vivo.*

We therefore generated transgenic murine lines with cardiac-directed expression of AC6mut. Our hope was that such lines would provide additional insight vis-à-vis differentiation of cAMP vs Ca^{2+} handling effects on the function of the intact normal heart. Furthermore, such studies might indicate whether AC6mut provides inotropic stimulation free from the potentially deleterious effects of increased cAMP. Our hypothesis was that LV function, despite marked diminution in cAMP generating capacity, would remain normal, through beneficial counterbalancing effects conferred by AC6 on Ca^{2+} handling.

Methods

Generation of AC6mut Transgenic Mice (Fig. 1A). The use of animals was in accordance with Association for Assessment and Accreditation of Laboratory Animal Care guidelines and was approved by the Institutional Animal Care and Use Committee of VA San Diego Healthcare System. To generate mice with cardiac-directed expression of AC6mut, the murine AC6mut cDNA [4] with an AU1 tag at the C-terminus, was subcloned between the α-myosin heavy chain promoter and SV40 polyA. A 9.2-kb fragment containing the expression cassette was used for pronuclear injection, carried out in the transgenic mouse facility at University of California, San Diego (inbred C57BL/6). Founder mice were identified by polymerase chain reaction (PCR) of genomic DNA prepared from tail tips.

The AC6mut gene was detected using a primer homologous to the α-MHC promoter (forward: 5' CACATAGAAGCCTAGCCCACACC) (SEQ ID NO:1); the reverse primer was for the AC6 region (5' CAGGAGGCCACTAAACCATGAC) (SEQ ID NO:2).

AC6mut mRNA was detected using the following primers: (forward: 5' TGGGCCTCTCTACTCTGCAT (SEQ ID NO:3); reverse: 5' TGGATGTAACCTCGGGTCTC) (SEQ ID NO:4) enabling quantification of fold increase of AC6mut mRNA over endogenous AC6 mRNA.

Endogenous AC6 mRNA was detected using primers homologous to its 3'-untranslated region (forward: 5' GGCATTGAGTGGGACTTTGT (SEQ ID NO:5); reverse: 5' TCTGCATCCAAACAAACGAA) (SEQ ID NO:6). This 3' untranslated region was not present in the AC6mut cDNA, enabling quantification of endogenous AC6.

Founder animals were crossbred with wild-type mice of the same strain, and selected animals were used for analysis of cardiac transgene expression. We documented variable transgene expression in independent lines and selected a line with a 17-fold increase in AC6mut protein expression (vs endogenous AC6) for our studies. LV expression levels of AC types 2 - 9 were determined using quantitative RT-PCR as previously described [5].

Echocardiography. Anesthesia was induced with 5% isoflurane (at a flow rate of 1 L/min oxygen) and maintained with 1% isoflurane in oxygen. Images were obtained using a 16L MHz linear probe and Sonos 5500® Echocardiograph system (Philips Medical Systems, Bothell, WA), as previously reported [7]. Data were acquired and analyzed without knowledge of group identity.

Isolated Perfused Hearts: LV Contractile Function. Cardiac function was assessed in isolated perfused hearts to assess LV contractile function in a manner unaffected by reflex activation or anesthesia, as previously reported [7]. An intraventricular balloon catheter was deployed to measure isovolumic LV pressure (LV end-diastolic pressure 10 mmHg; 1.7 mM ionized Ca^{2+}). Isoproterenol was delivered in bolus doses (from 0.1 nM to 300 nM) at five-minute intervals as LV pressure was recorded. Subsequently, the first derivative of the LV pressure (LV dP/dt) was derived and used as a surrogate of LV contractile function. Data were acquired and analyzed without knowledge of group identity.

Calcium Uptake. Initial rate of ATP-dependent sarcoplasmic reticulum (SR) Ca^{2+} uptake in LV homogenates was measured by the modified Millipore filtration technique as described previously [11].

Calcium Transient. Cytosolic calcium transients were measured using Indo-1, as described previously [19]. Cardiac myocytes were plated onto laminin-coated glass cover slips and loaded with indo-1/AM (3 μM, Calbiochem, La Jolla CA) and dispersing agent, pluronic F-127 (0.02 mg/ml, Calbiochem, La Jolla CA) for 30 min. Following dye loading, cover slips were mounted in a superfusion chamber, rinsed to remove excess indo-1/AM, and mounted on a Nikon DIAPHOT™ epifluorescence microscope equipped with a 40x objective interfaced to a Photon Technologies photometry system (Birmingham NJ) with the excitation wavelength set to 365 nm via a monochromator. Fluorescence emission was split and directed to two photomultiplier tubes through 20-nm band-pass filters centered at 405 and 485 nm, respectively. The ratio F405/F485

represents a measure for $[Ca^{2+}]i$. During these measurements, cardiac myocytes were superfused with 25 mM HEPES (pH 7.3) containing 2 mM $CaCl_2$. Myocytes were field-stimulated at 0.3 Hz. Isoproterenol-stimulated Ca^{2+} transient was determined by adding isoproterenol (10 μM) to the buffer. Calcium transients were recorded from at least 20 cells per heart and for at least 3 hearts per group. Diastolic and systolic intracellular Ca^{2+} levels were obtained from the basal and maximal F405/F485 ratio per cycle, respectively.

Cardiac Myocyte Isolation. Cardiac myocyte isolation was performed as previously described [4].

Cyclic AMP Measurement. Isolated cardiac myocytes were stimulated with isoproterenol (10 μM, 10 min) or the water-soluble forskolin analog NKH477 (10 μM, 10 min), and then lysed (2.5% dodecyltrimethylammonium bromide, 0.05 M sodium acetate, pH 5.8, and 0.02% bovine serum albumin). Cyclic AMP was measured using the cAMP BIOTRAK™ enzyme immunoassay system (GE Healthcare, Pittsburgh, PA) as previously reported [4].

PKA Activity Assay. Isolated cardiac myocytes were stimulated with isoproterenol (10 μM, 10 min) or NKH477 (10 μM, 10 min). Cardiac myocytes were homogenized in buffer A: 20 mM Tris-HCl (pH 7.4), 0.5 mM EGTA, 0.5 mM EDTA, and protease inhibitor cocktail from Invitrogen) and centrifuged (14,000 x *g*, 5 min, 4°C). The supernatant was incubated with PKA biotinylated peptide substrate (SignaTECT® (SIGNATECT®) cAMP-Dependent Protein Kinase Assay System (Promega, Madison WI)) in the presence of $[\gamma\text{-}^{32}P]ATP$. The ^{32}P-labeled, biotinylated substrate was recovered with a streptavidin matrix, and the specific activity of PKA determined.

Isoproterenol-Stimulated Phosphorylation of Ryanodine Receptor-2, PLB, and Troponin I in Cardiac Myocytes. To determine dynamic phosphorylation of key Ca^{2+} regulating proteins, we conducted studies of basal and isoproterenol-stimulated phosphorylation of RyR2, PLB and TnI in cultured cardiac myocytes isolated from each group (**Fig. 2C**). Cultured cardiac myocytes (100,000 cells per well) were used in these studies and immunoblotting performed before and after incubation with isoproterenol (10 μM, 10 min). Cells were lysed in lysis buffer: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin). Protein concentration was

measured using the Bradford method. Immunoblots were normalized to GAPDH and compared (**Fig. 2D**).

PDE Activity Assay. Phosphodiesterase activity was assayed using the Cyclic Nucleotide Phosphodiesterase Assay Kit (Enzo). LV tissues were homogenized in buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM PMSF, 10 mM activated orthovanadate, 1x protease inhibitor cocktail (Life Sciences) and centrifuged at 10,000 rpm (10 min) in a microfuge. Tissue homogenates were desalted by gel filtration using Desalting Column Resin (Enzo). Twenty μg of protein (Bradford) was added to each well and PDE activity measured.

Immunofluorescence. Isolated cardiac myocytes were attached to laminin coated 2-well chamber slides for 1 hr, washed, fixed (10% formalin, 15 min, 23°C), blocked with normal goat serum (1 hr) and incubated (4°C, overnight) with: anti-AU1 antibody (Fitzgerald, 1:300; for detecting AC6mut transgene protein); anti-Cav3 antibody (BD Pharmagen, 1:100; for detecting caveolae); anti-PDI antibody (Invitrogen, 1:1000; for detecting SR); anti-lamin A (Abcam, 1:200; for detecting nuclear envelope); anti-CREM-1 antibody (Santa Cruz, 1:50); or anti-phospho-CREB antibody (Upstate, 1:100). Cardiac myocytes were washed with PBS and then incubated with secondary antibodies (Alexia Fluo 488 or 594 conjugated, 1:1000 dilution) for 1 hr. To identify the nucleus, cells were incubated with Hoechst dye (1:1000 dilution, 20 min). Cardiac myocytes then were imaged as previously described [2].

Detection of mRNA and Immunoblotting. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was used to quantify mRNA and immunoblotting was used to quantify protein content [4]. The primers for RyR2 included (forward: 5'AACCTACCAGGCTGTGGATG) (SEQ ID NO:7); and (reverse: 5' GACTCGATGGGCAAGTCAAT) (SEQ ID NO:8).

We used the anti-AC5/6 antibody to identify endogenous AC6 and AC6mut (Santa Cruz, 1:200 dilution). The epitope for the AC5/6 antibody is at the C-terminus of AC6 and AC6mut (sequence: KGYQLECRGVVKVKGKGEMTTYFLNGGPSS (SEQ ID NO:9); protein accession #O43306 and #Q01234). We used AU1 antibody (Fitzgerald, 1:2,000) to detect AC6mut protein. Additional antibodies used included: calreticulin (ABR Affinity, 1:1,000); calsequestrin (Novus Biologicals, 1:1,000); GAPDH (Fitzgerald, 1:20,000); PDE3A (Advam); PKA catalytic subunit (BD Transduction, 1:1,000); p-PKA catalytic subunit (Cell Signaling, 1:1,000); PKA-RIIα and PKA-RIIβ

(BD Transduction, 1:1,000); phospho-PKA-RIIα (S96) and phospho-PKA-RIIβ (S114) (Santa Cruz, 1:200); PKCα catalytic subunit (Santa Cruz, 1:200); PLB (Affinity Bioreagents, 1:5,000); phospho S16-PLB (Badrilla, 1:3,000 dilution); phospho-RyR2 (S2808) (Abcam, 1:1,000); S100A1 (Epiyomics, 1:1,000); SERCA2a (Enzo, 1:1,000); troponin I and phospho-TnI (S22/23) (Cell Signaling, 1:1,000 each)

Statistical Analysis. Data represent mean ± SE; group differences were tested for statistical significance using either ANOVA, followed by Bonferroni *t*-testing, or, when appropriate, Student's *t* test (unpaired, 2-tailed). The null hypothesis was rejected when $p < 0.05$.

Results

AC6mut Transgenic Mice. AC6mut mice were physically indistinguishable from their transgene negative siblings. Necropsy of adult mice showed that body weight, tibial length, LV weight, and lung weight showed no group differences. (**Table 1**).

LV Expression of AC6mut. AC6mut mRNA was increased 62-fold and protein was increased 17-fold over the levels of endogenous AC6, which were detected using primers and antibody to the common regions on both endogenous AC6 and transgene AC6mut in RT-PCR and immunoblotting (**Figs. 1B and 1C**).

LV Expression of Endogenous AC Types. The mRNA of endogenous AC types 2 - 9 showed no group differences (data not shown).

LV cAMP Production. LV samples from AC6mut mice showed reduced cAMP production when stimulated with isoproterenol (74% reduction; $p<0.001$) or NKH477, a water-soluble forskolin analog (52% reduction; $p = 0.05$) (**Fig. 1D**); basal cAMP production was unchanged. Thus, the transgenic line was suited to test the overall effect of reduced βAR-stimulated cAMP production in the presence of increased AC6mut expression on LV function.

PKA Activity and Expression. Cardiac myocytes isolated from AC6mut mice showed a 48% reduction in basal PKA activity ($p=0.01$). In addition there were reductions in PKA activity stimulated by isoproterenol (38% reduction; $p=0.006$); and NKH477 (38% reduction; $p=0.001$) (**Fig. 2A, upper**). AC6mut expression did not alter LV expression of the PKA catalytic subunit (**Fig. 2A, lower**) or expression or phosphorylation of PKA-RII-α and β (phospho-PKA-RIIα: AC6mut, 0.32±0.04 du; Con, 0.30±0.03 du, $p=0.7$; phospho-PKA-RIIβ: AC6mut, 7.1±1.1 du; Con, 10.6±01.4 du;

p=0.09; **Fig. 2B**). PKC catalytic subunit expression also showed no group difference (PKCα: AC6mut, 0.8±0.1 du; Con, 0. 7±0.1 du, p=0.4; **Fig. 2B**)

Isoproterenol-Stimulated Phosphorylation of Ryanodine Receptor-2, PLB and Troponin I in Cardiac Myocytes. Basal phosphorylation of RyR2, PLB and TnI showed no group differences (P-RyR2: AC6mut, 4.4±0.6 vs Con, 2.4±0.5 du, p=0.06; P-PLB: AC6mut, 0.3±0.03 vs Con, 0.2±0.1 du, p=0.8; P-TnI: AC6mut, 0.8±0.2 vs Con, 1.0±0.01 du, p=0.24, **Fig. 2C**). Isoproterenol stimulation was associated with increased phosphorylation of RyR2, PLB, and TnI in both groups (vs un-stimulated), but the extent of phosphorylation generally was greater in LV from AC6mut mice (P-RyR2: AC6mut, 30.0±1.1vs Con, 7.4±1.1 du, p=0.001,; P-PLB: AC6mut, 16.8±2.4 vs Con, 5.3±0.1 du, p=0.01; P-TnI: AC6mut, 5.8±1.4 vs Con, 2.2±0.7 du, p=0.07; **Fig. 2C**). TnI protein expression was not different between groups (AC6mut, 0.9±0.1 vs Con, 0.7±0.2 du; p=0.5; **Fig. 2B.** RyR2 mRNA expression showed no group difference.

PDE Activity and PDE3A Expression. There was no group difference in PDE activity in LV samples (AC6mut: 1252±23 Units/mg, n=7; Control: 1293±39 Units/mg, n=6; p=0.38). LV PDE3A protein expression showed no group difference (AC6mut: 0.3±0.1 vs Con, 0.4±0.1 du, p=0.6. **Fig.2B**).

Intracellular Distribution of AC6mut. AC6mut protein was identified in association with caveolae (mainly associated with plasma membrane), SR, and nuclear envelope (**Fig. 1E**).

Echocardiography. Echocardiography showed that basal cardiac structure and function were unchanged by cardiac-directed expression of AC6mut. LV dimensions were not different between groups, and basal LV ejection fraction and the velocity of circumferential fiber shortening were similar (**Table 2**). Thus, despite marked diminution of LV cAMP generating capacity in AC6mut mice, LV structure and basal function were unaltered.

LV Contractile Function in Response to Isoproterenol. To assess cardiac contractility in a manner independent of autonomic nervous influence, endogenous catecholamines, and anesthesia, LV pressure development was measured in isolated perfused hearts. Basal and isoproterenol-stimulated LV dP/dt showed no group differences (**Fig. 3**), despite marked diminution in LV cAMP generating capacity.

Ca^{2+} Uptake and Ca^{2+} Related Proteins. ATP-dependent SR Ca^{2+} uptake rate in pooled LV homogenates from AC6mut and transgene-negative sibling control mice was

determined. Increased AC6mut expression was associated with increased SR Ca^{2+} uptake (**Fig. 4A, upper panel**). In addition, an increased affinity of SERCA2a for Ca^{2+} was reflected in a reduced Ca^{2+} concentration required for a half maximal effect (EC50: AC6mut 1.1 μmol/L; Control 3.7 μmol/L, n=6, **Fig. 4A, lower panel**).

Associated with these physiological changes in Ca^{2+} handling was altered LV expression of proteins that regulate SR Ca^{2+} uptake. For example, AC6mut expression was associated with a 43% reduction in LV PLB protein expression (p=0.01), and a 73% increase in LV S100A1 protein content (p=0.03) (**Figs. 4B and 4C**). The contents of LV SERCA2a, calreticulin, and calsequestrin were unchanged, and PLB phosphorylation at Ser16 was unchanged (**Fig. 4D**).

Transcription Factors. AC6mut expression was associated with a 2-fold increase in LV expression of CREM-1 (p=0.03, **Fig. 4B**) and a 1.7-fold increase in phosphorylation of CREB at Ser133 (p=0.01, **Fig. 4C**); total CREB protein content was unaltered. To determine whether increased CREM-1 and phospho-CREB were present in the nuclei, immunofluorescence staining of isolated cardiac myocytes was performed using anti-CREM-1 and anti-phospho-CREB (S133) antibodies. We detected increased nuclear localization of CREM-1 and phospho-CREB in AC6mut mice **(Fig. 4E).**

Calcium Transients: To determine whether increased SR Ca^{2+} uptake associated with AC6mut expression would affect cytosolic $[Ca^{2+}]i$, cardiac myocyte real-time $[Ca^{2+}]i$ was assessed using the ratiometric dye Indo-1. Basal Ca^{2+} release during contraction was unchanged (**Fig. 5A**). However, AC6mut expression was associated with increased peak systolic *Ca^{2+} transient* amplitude after isoproterenol stimulation (p=0.0001, **Figs. 5B and 5C**), and time to peak amplitude was decreased (p=0.03, **Fig. 5D**). In addition, time to 50% relaxation (tau) was decreased (p=0.04) in cardiac myocytes from AC6mut mice (**Fig. 5E**). Thus, SERCA2a activity, expression of PLB and S100A1, and isoproterenol-stimulated Ca^{2+} transients all were altered by AC6mut expression in a manner that would favorably influence LV function.

Discussion

The most surprising and important finding of this study is that cardiac-directed expression of a mutant AC6 molecule that markedly impairs βAR-stimulated cAMP production is associated with preserved LV function in response to isoproterenol stimulation. This was confirmed by echocardiography and studies of contractile function

in isolated perfused hearts. Marked diminution of cardiac cAMP generation in other settings is associated with proportional reductions in LV contractile function. For example, most models of heart failure, where cAMP impairment typically is 50% reduced, there is a similar reduction in LV dP/dt and in βAR-responsiveness [10],[11],[12],[13],[14]. Furthermore, deletion of AC6, which is associated with a 60% reduction in cAMP generating capacity, was also associated with a similar reduction in LV contractile function [5]. What then explains preservation of isoproterenol-stimulated LV contractile function?

The proximate mechanisms for preserved LV function despite markedly impaired cAMP generation in the AC6mut line were favorable changes on Ca^{2+} handling. We previously reported that cardiac-directed expression of AC6 increased function of the failing heart, but because of pronounced effects of AC6 on βAR signaling, it was impossible to determine the degree to which these beneficial effects reflected augmented βAR signaling per se vs Ca^{2+} handling [10],[11]. Supporting the link of AC6 to Ca^{2+} handling is the observation that AC6 deletion has striking adverse effects on Ca^{2+} handling [5], but since cAMP-generating capacity was reduced following AC6 deletion, the independent effects of AC6 on Ca^{2+} handling were difficult to ascertain. What is new in the present study, however, is the demonstration in TG mice that an AC6 mutant molecule appears to mimic the parent molecule's favorable effects on Ca^{2+} handling, thereby preserving LV function even whilst cAMP generating capacity is markedly diminished. It appears that the effects of AC6 on Ca^{2+} handling does not require cAMP generation, and must therefore occur through alternative mechanisms.

We found that AC6mut expression is associated with increased SR Ca^{2+} uptake in LV homogenates and increased Ca^{2+} transients with reduced time of relaxation in intact cardiac myocytes. Associated with these physiologically favorable effects of AC6mut expression was reduced PLB expression, a Ca^{2+} regulator that inhibits SERCA2a activity. Reduced PLB content or increased PLB phosphorylation at Ser16 is associated with reduction of its inhibitory effects, which increases SERCA2a activity [20],[21],[22]. We previously found that PLB expression is reduced in cultured cardiac myocytes expressing AC6 or AC6mut [4], but the current study is the first to demonstrate that this effect is also seen in vivo (**Fig. 4B**). Increases in the degree of isoproterenol-stimulated

phosphorylation of RyR2, PLB, and to a lesser extent, TnI (**Fig. 2C**) in cardiac myocytes isolated from AC6mut mice would be predicted also to increase LV contractile function.

AC6mut expression was associated with increased expression and nuclear translocation of CREM-1 (**Figs. 3B and 3E**), a transcriptional suppressor in the CREB/ATF family [23]. We previously identified that, in the setting of AC6 expression, the PLB promoter was negatively regulated by increased ATF3 in neonatal rat cardiac myocytes through the CRE site in the PLB promoter [2]. In the present study we did not see increased ATF3 expression associated with AC6mut expression. However, both ATF3 and CREM-1 recognize the same CRE sites, so it is plausible that the AC6mut-related increased CREM-1 may be mechanistically important in reduced PLB expression. This will require additional study.

AC6mut expression was associated with an unanticipated increase in LV expression of the Ca^{2+} sensitizing protein, S100A1, which increases contractile function through modulation of RyR2 and SERCA2a [24]. How might AC6mut expression be linked with increased LV S100A1 expression? AC6mut expression was associated with increased phosphorylation and nuclear translocation of CREB (**Figs. 4C and 4E**), processes that are required for CREB activation. CREB is a transcriptional activator that regulates many genes through CRE site(s) in their promoters [25]. The S100A1 promoter possesses a CRE site [26], indicating that S100A1 expression could plausibly have been activated by AC6mut expression. In addition, compartmentalization of PKA and cAMP may also be factors [27],[28].

The substantial improvements in Ca^{2+} handling appear to have preserved LV function despite marked diminution in cAMP generation. The precise pathways by which increased amounts of AC6mut influence transcriptional regulation and ultimately the physiological behavior of cardiac myocytes and LV function will require additional studies. Histological studies (**Fig. 1E**) confirm that substantial amounts of transgene AC6mut are present in multiple intracellular compartments, not just in the plasma membrane. This enables AC6mut protein to interact with important intracellular proteins that influence intracellular signaling and thereby affect physiological function.

The importance of AC6 vis-à-vis Ca^{2+} handling was recently underscored by AC6 deletion [5]. In this setting, cAMP generating capacity was reduced, albeit not by as much as in the present study, but Ca^{2+} handling was markedly impaired. In the present study, we see more marked impairment of cAMP generation, but Ca^{2+} handling is increased, not

decreased. This is because, unlike in AC6 deletion, the AC6 molecule, albeit one deficient in cAMP generating capacity, is present in the cytoplasm where it may influence Ca^{2+} handling.

We did not examine transgenic lines that expressed reduced amounts of AC6mut to determine if the physiological effects were proportional to level of AC6mut expression. One could argue that a 17-fold increase in AC6mut protein (vs endogenous AC6) might affect signaling in a non-specific manner. While our data cannot discount this possibility, it is important to recognize that endogenous AC6 is an exceedingly low abundance protein—approximately 100-fold less abundant, for example, than Gsα [29]. Therefore, even expressed at 17-fold higher level than endogenous AC6, it still is considerably less abundant than Gsα. Furthermore, similar increases in the catalytically active (normal) AC6 are associated with marked increases in recruitable cAMP production [30]. These observations suggest that the findings are specific.

Conclusions. Substantial improvements in Ca^{2+} handling appear to preserve LV function despite marked diminution in cAMP generation. Immunofluorescence indicates that AC6mut is located on the nuclear envelope, providing an opportunity for AC6mut to influence transcription factor expression and function. Increased CREM-1, a transcriptional suppressor and increased phospho-CREB (**Fig. 4E**) may be involved in altered expression of PLB and S100A1 respectively. We conclude that AC6mut preserves cardiac function through increased Ca^{2+} handling and altered protein expression, despite reduced cAMP generation. These results provide insight regarding the interplay between Ca^{2+} handling and βAR signaling vis-à-vis LV function, and indicate that AC6mut may provide inotropic stimulation free from the potentially deleterious effects of increased cAMP. Data indicated reduced cardiac myocyte apoptosis associated with AC6mut expression in the failing heart, which is a focus of an ongoing study in our laboratory.

FIGURE LEGENDS

Figure 1. AC6mut Design, Expression, Activity and Cellular Distribution

A. The diagram depicts the site of substitution of alanine (ala) for aspartic acid (asp) at position 426 (position number based on SEQ ID NO:16) in the C1 domain (intracellular loop) in the construction of AC6mut. The substitution inhibits Mg^{2+} binding and alters the efficiency of Gsα-mediated activation of the catalytic core, which impairs the enzymatic activity of AC6, resulting in reduced cAMP production.

M1 and M2, transmembrane domains of AC6; C1 and C2, cytoplasmic domains of AC6, which form the catalytic core; βAR, β-adrenergic receptor; βY and α, components of the guanosine 5'-triphosphate (GTP)-binding protein, Gs

B. AC6mut mRNA expression was assessed by qRT-PCR using primers common to endogenous AC6 and transgene AC6mut. Primers for detecting GAPDH mRNA were used for internal control of the qRT-PCR reaction. AC6mut mRNA was increased 62-fold vs endogenous AC6. Animal number in bars +SE; Student's t-test, unpaired, 2 tails

C. AC6mut protein was detected in immunoblotting using anti-AC5/6 antibody and confirmed using anti-AU1 tag antibody. AC6mut protein was increased 17-fold vs endogenous AC6.

D. Cyclic AMP production in isolated cardiac myocytes from AC6mut and control mice, before (Basal) and after stimulation with isoproterenol (Iso; 10 μM, 10 min) or NKH477 (NKH; 10 μM, 10 min); cAMP Enzymeimmunoassay. Cardiac myocytes from AC6mut mice (M vs C, control) showed impaired cAMP production in response to Iso and NKH477, a forskolin analog. Bars denote mean +SE; p values from 1-way ANOVA followed by Bonferroni post test (n=6, each group).

E. Double immunofluorescence staining of AC6mut protein in cardiac myocytes isolated from AC6mut vs control mice using anti-AU1 antibody (red); anti-caveolin 3 (Cav-3) antibody (green, for caveolae); anti-protein disulphide-isomerase (PDI) antibody (green, for sarcoplasmic reticulum); anti-lamin A antibody (green, for nuclear envelope), and anti-voltage dependent anion selective channel protein (VDAC) antibody (green, for mitochondria). Nucleus is blue. AC6mut transgene was detected in caveolae, SR, and nuclear envelope, but was not associated with mitochondria.

Figure 2. Activities and Expression of PKA, PKS and PDE

A. *Upper Graph:* PKA activity in isolated cardiac myocytes without stimulation (Basal) or stimulated with isoproterenol (Iso; 10 μM, 10 min) or NKH477 (NKH; 10 μM, 10 min). AC6mut expression reduced basal PKA activity (p=0.01) and both Iso (p=0.001) and NKH (p=0.001) activities were reduced as well (n=3, each group). *Lower Gel:* PKA protein in LV homogenates. LV PKA catalytic subunit protein expression was unaltered by AC6mut expression.

B. The expression of key signaling proteins and their phosphorylation are shown in immunoblots using left ventricular homogenates from AC6mut and control mice. No group differences were observed. Shown are phospho (P) and Total (T) PKA regulatory subunits II-α and II-β, PKCα, Phosphodiesterase type 3A (PDE3A), phospho-troponin I (P22/23-TnI), and total TnI.

C. Phosphorylation of RyR2, PLB and TnI before and after isoproterenol stimulation was assessed in cultured cardiac myocytes isolated from each group. Basal phosphorylation of RyR2, PLB and TnI showed no group differences. Isoproterenol stimulation was associated with increased phosphorylation of RyR2, PLB, and TnI in both groups, but was more extensive in cardiac myocytes from AC6mut mice (**Fig. 2C**).

D. The data from **Fig. 2C** indicating that isoproterenol stimulation was associated with increased phosphorylation of RyR2, PLB, and TnI in cardiac myocytes from AC6mut mice are shown in graphic format, normalized for loading (GAPDH). The increase in TnI phosphorylation was not statistically significant (p=0.07).

Figure 3. Left Ventricular Contractile Function

Isolated hearts from AC6mut TG mice (closed circle; n=11) showed preserved LV dP/dt in response to isoproterenol stimulation through a wide range of isoproterenol doses. Data were acquired and analyzed without knowledge of group identity. Open circles, transgene negative control mice (n=12). There was no group difference (2-way ANOVA). Data points denote mean ±SE.

Figure 4. SR Ca^{2+} uptake, Ca^{2+} signaling proteins, and transcriptional factors

A. Upper: Ca^{2+} uptake activity in pooled LV samples from AC6mut and TG negative sibling control mice (n=6, both groups)

Lower: Expression of AC6mut decreased SERCA2a affinity for Ca^{2+}. The effective concentration of Ca^{2+} for 50% maximal effect (EC_{50}) was calculated from the initial ATP-dependent Ca^{2+} uptake rate at different free Ca^{2+} concentrations.

B. Upper: AC6mut expression was associated with decreased LV phospholamban (PLB) expression.

Lower: AC6mut expression was associated with increased LV CREM-1 protein expression.

C. Upper: AC6mut expression was associated with increased LV S100A1 protein expression.

Lower: AC6mut expression was associated with increased LV P133-CREB protein expression. Total CREB expression was similar in both groups.

D. AC6mut expression did not affect LV expression of SERCA2a, calreticulin, calsequestrin or phospho-S16-PLB proteins. (n=4, both groups).

E. Double immunofluorescence staining of AC6mut protein in isolated cardiac myocytes from AC6mut and control mice using anti-AU1 antibody (red) and anti–CREM-1 antibody (green) or anti-AU1 and anti-phospho-CREB (S133, green). Nucleus was showing in blue. AC6mut expression increased nuclear localizations of CREM-1 and phospho-CREB.

In graphs (A,B,C), bars denote mean +SE; numbers in bars indicate group size; members above bars indicate p values from Student's t-test (unpaired, 2 tailed)

Figure 5. Cytosolic Ca^{2+} transients in isolated cardiac myocytes from AC6mut and control mice

A. Basal Ca^{2+} released (systolic-diastolic Ca^{2+}) showed no group difference.

B. Representative Indo-1 Ca^{2+} transient recordings in cardiac myocytes stimulated with isoproterenol (Iso; 10 μM) were higher in cardiac myocytes from AC6mut mice. Summary data are displayed in Panel C.

C. Ca^{2+} released in the presence of isoproterenol was increased in cardiac myocytes from AC6mut mice.

D. Time-to-peak Ca^{2+} transient in the presence of isoproterenol was decreased in cardiac myocytes from AC6mut mice.

E. Time to 50% relaxation (tau) in the presence of isoproterenol was decreased in cardiac myocytes from AC6mut mice.

Experiments were repeated four times. Bars denote mean +SE; numbers in bars indicate number of cardiac myocytes; numbers above bars indicate p values from Student's t-test (unpaired, 2-tailed).

REFERENCES

1. Cleland JG, Teerlink JR, Senior R, Nifontov EM, Mc Murray JJ, et al. (2011) The effects of the cardiac myosin activator, omecamtiv mecarbil, on cardiac function in systolic heart failure: a double-blind, placebo-controlled, crossover, dose-ranging phase 2 trial. Lancet 378: 676-683.

2. Gao, MH, Tang T, Guo T, Sun SQ, Feramisco JR, et al. (2004) Adenylyl cyclase type VI gene transfer reduces phospholamban expression in cardiac myocytes via activating transcription factor 3. J Biol Chem 279: 38797-3802.
3. Gao MH, Tang T, Guo T, Yajima T, Pestonjamasp K, et al. (2008) Adenylyl cyclase type VI increases Akt activity and phospholamban phosphorylation in cardiac myocytes. J Biol Chem 283: 33527-33535.
4. Gao MH, Tang T, Lai NC, Yajima T, Miyanohara A, et al. (2011) Beneficial effects of adenylyl cyclase type 6 (AC6) expression persist using a catalytically inactive AC6 mutant. Mol Pharmacol 79: 381-388.
5. Tang T, Gao MH, Lai NC, Firth AL, Takahashi T, et al. (2008) Adenylyl cyclase type 6 deletion decreases left ventricular function via impaired calcium handling. Circulation. 117: 61-69.
6. Ping P, Anzai T, Gao MH, Hammond HK. (1996) Adenylyl cyclase and G protein receptor kinase expression during development of heart failure. Am J Physiol Heart Circ Physiol 273: H707-717.
7. Roth DM, Bayat H, Drumm JD, Gao MH, Swaney JS, Ander A, et al. (2002) Adenylyl cyclase increases survival in cardiomyopathy. Circulation 105: 1989-1994.
8. Takahashi T, Tang T, Lai NC, Roth DM, Rebolledo B, Saito M, et al. (2006) Increased cardiac adenylyl cyclase expression is associated with increased survival after myocardial infarction. Circulation 114: 388-396.
9. Lai NC, Roth DM, Gao MH, Tang T, Dalton N, et al. (2004) Intracoronary adenovirus encoding adenylyl cyclase VI increases left ventricular function in heart failure. Circulation 110: 330-336.
10. Roth DM, Gao MH, Lai NC, Drumm J, Dalton N, et al. (1999) Cardiac-directed adenylyl cyclase expression improves heart function in murine cardiomyopathy. Circulation 99: 3099-3102.
11. Lai NC, Tang T, Gao MH, Saito M, Takahashi T, et al. (2008) Activation of cardiac adenylyl cyclase expression increases function of the failing ischemic heart in mice. J Am Coll Cardiol 51: 1490-1497.
12. Tang T, Gao MH, Roth DM, Guo T, Hammond HK. (2004) Adenylyl cyclase type VI corrects cardiac sarcoplasmic reticulum calcium uptake defects in cardiomyopathy. Am J Physiol 287: H1906-1912.

13. Gaudin C, Ishikawa Y, Wight DC, Mahdavi V, Nadal-Ginard B, et al. (1995) Overexpression of Gsα protein in the hearts of transgenic mice. J Clin Invest 95: 1676-1683.

14. Engelhardt S, Hein L, Wiesmann F, Lohse MJ. (1999) Progressive hypertrophy and heart failure in β1-adrenergic receptor transgenic mice. Proc Natl Acad Sci 96: 7059-7064.

15. Liggett SB, Tepe NM, Lorenz JN, Canning AM, Jantz TD, et al. (2000) Early and delayed consequences of β2-adrenergic receptor overexpression in mouse hearts: critical role for expression level. Circulation 101: 1707-1714.

16. Antos CL, Frey N, Marx SO, Reiken S, Gaburjakova M, et al. (2001) Dilated cardiomyopathy and sudden death resulting from constitutive activation of protein kinase A. Circ Res 89: 997-1004.

17. Packer M, Carver JR, Rodeheffer RJ, Ivanhoe RJ, Dibianco R, et al. (1991) Effect of oral milrinone on mortality in severe chronic heart failure. The PROMISE Study Research Group. N Engl J Med 325: 1468-1475.

18. Bristow MR, Ginsburg R, Minobe W, Cubicciotti RS, Sageman WS, et al. (1982) Decreased catecholamine sensitivity and beta-adrenergic-receptor density in failing human hearts. N Engl J Med 307: 205-211.

19. Suarez J, Scott B, Dillmann WH. (2008) Conditional increase in SERCA2a protein is able to reverse contractile dysfunction and abnormal calcium flux in established diabetic cardiomyopathy. Am J Physiol 295: R1439-1445.

20. Brittsan AG, Kranias EG. (2000) Phospholamban and cardiac contractile function. J Mol Cell Cardiol 32: 2131-2139.

21. Chu G, Lester JW, Young KB, Luo W, Zhai J, et al. (2000) A single site (Ser16) phosphorylation in phospholamban is sufficient in mediating its maximal cardiac responses to beta-agonists. J Biol Chem 275: 38938-38943.

22. Luo W, Chu G, Sato Y, Zhou Z, Kadambi VJ, et al. (1998) Transgenic approaches to define the functional role of dual site phospholamban phosphorylation. J Biol Chem 273: 4734-4739.

23. Foulkes NS, Borrelli E, Sassone-Corsi P. (1991) CREM gene: use of alternative DNA-binding domains generates multiple antagonists of cAMP-induced transcription. Cell 64: 739-749.

24. Most P, Remppis A, Pleger ST, Löffler E, Ehlermann P, et al. (2003) Transgenic overexpression of the Ca2+ -binding protein S100A1 in the heart leads to increase in vivo myocardial contractile performance. J Biol Chem 278: 33809-33817.

25. Mayr B, Montminy M. (2001) Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol 2: 599-609.

26. Song W, Zimmer DB. (1996) Expression of the rat S100A1 gene in neurons, glia, and skeletal muscle. Brain Res 721: 204-216.

27. Iancu RV, Ramamurthy G, Harvey RD. (2008) Spatial and temporal aspects of cAMP signaling in cardiac myocytes. Clin Exp Pharmacol Physiol 35: 1343-1348.

28. Efendiev R, Samelson BK, Nguyen BT, Phatarpekar PV, Baameur F, et al. (2010) AKAP79 interacts with multiple adenylyl cyclase (AC) isoforms and scaffolds AC5 and AC6 to α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors. J Biol Chem 285: 14450-14458.

29. Ostrom RS, Post SR, Insel PA. (2000). Stoichiometry and compartmentation in G protein-coupled receptor signaling: implications for therapeutic interventions involving Gs. J Pharmacol Exp Ther 294:407-412.

30. Gao MH, Lai NC, Roth DM, Zhou J, Zhu J, Anzai T, Dalton N, Hammond HK. (1999) Adenylylcyclase increases responsiveness to catecholamine stimulation in transgenic mice. Circulation 99:1618-1622.

Table 1. Body, LV, and Lung Weight

	AC6mut (23)	TG- Control (16)	p
Body (g)	25.5±0.7	25.0±1.2	0.7
LV (mg)	91±2.7	89±3.4	0.6
Tibial Length (mm)	17±0.1	16.7±0.2	0.3
LV/Body (mg/g)	3.6±0.1	3.6±0.1	0.9
LV/TL (mg/mm)	5.4±0.1	5.3±0.2	0.7
Lung (mg)	150±4.9	149±6.7	0.9
Lung/Body (mg/g)	6.0±0.2	6.0±0.2	0.9

LV, left ventricle; TL, tibial length. Values represent mean ± SE; Student's *t* test (unpaired, 2-tailed).

Table 2. Echocardiography (Basal)

	AC6mut (8)	TG- Control (12)	p
HR (bpm)	501±26	506±17	0.9
EDD (mm)	4.2±0.2	4.3±0.1	0.7
ESD (mm)	2.9±0.2	3.0±0.1	0.4
PW Thickness (mm)	0.6±0.1	0.6±0.1	0.5
Septal Thickness (mm)	0.6±0.1	0.6±0.1	0.4
EDV (μL)	76±7	78±4	0.8
ESV (μL)	25±4	27±2	0.6
EF (%)	69±3	65±2	0.2
CO (μL/min)	26±2	26±2	0.8
Vcf (circ/sec)	7.0±0.7	6.2±0.3	0.2

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method, or an *in vivo* method for or method of:

(1) treating a subject having or at risk of having a heart disease or a heart failure;

(2) treating, ameliorating, reversing the effects of, protecting or preventing an individual or a patient against:

a heart disease,

a heart failure,

a decrease in heart function or cardiac output,

a decrease in heart function or cardiac output due to a heart infection or a heart condition,

(3) enhancing calcium handling in intact cardiac myocytes by increasing sarcoplasmic reticulum (SR) Ca^{2+} uptake and/or increased Ca^{2+} transients with reduced time of relaxation in intact cardiac myocytes,

(4) inhibiting the generation of intracellular cAMP levels in cardiac myocytes,

(5) protecting a cardiac myocyte from a programmed cell death (apoptosis) signal, or decreasing the number of cardiac myocytes signaled to programmed cell death (apoptosis) subsequent to an apoptotic signal, or

(6) in heart failure patients or in individuals having a heart infection or a heart condition resulting in a decrease in heart function or cardiac output: increasing heart function or cardiac output, reducing symptom and/or decreasing mortality; or reducing the frequency of hospitalizations for heart failure,

comprising:

(a) providing:

(i) a cyclic adenosine monophosphate-incompetent (cAMP-incompetent) adenylyl cyclase type 6 (AC6) protein or polypeptide (also called "an AC6mut"),

wherein optionally the AC6mut is a recombinant, a synthetic, a peptidomimetic or an isolated AC6mut polypeptide or peptide; or

(ii) a AC6mut-encoding nucleic acid or gene:

wherein optionally the AC6mut-encoding nucleic acid or gene is operatively linked to a transcriptional regulatory sequence, wherein optionally the transcriptional regulatory sequence is a promoter and/or an enhancer, or a cardiac cell-specific promoter or a myocyte-specific promoter; or

wherein optionally the AC6mut-encoding nucleic acid or gene is operatively linked to a transcriptional regulatory sequence, and optionally the AC6mut-encoding nucleic acid or gene is contained in a delivery vehicle, a vector, an expression vector, a recombinant virus, or an equivalent, and the delivery vehicle, expression vehicle, vector, recombinant virus, or equivalent can express the AC6mut-encoding nucleic acid or gene in a cell or *in vivo,*

wherein optionally the cell is a cardiac cell or a myocyte;

wherein the AC6mut does not catalyze the breakdown of ATP to cAMP, or has impaired ability to catalyze the breakdown of ATP to cAMP, and optionally the impaired ability to catalyze the breakdown of ATP to cAMP is defined as the AC6mut having only about 1%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% of the ATP to cAMP catalytic activity of wild type AC6,

and when the AC6mut is expressed in a cardiac myocyte *in vivo* left ventricular (LV) function is not affected or does not decrease or LV function is substantially not affected or decreased,

and optionally AC6mut expression in a cardiac myocyte increases sarcoplasmic reticulum Ca^{2+} uptake,

and optionally AC6mut expression in a cardiac myocyte reduces the EC50 for SERCA2a activation,

and optionally AC6mut expression in a cardiac myocyte reduces expression of a phospholamban protein, ,

and optionally the substitution inhibits Mg^{2+} binding and alters the efficiency of Gsα-mediated activation of the catalytic core;

(b) delivering or administering the AC6mut, or the AC6mut-encoding nucleic acid or gene, to a cardiac cell or a cardiac myocyte, or expressing the AC6mut in a cardiac cell or a cardiac myocyte, or expressing the AC6mut-encoding nucleic acid or gene in a cardiac cell or a cardiac myocyte,

wherein optionally the AC6mut-encoding nucleic acid is operatively linked to a transcriptional regulatory sequence, or optionally the delivery vehicle, vector, expression vector, recombinant virus, or equivalent, is delivered or administered to a cardiac myocyte cell, or to an individual or a patient in need thereof,

and optionally the delivering or administering of the AC6mut-encoding nucleic acid or gene to the cardiac cell or myocyte *in vivo* is a targeted delivery to a heart muscle

or a cardiac myocyte, or comprises direct delivery or administration to a heart, or comprises an intracardiac injection or an infusion,

thereby:

treating the subject having or at risk of having a heart disease or a heart failure,

treating, ameliorating or protecting (preventing) an individual or a patient against a heart disease, a heart failure, a decrease in heart function or cardiac output, a decrease in heart function or cardiac output due to a heart infection or a heart condition,

enhancing calcium handling in intact cardiac myocytes by increasing sarcoplasmic reticulum (SR) Ca^{2+} uptake and/or increased Ca^{2+} transients with reduced time of relaxation in intact cardiac myocytes,

inhibiting the generation of intracellular cAMP levels in cardiac myocytes,

protecting a cardiac myocyte from a programmed cell death (apoptosis) signal, or decreasing the number of cardiac myocytes signaled to programmed cell death (apoptosis) subsequent to an apoptotic signal, or

in heart failure patients or in individuals having a heart infection or a heart condition resulting in a decrease in heart function or cardiac output: increasing heart function or cardiac output, reducing symptom and/or decreasing mortality.

2. The method of claim 1, wherein the AC6mut comprises an adenylyl cyclase (AC) polypeptide having a substitution of an uncharged or non-polar amino acid for a charged or an acidic amino acid in the catalytic core of the AC polypeptide,

wherein optionally the uncharged or non-polar amino acid is an alanine (Ala), and optionally the acidic amino acid is an aspartic acid (Asp), or optionally the uncharged or non-polar amino acid is an Ala and the acidic amino acid is an Asp.

3. The method of claim 2, wherein the AC6mut comprises:

a murine adenylyl cyclase (AC) polypeptide having a substitution of an Ala for an Asp at position 426 in the catalytic core of the AC polypeptide based on SEQ ID NO:16, where SEQ ID NO:17 is the polypeptide amino acid sequence after the D => A substitution (SEQ ID NO:16 is the amino acid sequence before the D => A substitution); or

a murine AC6mut polypeptide having a substitution of an alanine, or Ala for an Asp at position 436 in the catalytic core of the AC polypeptide based on SEQ ID NO:11,

where SEQ ID NO:12 is the polypeptide amino acid sequence after the D => A substitution (SEQ ID NO:11 is the amino acid sequence before the D => A substitution).

4. The method of claim 1, wherein the AC6 is a mammalian AC6 polypeptide.

5. The method of claim 4, wherein the AC6 is a human AC6 polypeptide.

6. The method of claim 5, wherein the human AC6 polypeptide comprises a human AC6 polypeptide having a substitution of an Ala for an Asp at position 426 in the catalytic core of the AC polypeptide based on SEQ ID NO:10, where SEQ ID NO:13 is the polypeptide amino acid sequence after the D => A substitution (SEQ ID NO:10 is the amino acid sequence before the D => A substitution).

7. The method of claim 1, wherein:

(a) the AC6mut-encoding nucleic acid or gene is stably inserted into a chromosome of a cell;

(b) the delivery vehicle, vector, expression vector, recombinant virus, or equivalent, is or comprises: an adeno-associated virus (AAV); a recombinant AAV virus or vector; an AAV virion, or an adenovirus vector, or any pseudotype, hybrid or derivative thereof;

(c) the method of (b), wherein the adeno-associated virus (AAV), recombinant AAV virus or vector, AAV virion, or adenovirus vector, is or comprises: an AAV serotype AAV5, AAV6, AAV7, AAV8 or AAV9; a rhesus macaque AAV (AAVrh), or an AAVrh10; or any hybrid or derivative thereof;

(d) the AC6mut -encoding nucleic acid or gene is operatively linked to a regulated or inducible transcriptional regulatory sequence;

(e) the method of (d), wherein the regulated or inducible transcriptional regulatory sequence is a regulated or inducible promoter;

(f) the method of any of (a) to (e), wherein administering the AC6mut -encoding nucleic acid or gene operatively linked to a transcriptional regulatory sequence, or the delivery vehicle, vector, expression vector, recombinant virus, or equivalent, to an individual or a patient in need thereof results in: targeted delivery and expression of the

AC6mut in a cardiac myocyte, or a AC6mut being released into the bloodstream or general circulation; or

(g) the method of any of (a) to (f), wherein a disease, infection or condition responsive to an increased AC6mut level *in vivo* is a cardiac contractile dysfunction; a congestive heart failure (CHF); a cardiac fibrosis; a cardiac myocyte disease; a cardiac myocyte dysfunction or a cardiac myocyte apoptosis.

8. The method of claim 1, wherein:

(a) the AC6mut -encoding nucleic acid or gene operatively linked to the transcriptional regulatory sequence; or the delivery vehicle, vector, expression vector, recombinant virus, or equivalent, is administered or delivered to the individual or a patient in need thereof, by oral administration, by intramuscular (IM) injection, by intravenous (IV) injection, by subcutaneous (SC) injection, by intradermal injection, by intrathecal injection, by intra-arterial (IA) injection, by intracoronary or intracardiac injection, by intraocular injection or application, by inhalation, or by a biolistic particle delivery system, or by using a "gene gun", air pistol or a HELIOS™ gene gun (Bio-Rad Laboratories, Hercules, CA),

wherein optionally the AC6mut -encoding nucleic acid or gene is delivered by intravenous (IV) injection of an AAV vector, or AAV-9 vector; or

(b) the AC6mut -encoding nucleic acid or gene operatively linked to the transcriptional regulatory sequence; or the expression vehicle, vector, recombinant virus, or equivalent, is administered or delivered to the individual or a patient in need thereof, by introduction into any cell, organ, tissue or fluid space within the body that is adjacent to or is drained by the bloodstream, such that the encoded AC6mut protein may be secreted from cells in the tissue and released into the bloodstream.

9. The method of any of claims 1 to 8, wherein:

(a) the individual, patient or subject is administered a stimulus or signal that induces expression of the AC6mut -expressing nucleic acid or gene, or induces or activates a promoter (e.g., a promoter operably linked to the AC6mut -expressing nucleic acid or gene) that induces expression of or up-regulates expression of the AC6mut -expressing nucleic acid or gene;

(b) the individual, patient or subject is administered a stimulus or signal that induces synthesis of an activator of a promoter, wherein optionally the promoter is an AC gene promoter, or a myocyte cell-specific promoter;

(c) the individual, patient or subject is administered a stimulus or signal that induces synthesis of a natural or a synthetic activator of the AC6mut -expressing nucleic acid or gene or the AC6mut -expressing nucleic acid or gene-specific promoter,

wherein optionally the natural activator is an endogenous transcription factor;

(d) the method of (c), wherein the synthetic activator is a zinc-finger DNA binding protein designed to specifically and selectively turn on an endogenous or exogenous target gene, wherein optionally the endogenous target is an AC6mut -expressing nucleic acid or gene or an activator of an AC6mut, or a AC6mut -expressing nucleic acid or gene, or an activator of a promoter operatively linked to a AC6mut -expressing nucleic acid or gene;

(e) the method of any of (a) to (c), wherein the stimulus or signal comprises a biologic, a light, a chemical or a pharmaceutical stimulus or signal;

(f) the individual, patient or subject is administered a stimulus or signal that stimulates or induces expression of a post-transcriptional activator of an AC6mut, or a AC6mut -expressing nucleic acid or gene, or an activator of a promoter operatively linked to a AC6mut -expressing nucleic acid or gene, or

(g) the individual, patient or subject is administered a stimulus or signal that inhibits or induces inhibition of a transcriptional repressor or a post-transcriptional repressor of a AC6-expressing nucleic acid or gene.

10. The method of claim 9, wherein the chemical or pharmaceutical that induces expression of the AC6mut, or the AC6mut -expressing nucleic acid or gene, or induces expression of the regulated or inducible promoter operatively linked to the AC6mut -expressing nucleic acid or gene, is or comprises an oral antibiotic, a doxycycline or a rapamycin; or a tet-regulation system using doxycycline is used to induce expression of the AC6mut, or the AC6mut -expressing nucleic acid or gene, or an equivalent thereof.

11. The method of any of claims 1 to 10, wherein the AC6mut, or the AC6mut -expressing nucleic acid or gene, or the delivery vehicle, vector, expression vector,

recombinant virus, or equivalent, is formulated in or as a lyophilate, a liquid, a gel, a hydrogel, a powder, a spray, an ointment, or an aqueous or a saline formulation.

12. The method of any of claims 1 to 11, wherein the AC6mut, or the AC6mut -expressing nucleic acid or gene or the delivery vehicle, vector, expression vector, recombinant virus, or equivalent, comprises, or is formulated in, a vesicle, a hydrogel, a gel, a liposome, a nanoliposome, a nanoparticle or a nanolipid particle (NLP).

13. The method of any of claims 1 to 11, wherein the AC6mut, or the AC6mut -expressing nucleic acid or gene or the delivery vehicle, vector, expression vector, recombinant virus, or equivalent, is formulated in an isolated or cultured cell, and optionally the cell is a mammalian cell, a cardiac cell, or a human cell, a non-human primate cell, a monkey cell, a mouse cell, a rat cell, a guinea pig cell, a rabbit cell, a hamster cell, a goat cell, a bovine cell, an equine cell, an ovine cell, a canine cell or a feline cell.

14. The method of any of claims 1 to 13, wherein the AC6mut, or the AC6mut -expressing nucleic acid or gene, or the delivery vehicle, vector, expression vector, recombinant virus, or equivalent, is formulated as a pharmaceutical or sterile.

15. The method of any of claims 1 to 14, wherein the AC6mut, or the AC6mut -expressing nucleic acid or gene or the delivery vehicle, vector, expression vector, recombinant virus, or equivalent, is formulated or delivered with, on, or in conjunction with a product of manufacture, an artificial organ or an implant.

16. The method of any of claims 1 to 15, wherein the wherein the AC6mut, or the AC6mut -expressing nucleic acid or gene or the delivery vehicle, vector, expression vector, recombinant virus, or equivalent expresses a AC6mut polypeptide *in vitro* or *ex vivo*.

17. A method for treating, ameliorating, reversing, protecting or preventing an individual or a patient against a AC6mut -responsive pathology, infection, disease, illness, or condition, comprising practicing the method of any of claims 1 to 16.

18. A method for treating, ameliorating, reversing, protecting or preventing a cardiopathy or a cardiovascular disease in an individual or a patient in need thereof, comprising practicing the method of any of claims 1 to 16.

19. The method of claim 18, wherein the cardiopathy or cardiovascular disease comprises: a coronary artery disease (CAD); an atherosclerosis; a thrombosis; a restenosis; a vasculitis, an autoimmune or a viral vasculitis; a polyarteritis nodosa; a Churg-Strass syndrome; a Takayasu's arteritis; a Kawasaki Disease; a Rickettsial vasculitis; an atherosclerotic aneurism; a myocardial hypertrophy; a congenital heart disease (CHD); an ischemic heart disease; an angina; an acquired valvular or an endocardial disease; a primary myocardial disease; a myocarditis; an arrhythmia; a transplant rejection; a metabolic myocardial disease; a myocardiomyopathy; a congestive, a hypertrophic or a restrictive cardiomyopathy; and/or, a heart transplant.

20. Use of:

an AC6mut; an AC6mut -expressing nucleic acid or gene; a delivery vehicle, a vector, an expression vector, a recombinant virus, or equivalent; an adeno-associated virus (AAV); a recombinant AAV virus or vector; or an adenovirus vector, or any pseudotype, hybrid or derivative thereof, as set forth in any of claims 1 to 16,

wherein optionally the AAV or recombinant AAV virus or vector comprises an AAV serotype AAV5, AAV6, AAV7, AAV8 or AAV9; a rhesus macaque AAV (AAVrh), or an AAVrh10; or any hybrid or derivative thereof, or an AC6mut-expressing cell or cardiac myocyte,

in the preparation of a medicament for:

(1) treating a subject having or at risk of having a heart disease or a heart failure;

(2) treating, ameliorating, reversing the effects of, protecting or preventing an individual or a patient against:

a heart disease,

a heart failure,

a decrease in heart function or cardiac output,

a decrease in heart function or cardiac output due to a heart infection or a heart condition,

(3) enhancing calcium handling in intact cardiac myocytes by increasing sarcoplasmic reticulum (SR) Ca^{2+} uptake and/or increased Ca^{2+} transients with reduced time of relaxation in intact cardiac myocytes,

(4) inhibiting the generation of intracellular cAMP levels in cardiac myocytes,

(5) protecting a cardiac myocyte from a programmed cell death (apoptosis) signal, or decreasing the number of cardiac myocytes signaled to programmed cell death (apoptosis) subsequent to an apoptotic signal,

(6) in heart failure patients or in individuals having a heart infection or a heart condition resulting in a decrease in heart function or cardiac output: increasing heart function or cardiac output, reducing symptom and/or decreasing mortality; or reducing the frequency of hospitalizations for heart failure;

(7) a cardiopathy or a cardiovascular disease; or

(8) a coronary artery disease (CAD); an atherosclerosis; a thrombosis; a restenosis; a vasculitis, an autoimmune or a viral vasculitis; a polyarteritis nodosa; a Churg-Strass syndrome; a Takayasu's arteritis; a Kawasaki Disease; a Rickettsial vasculitis; an atherosclerotic aneurism; a myocardial hypertrophy; a congenital heart disease (CHD); an ischemic heart disease; an angina; an acquired valvular or an endocardial disease; a primary myocardial disease; a myocarditis; an arrhythmia; a transplant rejection; a metabolic myocardial disease; a myocardiomyopathy; a congestive, a hypertrophic or a restrictive cardiomyopathy; and/or, a heart transplant.

21. A therapeutic formulation as used or set forth in any of claims 1 to 16, for use in the treatment of or for:

(1) a heart disease, a heart failure, a decrease in heart function or cardiac output, a decrease in heart function or cardiac output due to a heart infection or a heart condition,

(2) enhancing calcium handling in intact cardiac myocytes by increasing sarcoplasmic reticulum (SR) Ca^{2+} uptake and/or increased Ca^{2+} transients with reduced time of relaxation in intact cardiac myocytes,

(3) inhibiting the generation of intracellular cAMP levels in cardiac myocytes,

(4) protecting a cardiac myocyte from a programmed cell death (apoptosis) signal, or decreasing the number of cardiac myocytes signaled to programmed cell death (apoptosis) subsequent to an apoptotic signal,

(5) in heart failure patients or in individuals having a heart infection or a heart condition resulting in a decrease in heart function or cardiac output: increasing heart function or cardiac output, reducing symptom and/or decreasing mortality; or reducing the frequency of hospitalizations for heart failure;

(6) a cardiopathy or a cardiovascular disease; or

(7) a coronary artery disease (CAD); an atherosclerosis; a thrombosis; a restenosis; a vasculitis, an autoimmune or a viral vasculitis; a polyarteritis nodosa; a Churg-Strass syndrome; a Takayasu's arteritis; a Kawasaki Disease; a Rickettsial vasculitis; an atherosclerotic aneurism; a myocardial hypertrophy; a congenital heart disease (CHD); an ischemic heart disease; an angina; an acquired valvular or an endocardial disease; a primary myocardial disease; a myocarditis; an arrhythmia; a transplant rejection; a metabolic myocardial disease; a myocardiomyopathy; a congestive, a hypertrophic or a restrictive cardiomyopathy; and/or, a heart transplant.

Figure 1

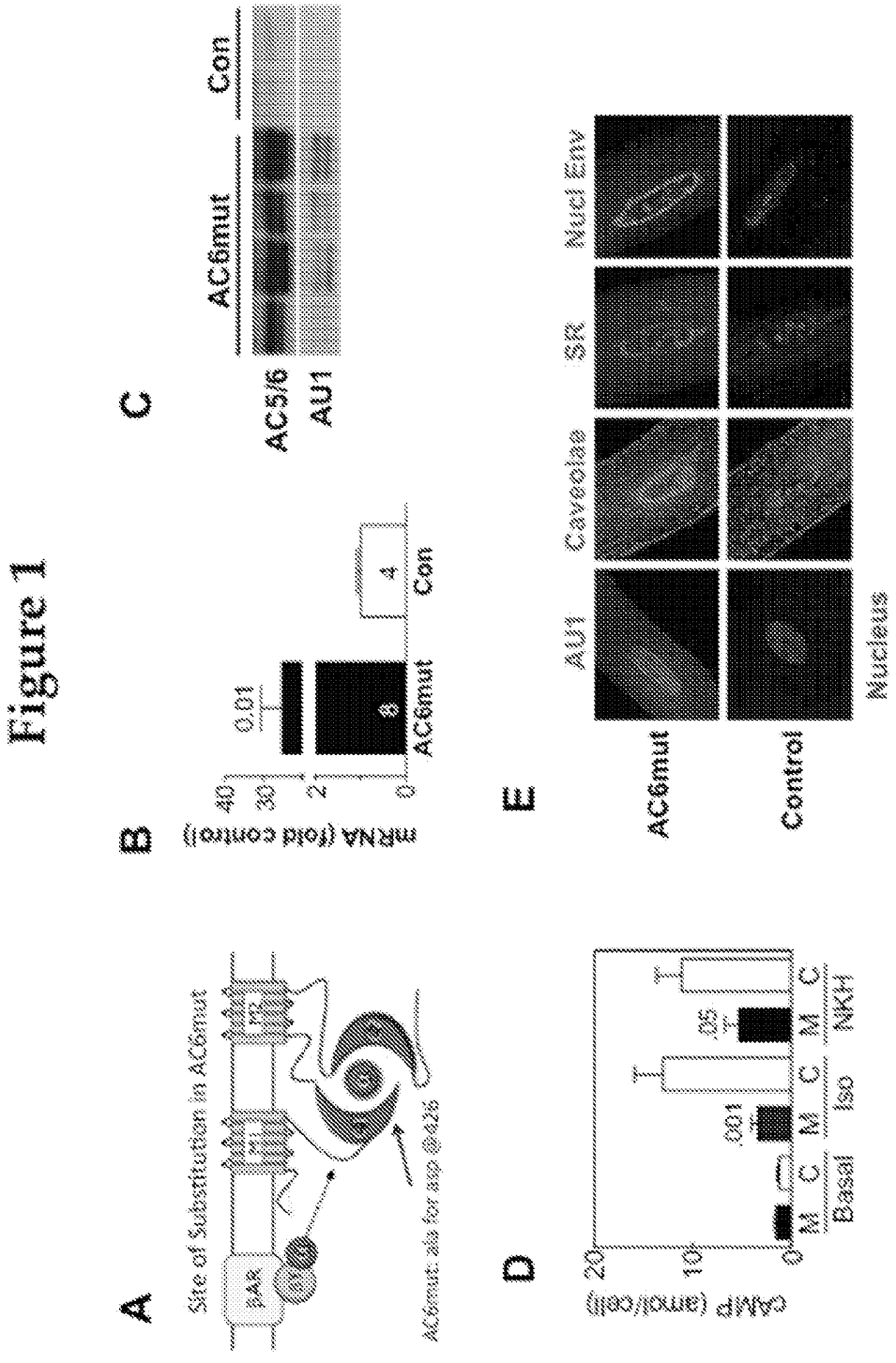

A
Site of Substitution in AC6mut
PAR
C1
C2
R1
R2
AC6mut: ala for asp @426
B
mRNA (fold control)
40
30
2
0
0.01
AC6mut
4
Con
C
AC6mut
Con
AC5/6
AU1
D
cAMP (amol/cell)
20
10
0
M C M C M C
Basal Iso NKH
.001
.05
1
1
E
AU1
Caveolae
SR
Nucl Env
AC6mut
Control
Nucleus

Figure 2

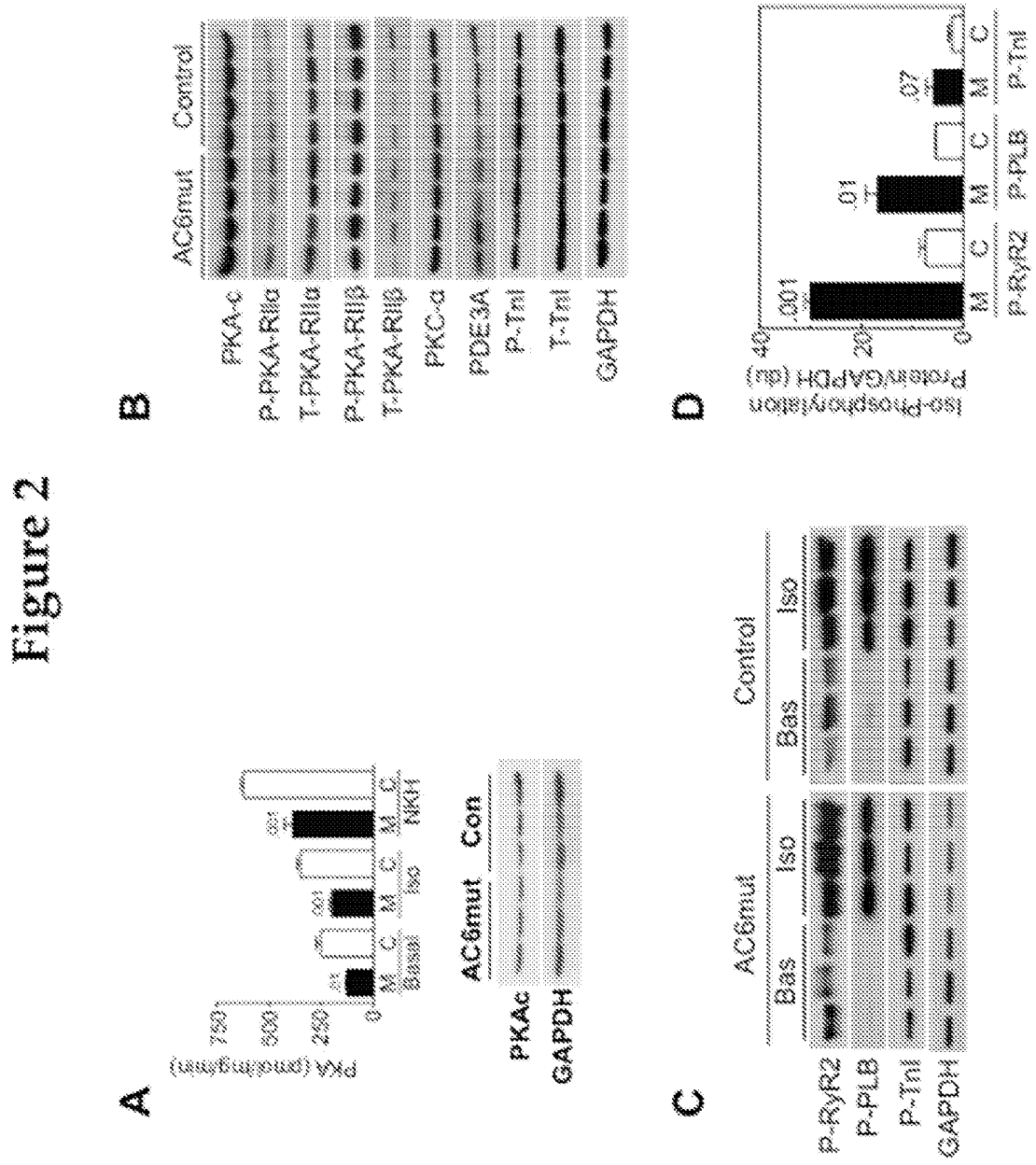

A
PKA (pmol/mg/mn)
750
500
250
0
M C M C M C
Basal Iso NKH
AC6mut Con
PKAc
GAPDH
B
AC6mut Control
PKA-c
P-PKA-RIIα
T-PKA-RIIα
P-PKA-RIIβ
T-PKA-RIIβ
PKC-α
PDE3A
P-TnI
T-TnI
GAPDH
C
AC6mut Control
Bas Iso Bas Iso
P-RyR2
P-PLB
P-TnI
GAPDH
D
Iso-Phosphorylation
Protein/GAPDH (du)
40
20
0
.001
.01
.07
M C M C M C
P-RyR2 P-PLB P-TnI

Figure 3

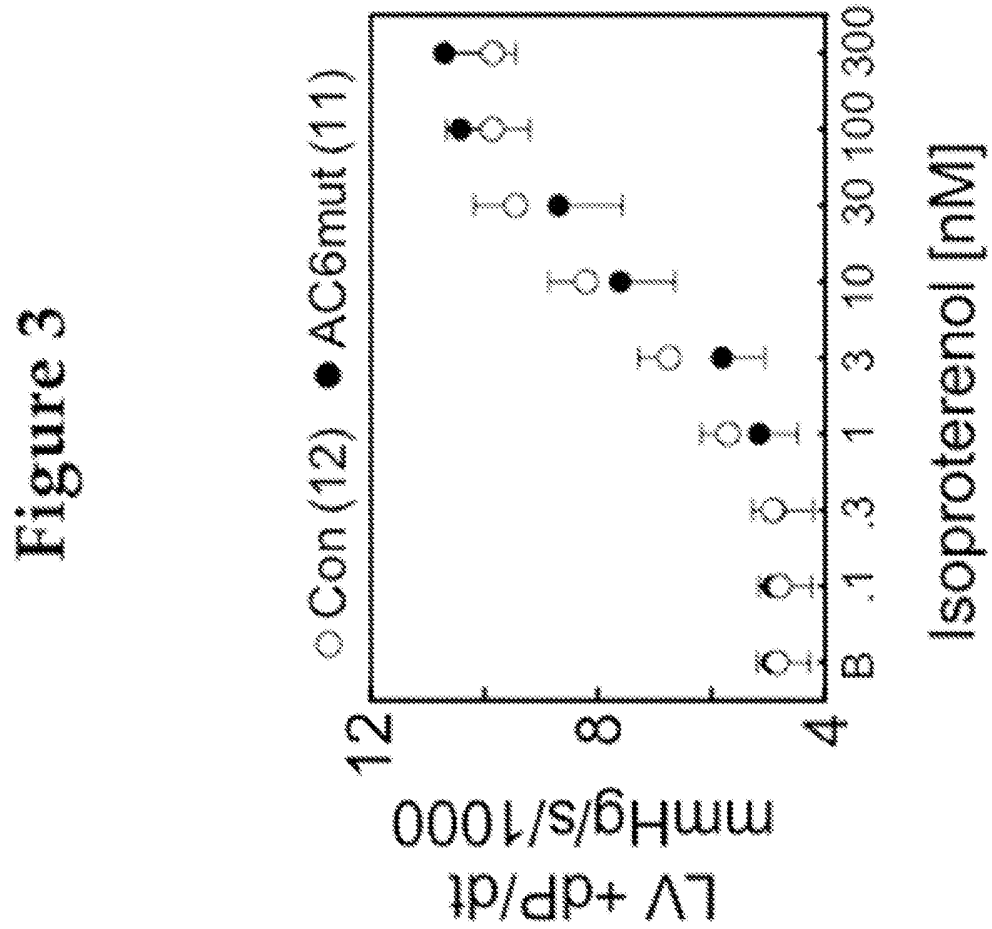

○ Con (12) ● AC6mut (11)
LV +dP/dt
mmHg/s/1000
12
8
4
B .1 .3 1 3 10 30 100 300
Isoproterenol [nM]

Figure 4

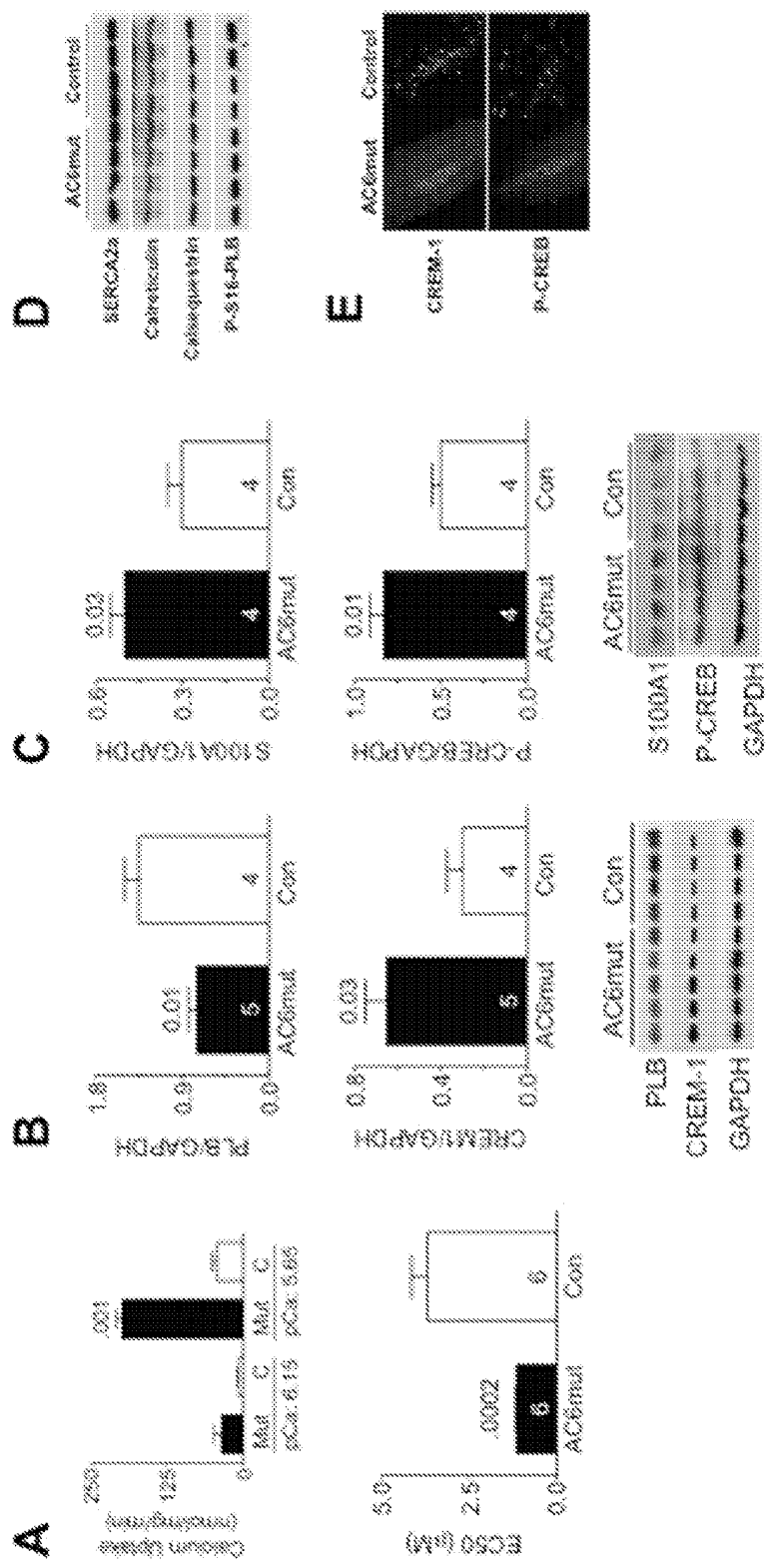
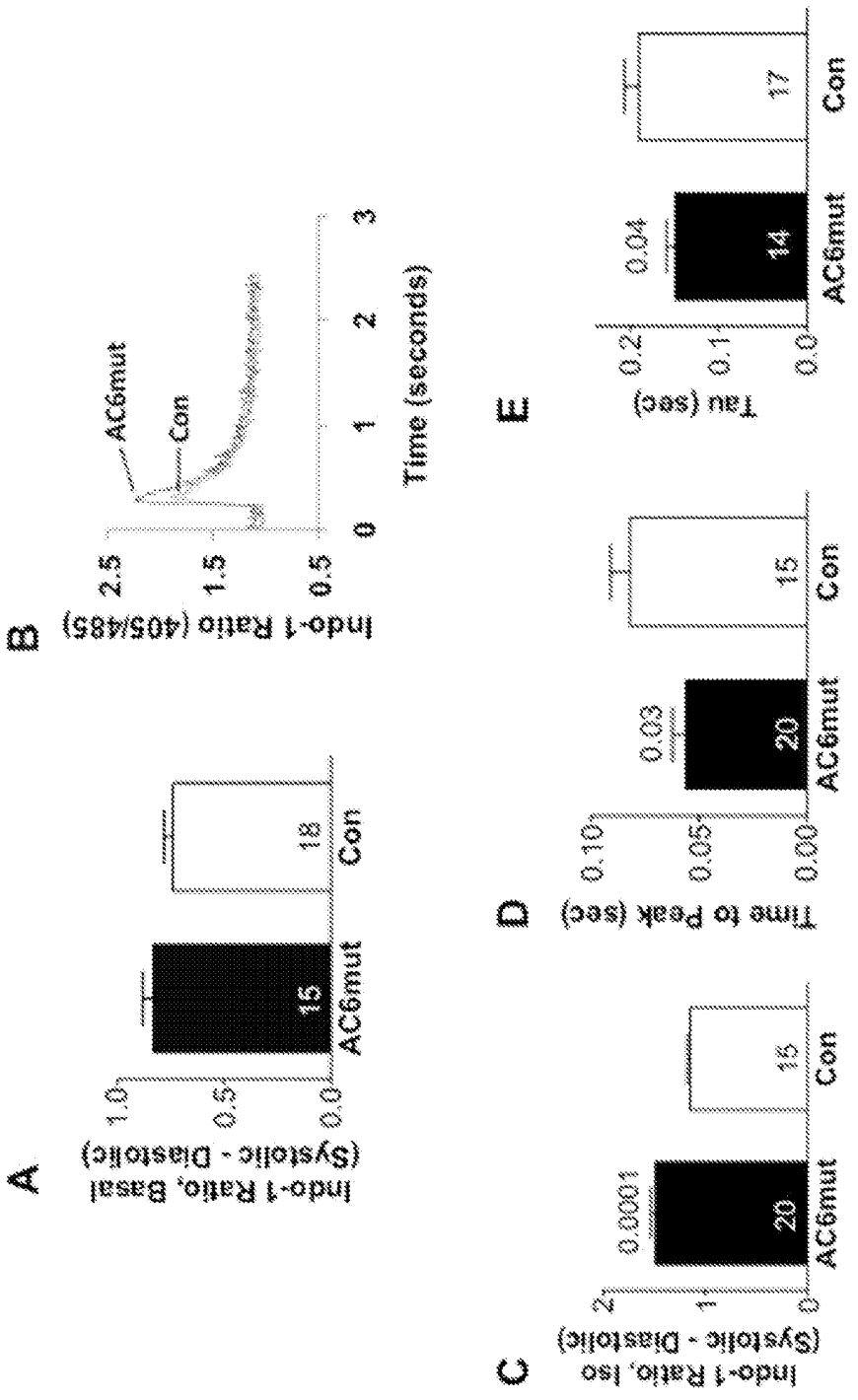

A
Calcium Uptake (nmol/mg/min)
250
125
0
.001
Mut
C
pCa: 6.15
Mut
C
pCa: 6.85
ECS0 (μM)
5.0
2.5
0.0
.0002
6
6
AC6mut
Con
B
PLB/GAPDH
1.8
0.9
0.0
0.01
5
4
AC6mut
Con
CREM/GAPDH
0.8
0.4
0.0
0.03
5
4
AC6mut
Con
AC6mut
Con
PLB
CREM-1
GAPDH
C
S100A1/GAPDH
0.6
0.3
0.0
0.02
4
4
AC6mut
Con
P-CREB/GAPDH
1.0
0.5
0.0
0.01
4
4
AC6mut
Con
AC6mut
Con
S100A1
P-CREB
GAPDH
D
AC6mut
Control
SERCA2a
Calreticulin
Calsequestrin
P-S16-PLB
E
AC6mut
Control
CREM-1
P-CREB

Figure 5


A
Indo-1 Ratio, Basal (Systolic - Diastolic)
1.0
0.5
0.0
15
18
AC6mut
Con
B
Indo-1 Ratio (405/485)
2.5
1.5
0.5
AC6mut
Con
0
1
2
3
Time (seconds)
C
Indo-1 Ratio, Iso (Systolic - Diastolic)
2
1
0
0.0001
20
15
AC6mut
Con
D
Time to Peak (sec)
0.10
0.05
0.00
0.03
20
15
AC6mut
Con
E
Tau (sec)
0.2
0.1
0.0
0.04
14
17
AC6mut
Con

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/040948

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 9/88 (2014.01)
CPC - C12N 9/88 (2014.09)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61K 38/17, 48/00; C12N 5/10, 9/12, 9/88, 15/60 (2014.01)
USPC - 435/232, 375; 514/44R; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - A01K 2217/056; A61K 38/17, 48/00; C12N 5/10, 9/12, 9/88 (2014.09) (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Orbit, Google Patents, Google Scholar

Seach terms used: adenylyl, adenylate, VI, type 6, heart, cardiac, myocardial, mutant, mutation, mutated, mutein, AC6mut, tet, inducible

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SUGANO et al. "Activated expression of cardiac adenylyl cyclase 6 reduces dilation and dysfunction of the pressure-overloaded heart," Biochem Biophys Res Commun. 30 December 2010 (30.12.2010), Vol. 405, No. 3, Pgs. 349-355. entire document	1, 2, 4, 5, 7-10
Y	GAO et al. "Beneficial effects of adenylyl cyclase type 6 (AC6) expression persist using a catalytically inactive AC6 mutant," Mol Pharmacol. 02 December 2010 (02.12.2010), Vol. 79, No. 3, Pgs. 381-388. entire document	1, 2, 4, 5, 7-10
A	TAKAHASHI et al. "Increased cardiac adenylyl cyclase expression is associated with increased survival after myocardial infarction," Circulation, 24 July 2006 (24.07.2006), Vol. 114, No. 5, Pgs. 388-396. entire document	1-10
A	LAI et al. "Activation of cardiac adenylyl cyclase expression increases function of the failing ischemic heart in mice," J Am Coll Cardiol. 15 April 2008 (15.04.2008), Vol. 51, No. 15, Pgs. 1490-1497. entire document	1-10
A	US 2002/0103147 A1 (HAMMOND et al) 01 August 2002 (01.08.2002) entire document	1-10

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
29 October 2014	12 NOV 2014

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/040948

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

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

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

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

3. Additional comments:

SEQ ID NOs: 1-17 were searched.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/040948

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 11-21
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

(19)中华人民共和国国家知识产权局

(12)发明专利申请

(10)申请公布号 CN 105452458 A
(43)申请公布日 2016.03.30

(21)申请号 201480044190.5
(22)申请日 2014.06.04
(30)优先权数据
61/832,759 2013.06.07 US
(85)PCT国际申请进入国家阶段日
2016.02.04
(86)PCT国际申请的申请数据
PCT/US2014/040948 2014.06.04
(87)PCT国际申请的公布数据
WO2014/197624 EN 2014.12.11
(71)申请人 加利福尼亚大学董事会
地址 美国加利福尼亚州
(72)发明人 H·K·哈蒙德 高美华
(74)专利代理机构 北京安信方达知识产权代理有限公司 11262
代理人 陈建芳 阎娴斌
(51)Int.Cl.
C12N 9/88(2006.01)

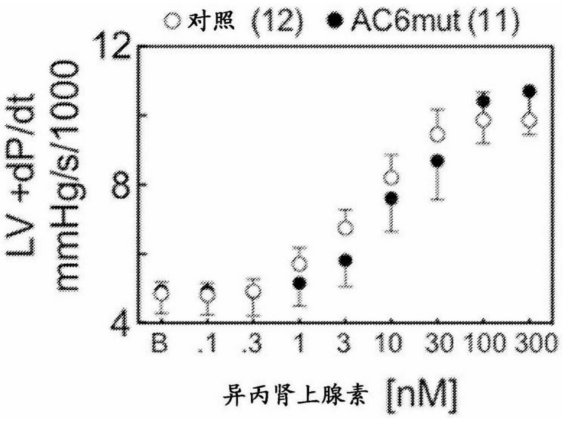
权利要求书6页 说明书37页
序列表29页 附图5页

(54)发明名称

用于治疗心力衰竭和增加心脏功能的不能产生环腺苷酸的腺苷酸环化酶和组合物和方法

(57)摘要

本发明提供用于治疗、改善或保护(预防)患有心脏疾病或心力衰竭或降低的心脏功能或具有患心脏疾病或心力衰竭或降低的心脏功能的风险的个体或患者的方法,其包括:提供不能产生环腺苷酸的(不能产生cAMP的)腺苷酸环化酶6型(AC6)蛋白质或多肽(也被称为"AC6mut")或可操作地连接到转录调控序列的编码AC6mut的核酸或基因。



CN 105452458 A

1.一种用于以下的方法或体内方法或以下的方法：

(1)治疗患有心脏疾病或心力衰竭或具有患心脏疾病或心力衰竭的风险的受试者；

(2)针对以下来治疗、改善、逆转其效应、保护或预防个体或患者：

心脏疾病、

心力衰竭、

心脏功能或心输出量的降低、

由于心脏感染或心脏病况所致的心脏功能或心输出量的降低，

(3)通过增加完整心肌细胞中肌浆网(SR)Ca^{2+}摄取和/或具有缩短的松驰时间的增加的Ca^{2+}瞬变来增强完整心肌细胞中的钙处置，

(4)抑制心肌细胞中细胞内cAMP水平的生成，

(5)保护心肌细胞免受程序化细胞死亡(凋亡)信号影响，或降低在凋亡信号之后被信号传导至程序化细胞死亡(凋亡)的心肌细胞数量，或

(6)在心力衰竭患者中或在患有导致心脏功能或心输出量降低的心脏感染或心脏病况的个体中：增加心脏功能或心输出量，降低症状和/或降低死亡率；或降低心力衰竭的住院频率，

所述方法包括：

(a)提供：

(i)不能产生环腺苷酸的(不能产生cAMP的)腺苷酸环化酶6型(AC6)蛋白质或多肽(也被称为“AC6mut”)，

其中任选地，所述AC6mut是重组的、合成的、拟肽的或分离的AC6mut多肽或肽；或

(ii)编码AC6mut的核酸或基因：

其中任选地，所述编码AC6mut的核酸或基因可操作地连接到转录调控序列，其中任选地，所述转录调控序列是启动子和/或增强子或心细胞特异性启动子或肌细胞特异性启动子；或

其中任选地，所述编码AC6mut的核酸或基因可操作地连接到转录调控序列，且任选地，所述编码AC6mut的核酸或基因包含于递送运载体、载体、表达载体、重组病毒，或等同物中，并且所述递送运载体、表达运载体、载体、重组病毒或等同物可以在细胞中或体内表达所述编码AC6mut的核酸或基因，

其中任选地，所述细胞是心细胞或肌细胞；

其中所述AC6mut不催化ATP分解为cAMP，或具有催化ATP分解为cAMP的受损能力，并且任选地，所述催化ATP分解为cAMP的受损能力被定义为所述AC6mut仅具有野生型AC6的ATP至cAMP的催化活性的约1％、10％、20％、30％、40％、50％、60％、70％、80％、90％或95％，

并且当所述AC6mut在体内心肌细胞中表达时，左心室(LV)功能不受影响或不降低，或LV功能基本上不受影响或基本上不降低，

并且任选地，心肌细胞中的AC6mut表达增加肌浆网Ca^{2+}摄取，

并且任选地，心肌细胞中的AC6mut表达降低用于SERCA2a激活的EC50，

并且任选地，心肌细胞中的AC6mut表达降低受磷蛋白蛋白质的表达，

并且任选地，所述取代抑制Mg^{2+}结合并且改变Gsα介导的催化核心的激活的效率；

(b)向心细胞或心肌细胞递送或施用所述AC6mut或所述编码AC6mut的核酸或基因，或

在心细胞或心肌细胞中表达所述AC6mut，或在心细胞或心肌细胞中表达所述编码AC6mut的核酸或基因，

其中任选地，所述编码AC6mut的核酸可操作地连接到转录调控序列，或任选地，向心肌细胞或向需要其的个体或患者递送或施用所述递送运载体、载体、表达载体、重组病毒或等同物，

并且任选地，向体内所述心细胞或肌细胞递送或施用所述编码AC6mut的核酸或基因是向心肌或心肌细胞的靶向递送，或包括向心脏的直接递送或施用，或包括心内注射或输注，

由此：

治疗所述患有心脏疾病或心力衰竭或具有患心脏疾病或心力衰竭的风险的受试者，

针对心脏疾病、心力衰竭、心脏功能或心输出量的降低、由于心脏感染或心脏病况所致的心脏功能或心输出量的降低治疗、改善或保护(预防)个体或患者，

通过增加完整心肌细胞中肌浆网(SR)Ca^{2+}摄取和/或具有缩短的松驰时间的增加的Ca^{2+}瞬变来增强完整心肌细胞中的钙处置，

抑制心肌细胞中细胞内cAMP水平的生成，

保护心肌细胞免受程序化细胞死亡(凋亡)信号影响，或降低在凋亡信号之后被信号传导至程序化细胞死亡(凋亡)的心肌细胞数量，或

在心力衰竭患者中或在患有导致心脏功能或心输出量降低的心脏感染或心脏病况的个体中：增加心脏功能或心输出量，降低症状和/或降低死亡率。

2.根据权利要求1所述的方法，其中所述AC6mut包含在腺苷酸环化酶(AC)多肽的所述催化核心中具有不带电荷的氨基酸或非极性氨基酸对带电荷的氨基酸或酸性氨基酸的取代的AC多肽，

其中任选地，所述不带电荷的氨基酸或非极性氨基酸是丙氨酸(Ala)，并且任选地，所述酸性氨基酸是天冬氨酸(Asp)，或任选地，所述不带电荷的氨基酸或非极性氨基酸是Ala并且所述酸性氨基酸是Asp。

3.根据权利要求2所述的方法，其中所述AC6mut包含：

在基于SEQ ID NO:16的腺苷酸环化酶(AC)多肽的催化核心中的第426位具有Ala对Asp的取代的鼠AC多肽，其中SEQ ID NO:17是D＝>A取代之后的多肽氨基酸序列(SEQ ID NO:16是D＝>A取代之前的氨基酸序列)；或

在基于SEQ ID NO:11的AC多肽的催化核心中的第436位具有丙氨酸或Ala对Asp的取代的鼠AC6mut多肽，其中SEQ ID NO:12是D＝>A取代之后的多肽氨基酸序列(SEQ ID NO:11是D＝>A取代之前的氨基酸序列)。

4.根据权利要求1所述的方法，其中所述AC6是哺乳动物AC6多肽。

5.根据权利要求4所述的方法，其中所述AC6是人AC6多肽。

6.根据权利要求5所述的方法，其中所述人AC6多肽包含在基于SEQ ID NO:10的腺苷酸环化酶(AC)多肽的催化核心中的第426位具有Ala对Asp的取代的人AC6多肽，其中SEQ ID NO:13是D＝>A取代之后的多肽氨基酸序列(SEQ ID NO:10是D＝>A取代之前的氨基酸序列)。

7.根据权利要求1所述的方法，其中：

(a)将所述编码AC6mut的核酸或基因稳定地插入到细胞的染色体中；

(b)所述递送运载体、载体、表达载体、重组病毒或等同物是或包括:腺相关病毒(AAV);重组AAV病毒或载体;AAV病毒粒子或腺病毒载体或其任何假型、杂合体或衍生物;

(c)(b)的方法,其中所述腺相关病毒(AAV)、重组AAV病毒或载体、AAV病毒粒子或腺病毒载体是或包括:AAV血清型AAV5、AAV6、AAV7、AAV8或AAV9;恒河猴AAV(AAVrh)或AAVrh10;或其任何杂合体或衍生物;

(d)所述编码AC6mut的核酸或基因可操作地连接到受调控的或可诱导的转录调控序列;

(e)(d)的方法,其中所述受调控的或可诱导的转录调控序列是受调控的或可诱导的启动子;

(f)(a)到(e)中任一个的方法,其中向需要其的个体或患者施用可操作地连接到转录调控序列的所述编码AC6mut的核酸或基因或所述递送运载体、载体、表达载体、重组病毒或等同物导致:所述AC6mut在心肌细胞中的靶向递送和表达、或AC6mut被释放到血流或全身循环中;或

(g)(a)到(f)中任一个的方法,其中响应体内增加的AC6mut水平的疾病、感染或病况是心脏收缩功能障碍;充血性心力衰竭(CHF);心脏纤维化;心肌细胞疾病;心肌细胞功能障碍或心肌细胞凋亡。

8.根据权利要求1所述的方法,其中:

(a)通过口服施用、通过肌内(IM)注射、通过静脉内(IV)注射、通过皮下(SC)注射、通过皮内注射、通过鞘内注射、通过动脉内(IA)注射、通过冠状动脉内或心内注射、通过眼内注射或施加、通过吸入或通过生物弹射击粒子递送系统或通过使用“基因枪”、气手枪或HELIOS™基因枪(Bio-Rad Laboratories,Hercules,CA),向需要其的所述个体或患者施用或递送可操作地连接到所述转录调控序列的所述编码AC6mut的核酸或基因;或所述递送运载体、载体、表达载体、重组病毒或等同物,

其中任选地,通过静脉内(IV)注射AAV载体或AAV-9载体来递送所述编码AC6mut的核酸或基因;或

(b)通过引入到身体内邻接血流或被血流排放的任何细胞、器官、组织或流体空间中,向需要其的所述个体或患者施用或递送可操作地连接到所述转录调控序列的所述编码AC6mut的核酸或基因;或所述表达运载体、载体、重组病毒或等同物,使得被编码的AC6mut蛋白质可以从所述组织中的细胞被分泌并释放到所述血流中。

9.根据权利要求1-8中任一项所述的方法,其中:

(a)向所述个体、患者或受试者施用刺激或信号,所述刺激或信号诱导所述表达AC6mut的核酸或基因的表达,或诱导或激活诱导或上调所述表达AC6mut的核酸或基因的表达的启动子(例如,可操作地连接到所述表达AC6mut的核酸或基因的启动子);

(b)向所述个体、患者或受试者施用刺激或信号,所述刺激或信号诱导启动子的激活子的合成,其中任选地,所述启动子是AC基因启动子或肌细胞细胞特异性启动子;

(c)向所述个体、患者或受试者施用刺激或信号,所述刺激或信号诱导所述表达AC6mut的核酸或基因或所述表达AC6mut的核酸或基因特异性启动子的天然激活子或合成激活子的合成,

其中任选地,所述天然激活子是内源性转录因子;

(d)(c)的方法,其中所述合成激活子是被设计成特异性地且选择性地开启内源性或外源性靶基因的锌指DNA结合蛋白质,其中任选地,所述内源性靶标是表达AC6mut的核酸或基因、或AC6mut或表达AC6mut的核酸或基因的激活子、或可操作地连接到表达AC6mut的核酸或基因的启动子的激活子;

(e)(a)到(c)中任一个的方法,其中所述刺激或信号包括生物剂刺激或信号、光刺激或信号、化学剂刺激或信号或药物刺激或信号;

(f)向所述个体、患者或受试者施用刺激或信号,所述刺激或信号刺激或诱导AC6mut或表达AC6mut的核酸或基因的转录后激活子的表达、或可操作地连接到表达AC6mut的核酸或基因的启动子的激活子的表达,或

(g)向所述个体、患者或受试者施用刺激或信号,所述刺激或信号抑制表达AC6的核酸或基因的转录阻遏物或转录后阻遏物、或诱导对表达AC6的核酸或基因的转录阻遏物或转录后阻遏物的抑制。

10.根据权利要求9所述的方法,其中诱导所述AC6mut或所述表达AC6mut的核酸或基因的表达、或诱导可操作地连接到所述表达AC6mut的核酸或基因的所述受调控的或可诱导的启动子的表达的所述化学剂或药物是或包括口服抗生素、强力霉素或雷帕霉素;或使用利用强力霉素的tet-调控系统来诱导所述AC6mut或所述表达AC6mut的核酸或基因或其等同物的表达。

11.根据权利要求1-10中任一项所述的方法,其中所述AC6mut或所述表达AC6mut的核酸或基因或所述递送运载体、载体、表达载体、重组病毒或等同物被配制在冻干制剂、液体、凝胶、水凝胶、粉末、喷剂、软膏、或水性或盐水制剂中,或被配制为冻干制剂、液体、凝胶、水凝胶、粉末、喷剂、软膏、或水性或盐水制剂。

12.根据权利要求1-11中任一项所述的方法,其中所述AC6mut或所述表达AC6mut的核酸或基因或所述递送运载体、载体、表达载体、重组病毒或等同物包括囊泡、水凝胶、凝胶、脂质体、纳米脂质体、纳米粒子或纳米脂质粒子(NLP)或被配制在囊泡、水凝胶、凝胶、脂质体、纳米脂质体、纳米粒子或纳米脂质粒子(NLP)。

13.根据权利要求1-11中任一项所述的方法,其中所述AC6mut或所述表达AC6mut的核酸或基因或所述递送运载体、载体、表达载体、重组病毒或等同物被配制在分离的细胞或培养的细胞中,并且任选地,所述细胞是哺乳动物细胞、心细胞、或人细胞、非人灵长类动物细胞、猴细胞、小鼠细胞、大鼠细胞、豚鼠细胞、兔细胞、仓鼠细胞、山羊细胞、牛科动物细胞、马科动物细胞、绵羊细胞、犬科动物细胞或猫科动物细胞。

14.根据权利要求1-13中任一项所述的方法,其中所述AC6mut或所述表达AC6mut的核酸或基因或所述递送运载体、载体、表达载体、重组病毒或等同物被配制为药物或无菌的。

15.根据权利要求1-14中任一项所述的方法,其中所述AC6mut或所述表达AC6mut的核酸或基因或所述递送运载体、载体、表达载体、重组病毒或等同物与制品、人工器官或植入物被配制或递送,在制品、人工器官或植入物上被配制或递送,或结合制品、人工器官或植入物被配制或递送。

16.根据权利要求1-15中任一项所述的方法,其中所述AC6mut或所述表达AC6mut的核酸或基因或所述递送运载体、载体、表达载体、重组病毒或等同物在体外或离体表达AC6mut多肽。

17.一种用于针对AC6mut响应性病理、感染、疾病、病患或病况治疗、改善、逆转、保护或预防个体或患者的方法，其包括实施根据权利要求1-16中任一项所述的方法。

18.一种用于治疗、改善、逆转、保护或预防需要其的个体或患者的心脏病变或心血管疾病的方法，其包括实施根据权利要求1-16中任一项所述的方法。

19.根据权利要求18所述的方法，其中所述心脏病变或心血管疾病包括：冠状动脉疾病(CAD)；动脉粥样硬化；血栓形成；再狭窄；血管炎、自身免疫性血管炎或病毒性血管炎；结节性多动脉炎；变应性肉芽肿血管炎；高安动脉炎；川崎病；立克次体血管炎；动脉粥样硬化性动脉瘤；心肌肥大；先天性心脏疾病(CHD)；缺血性心脏疾病；绞痛；获得性瓣膜疾病或心内膜疾病；原发性心肌病；心肌炎；心律不齐；移植排斥；代谢性心肌病；心肌病变；充血性心肌病变、肥大性心肌病变或限制性心肌病变；和/或心脏移植。

20.如权利要求1-16中任一项所述的AC6mut；表达AC6mut的核酸或基因；递送运载体、载体、表达载体、重组病毒或等同物；腺相关病毒(AAV)；重组AAV病毒或载体；或腺病毒载体，或其任何假型、杂合体或衍生物，

其中任选地，所述AAV或重组AAV病毒或载体包括AAV血清型AAV5、AAV6、AAV7、AAV8或AAV9；恒河猴AAV(AAVrh)或AAVrh10；或其任何杂合体或衍生物、或表达AC6mut的细胞或心肌细胞，

在制备用于以下目的的药物中的用途：

(1)治疗患有心脏疾病或心力衰竭或具有患心脏疾病或心力衰竭的风险的受试者；

(2)针对以下来治疗、改善、逆转其效应、保护或预防个体或患者：

心脏疾病、

心力衰竭、

心脏功能或心输出量的降低、

由于心脏感染或心脏病况所致的心脏功能或心输出量的降低，

(3)通过增加完整心肌细胞中肌浆网(SR)Ca^{2+}摄取和/或具有缩短的松驰时间的增加的Ca^{2+}瞬变来增强完整心肌细胞中的钙处置，

(4)抑制心肌细胞中细胞内cAMP水平的生成，

(5)保护心肌细胞免受程序化细胞死亡(凋亡)信号影响，或降低在凋亡信号之后被信号传导至程序化细胞死亡(凋亡)的心肌细胞数量，

(6)在心力衰竭患者中或在患有导致心脏功能或心输出量降低的心脏感染或心脏病况的个体中：增加心脏功能或心输出量，降低症状和/或降低死亡率；或降低心力衰竭的住院频率；

(7)心脏病变或心血管疾病；或

(8)冠状动脉疾病(CAD)；动脉粥样硬化；血栓形成；再狭窄；血管炎、自身免疫性血管炎或病毒性血管炎；结节性多动脉炎；变应性肉芽肿血管炎；高安动脉炎；川崎病；立克次体血管炎；动脉粥样硬化性动脉瘤；心肌肥大；先天性心脏疾病(CHD)；缺血性心脏疾病；绞痛；获得性瓣膜疾病或心内膜疾病；原发性心肌病；心肌炎；心律不齐；移植排斥；代谢性心肌病；心肌病变；充血性心肌病变、肥大性心肌病变或限制性心肌病变；和/或心脏移植。

21.如权利要求1-16中任一项所用的或所述的治疗制剂，其用于治疗以下疾病或用于以下目的：

(1)心脏疾病、心力衰竭、心脏功能或心输出量的降低、由于心脏感染或心脏病况所致的心脏功能或心输出量的降低，

(2)通过增加完整心肌细胞中肌浆网(SR)Ca^{2+}摄取和/或具有缩短的松驰时间的增加的Ca^{2+}瞬变来增强完整心肌细胞中的钙处置，

(3)抑制心肌细胞中细胞内cAMP水平的生成，

(4)保护心肌细胞免受程序化细胞死亡(凋亡)信号影响，或降低在凋亡信号之后被信号传导至程序化细胞死亡(凋亡)的心肌细胞数量，

(5)在心力衰竭患者中或在患有导致心脏功能或心输出量降低的心脏感染或心脏病况的个体中：增加心脏功能或心输出量，降低症状和/或降低死亡率；或降低心力衰竭的住院频率；

(6)心脏病变或心血管疾病；或

(7)冠状动脉疾病(CAD)；动脉粥样硬化；血栓形成；再狭窄；血管炎、自身免疫性血管炎或病毒性血管炎；结节性多动脉炎；变应性肉芽肿血管炎；高安动脉炎；川崎病；立克次体血管炎；动脉粥样硬化性动脉瘤；心肌肥大；先天性心脏疾病(CHD)；缺血性心脏疾病；绞痛；获得性瓣膜疾病或心内膜疾病；原发性心肌病；心肌炎；心律不齐；移植排斥；代谢性心肌病；心肌病变；充血性心肌病变、肥大性心肌病变或限制性心肌病变；和/或心脏移植。

用于治疗心力衰竭和增加心脏功能的不能产生环腺苷酸的腺苷酸环化酶和组合物和方法

[0001] 相关申请

[0002] 本专利合作条约(PCT)国际申请根据35U.S.C.§119(e)要求2013年6月7日提交的美国临时申请号61/832,759的优先权权益。上述申请的全文出于所有目的以引用方式明确并入本文中。

技术领域

[0003] 本发明大体上涉及细胞和分子生物学、基因疗法和医学;且更具体地涉及通过施用不能产生环腺苷酸的(不能产生cAMP的)腺苷酸环化酶6型(AC6)蛋白质或多肽(也被称为“AC6mut”)或编码AC6mut的核酸序列治疗患有心力衰竭或心脏疾病或具有患心力衰竭或心脏疾病的风险的受试者的组合物方法。

背景技术

[0004] 腺苷酸环化酶(心肌细胞和其它细胞中的跨膜蛋白质)是通过生成细胞内cAMP转导p-肾上腺素能信号传导的关键效应分子。环-AMP是包括蛋白质激酶A激活在内的下游事件的第二信使。心力衰竭与受损的cAMP产生相关,cAMP产生与心脏功能紧密关联。已显示增加的心AC 6型(AC6)(在哺乳动物心肌细胞中表达的显性AC异型体)对衰竭的左心室(LV)具有多种有益作用。这些包括:1)在心肌病变和急性心肌梗塞中的延长存活、2)与AV阻滞降低相关的减少的动作电位时程和房室传导的易化、3)LV扩张和病理性肥大两者的减小、4)通过改善的SERCA2a活性、增加的受磷蛋白活性对钙处置的有效作用和5)增加的心肌肌钙蛋白I磷酸化。

[0005] 因此,已经生成了增加细胞内cAMP水平的若干药物,并且已经在患有心力衰竭的患者中对其进行了测试。然而,这些药物通常增加死亡率。当前的看法认定增加细胞内cAMP水平的药物和蛋白质对衰竭的心脏有害,且因此不适于治疗心力衰竭。

发明内容

[0006] 在替代性实施方案中,本发明提供针对心脏疾病或降低的心脏功能治疗、改善或保护(预防)个体或患者的方法,其包括:提供不能产生环腺苷酸的(不能产生cAMP的)腺苷酸环化酶6型(AC6)蛋白质或多肽(也被称为“AC6mut”)或可操作地连接到转录调控序列的编码AC6mut的核酸或基因;或其中含有编码AC6mut的核酸或基因的表达运载体、载体、重组病毒或等同物,并且所述表达运载体、载体、重组病毒或等同物可以在细胞中或体内表达所述编码AC6mut的核酸或基因;和向需要其的个体或患者施用或递送所述AC6mut或所述可操作地连接到转录调控序列的编码AC6mut的核酸或基因或所述表达运载体、载体、重组病毒或等同物,由此针对所述心脏疾病或降低的心脏功能治疗、改善或保护(预防)所述个体或患者。在替代性实施方案中,所述AC6mut包含在腺苷酸环化酶(AC)多肽的催化核心中具有不带电荷的氨基酸或非极性氨基酸对带电荷的氨基酸或酸性氨基酸的取代的AC多肽。

[0007] 在替代性实施方案中，本发明提供用于以下的方法和体内方法或以下的方法：

[0008] (1)治疗患有心脏疾病或心力衰竭或具有患心脏疾病或心力衰竭的风险的受试者；

[0009] (2)针对以下来治疗、改善、逆转其效应、保护或预防个体或患者：

[0010] 心脏疾病、

[0011] 心力衰竭、

[0012] 心脏功能或心输出量的降低、

[0013] 由于心脏感染或心脏病况所致的心脏功能或心输出量的降低，

[0014] (3)通过增加完整心肌细胞中肌浆网(SR)Ca^{2+}摄取和/或具有缩短的松弛时间的增加的Ca^{2+}瞬变来增强完整心肌细胞中的钙处置，

[0015] (4)抑制心肌细胞中细胞内cAMP水平的生成，

[0016] (5)保护心肌细胞免受程序化细胞死亡(凋亡)信号影响，或降低在凋亡信号之后被信号传导至程序化细胞死亡(凋亡)的心肌细胞数量，或

[0017] (6)在心力衰竭患者中或在患有导致心脏功能或心输出量降低的心脏感染或心脏病况的个体中：增加心脏功能或心输出量，降低症状和/或降低死亡率；或降低心力衰竭的住院频率，

[0018] 所述方法包括：

[0019] (a)提供：

[0020] (i)不能产生环腺苷酸的(不能产生cAMP的)腺苷酸环化酶6型(AC6)蛋白质或多肽(也被称为“AC6mut”)，

[0021] 其中任选地，所述AC6mut是重组的、合成的、拟肽的或分离的AC6mut多肽或肽；或

[0022] (ii)编码AC6mut的核酸或基因：

[0023] 其中任选地，所述编码AC6mut的核酸或基因可操作地连接到转录调控序列，其中任选地，所述转录调控序列是启动子和/或增强子或心细胞特异性启动子或肌细胞特异性启动子；或

[0024] 其中任选地，所述编码AC6mut的核酸或基因可操作地连接到转录调控序列，且任选地，所述编码AC6mut的核酸或基因被包含于递送运载体、载体、表达载体、重组病毒或等同物中，并且所述递送运载体、表达运载体、载体、重组病毒或等同物可以在细胞中或体内表达所述编码AC6mut的核酸或基因，

[0025] 其中任选地，所述细胞是心细胞或肌细胞；

[0026] 其中所述AC6mut不催化ATP分解为cAMP，或具有催化ATP分解为cAMP的受损能力，并且任选地，所述催化ATP分解为cAMP的受损能力被定义为所述AC6mut仅具有野生型AC6的ATP至cAMP的催化活性的约1％、10％、20％、30％、40％、50％、60％、70％、80％、90％或95％，

[0027] 并且当所述AC6mut在体内心肌细胞中表达时，左心室(LV)功能不受影响或不降低，或LV功能基本上不受影响或基本上不降低，

[0028] 并且任选地，心肌细胞中的AC6mut表达增加肌浆网Ca^{2+}摄取，

[0029] 并且任选地，心肌细胞中的AC6mut表达降低用于SERCA2a激活的EC50，

[0030] 并且任选地，心肌细胞中的AC6mut表达降低受磷蛋白蛋白质的表达，

[0031] 并且任选地，所述取代抑制Mg^{2+}结合并且改变Gsα介导的催化核心的激活的效率；

[0032] (b)向心细胞或心肌细胞递送或施用所述AC6mut或所述编码AC6mut的核酸或基因，或在心细胞或心肌细胞中表达所述AC6mut，或在心细胞或心肌细胞中表达所述编码AC6mut的核酸或基因，

[0033] 其中任选地，所述编码AC6mut的核酸可操作地连接到转录调控序列，或任选地，向心肌细胞或向需要其的个体或患者递送或施用所述递送运载体、载体、表达载体、重组病毒或等同物，

[0034] 并且任选地，向体内心细胞或肌细胞递送或施用所述编码AC6mut的核酸或基因是向心肌或心肌细胞的靶向递送，或包括向心脏的直接递送或施用，或包括心内注射或输注，

[0035] 由此：

[0036] 治疗所述患有心脏疾病或心力衰竭或具有患心脏疾病或心力衰竭的风险的受试者，

[0037] 针对心脏疾病、心力衰竭、心脏功能或心输出量的降低、由于心脏感染或心脏病况所致的心脏功能或心输出量的降低治疗、改善或保护(预防)个体或患者，

[0038] 通过增加完整心肌细胞中肌浆网(SR)Ca^{2+}摄取和/或具有缩短的松驰时间的增加的Ca^{2+}瞬变来增强完整心肌细胞中的钙处置，

[0039] 抑制心肌细胞中细胞内cAMP水平的生成，

[0040] 保护心肌细胞免受程序化细胞死亡(凋亡)信号影响，或降低在凋亡信号之后被信号传导至程序化细胞死亡(凋亡)的心肌细胞数量，或

[0041] 在心力衰竭患者中或在患有导致心脏功能或心输出量降低的心脏感染或心脏病况的个体中：增加心脏功能或心输出量，降低症状和/或降低死亡率。

[0042] 在替代性实施方案中，所述AC6mut包含在腺苷酸环化酶(AC)多肽的催化核心中具有不带电荷的氨基酸或非极性氨基酸对带电荷的氨基酸或酸性氨基酸的取代的AC多肽，

[0043] 其中任选地，所述不带电荷的氨基酸或非极性氨基酸是丙氨酸(Ala)，并且任选地，所述酸性氨基酸是天冬氨酸(Asp)，或任选地，所述不带电荷的氨基酸或非极性氨基酸是Ala并且所述酸性氨基酸是Asp。

[0044] 在替代性实施方案中，所述AC6mut包含：

[0045] 在基于SEQ ID NO:16的腺苷酸环化酶(AC)多肽的催化核心中的第426位具有Ala对Asp的取代的鼠AC多肽，其中SEQ ID NO:17是D＝>A取代之后的多肽氨基酸序列(SEQ ID NO:16是D＝>A取代之前的氨基酸序列)；或

[0046] 在基于SEQ ID NO:11的AC多肽的催化核心中的第436位具有丙氨酸或Ala对Asp的取代的鼠AC6mut多肽，其中SEQ ID NO:12是D＝>A取代之后的多肽氨基酸序列(SEQ ID NO:11是D＝>A取代之前的氨基酸序列)。

[0047] 在替代性实施方案中，所述AC6是哺乳动物AC6多肽，或所述AC6是人AC6多肽。在替代性实施方案中，所述人AC6多肽包含在基于SEQ ID NO:10的AC多肽的催化核心中的第426位具有Ala对Asp的取代的人AC6多肽，其中SEQ ID NO:13是D＝>A取代之后的多肽氨基酸序列(SEQ ID NO:10是D＝>A取代之前的氨基酸序列)。

[0048] 在所述方法的替代性实施方案中：

[0049] (a)将所述编码AC6mut的核酸或基因稳定地插入到细胞的染色体中；

[0050] (b)所述递送运载体、载体、表达载体、重组病毒或等同物是或包括:腺相关病毒(AAV);重组AAV病毒或载体;AAV病毒粒子或腺病毒载体或其任何假型、杂合体或衍生物;

[0051] (c)(b)的方法,其中所述腺相关病毒(AAV)、重组AAV病毒或载体、AAV病毒粒子或腺病毒载体是或包括:AAV血清型AAV5、AAV6、AAV7、AAV8或AAV9;恒河猴AAV(AAVrh)或AAVrh10;或其任何杂合体或衍生物;

[0052] (d)所述编码AC6mut的核酸或基因可操作地连接到受调控的或可诱导的转录调控序列;

[0053] (e)(d)的方法,其中所述受调控的或可诱导的转录调控序列是受调控的或可诱导的启动子;

[0054] (f)(a)到(e)中任一个的方法,其中向需要其的个体或患者施用可操作地连接到转录调控序列的所述编码AC6mut的核酸或基因或所述递送运载体、载体、表达载体、重组病毒或等同物导致:所述AC6mut在心肌细胞中的靶向递送和表达、或AC6mut被释放到血流或全身循环中;或

[0055] (g)(a)到(f)中任一个的方法,其中响应体内增加的AC6mut水平的疾病、感染或病况是心脏收缩功能障碍;充血性心力衰竭(CHF);心脏纤维化;心肌细胞疾病;心肌细胞功能障碍或心肌细胞凋亡。

[0056] 在所述方法的替代性实施方案中:

[0057] (a)通过口服施用、通过肌内(IM)注射、通过静脉内(IV)注射、通过皮下(SC)注射、通过皮内注射、通过鞘内注射、通过动脉内(IA)注射、通过冠状动脉内或心内注射、通过眼内注射或施加、通过吸入或通过生物弹射击(biolistic)粒子递送系统或通过使用“基因枪”、气手枪或HELIOS™基因枪(Bio-Rad Laboratories,Hercules,CA),向需要其的所述个体或患者施用或递送可操作地连接到所述转录调控序列的所述编码AC6mut的核酸或基因;或所述递送运载体、载体、表达载体、重组病毒或等同物,

[0058] 其中任选地,通过静脉内(IV)注射AAV载体或AAV-9载体来递送所述编码AC6mut的核酸或基因;或

[0059] (b)通过引入到身体内邻接血流或被血流排放的任何细胞、器官、组织或流体空间中,向需要其的所述个体或患者施用或递送可操作地连接到所述转录调控序列的所述编码AC6mut的核酸或基因;或所述表达运载体、载体、重组病毒或等同物,使得被编码的AC6mut蛋白质可以从所述组织中的细胞被分泌并释放到所述血流中。

[0060] 在所述方法的替代性实施方案中:

[0061] (a)向所述个体、患者或受试者施用刺激或信号,所述刺激或信号诱导所述表达AC6mut的核酸或基因的表达或诱导或激活(诱导或上调所述表达AC6mut的核酸或基因的表达的)启动子(例如,可操作地连接到所述表达AC6mut的核酸或基因的启动子);

[0062] (b)向所述个体、患者或受试者施用刺激或信号,所述刺激或信号诱导启动子的激活子的合成,其中任选地,所述启动子是AC基因启动子或肌细胞细胞特异性启动子;

[0063] (c)向所述个体、患者或受试者施用刺激或信号,所述刺激或信号诱导所述表达AC6mut的核酸或基因或所述表达AC6mut的核酸或基因特异性启动子的天然激活子或合成激活子的合成,

[0064] 其中任选地,所述天然激活子是内源性转录因子;

[0065] (d)(c)的方法，其中所述合成激活子是被设计成特异性地且选择性地开启内源性或外源性靶基因的锌指DNA结合蛋白质，其中任选地，所述内源性靶标是表达AC6mut的核酸或基因、或AC6mut或表达AC6mut的核酸或基因的激活子、或可操作地连接到表达AC6mut的核酸或基因的启动子的激活子；

[0066] (e)(a)到(c)中任一个的方法，其中所述刺激或信号包括生物剂刺激或信号、光刺激或信号、化学剂刺激或信号或药物刺激或信号；

[0067] (f)向所述个体、患者或受试者施用刺激或信号，所述刺激或信号刺激或诱导AC6mut或表达AC6mut的核酸或基因的转录后激活子的表达、或可操作地连接到表达AC6mut的核酸或基因的启动子的激活子的表达，或

[0068] (g)向所述个体、患者或受试者施用刺激或信号，所述刺激或信号抑制表达AC6的核酸或基因的转录阻遏物或转录后阻遏物、或诱导对表达AC6的核酸或基因的转录阻遏物或转录后阻遏物的抑制。

[0069] 在替代性实施方案中：诱导所述AC6mut或所述表达AC6mut的核酸或基因的表达、或诱导可操作地连接到所述表达AC6mut的核酸或基因的所述受调控的或可诱导的启动子的表达的所述化学剂或药物是或包括口服抗生素、强力霉素(doxycycline)或雷帕霉素(rapamycin)；或使用利用强力霉素的tet-调控系统来诱导所述AC6mut或所述表达AC6mut的核酸或基因或其等同物的表达。

[0070] 在替代性实施方案中：所述AC6mut或所述表达AC6mut的核酸或基因或所述递送运载体、载体、表达载体、重组病毒或等同物被配制在冻干制剂(lyophilate)、液体、凝胶、水凝胶、粉末、喷剂、软膏、或水性或盐水制剂中，或被配制为冻干制剂、液体、凝胶、水凝胶、粉末、喷剂、软膏、或水性或盐水制剂。

[0071] 在替代性实施方案中：所述AC6mut或所述表达AC6mut的核酸或基因或所述递送运载体、载体、表达载体、重组病毒或等同物包括囊泡、水凝胶、凝胶、脂质体、纳米脂质体、纳米粒子或纳米脂质粒子(NLP)或被配制在囊泡、水凝胶、凝胶、脂质体、纳米脂质体、纳米粒子或纳米脂质粒子(NLP)中。

[0072] 在替代性实施方案中：所述AC6mut或所述表达AC6mut的核酸或基因或所述递送运载体、载体、表达载体、重组病毒或等同物被配制在分离的细胞或培养的细胞中，并且任选地，所述细胞是哺乳动物细胞、心细胞、或人细胞、非人灵长类动物细胞、猴细胞、小鼠细胞、大鼠细胞、豚鼠细胞、兔细胞、仓鼠细胞、山羊细胞、牛科动物细胞、马科动物细胞、绵羊细胞、犬科动物细胞或猫科动物细胞。

[0073] 在替代性实施方案中：所述AC6mut或所述表达AC6mut的核酸或基因或所述递送运载体、载体、表达载体、重组病毒或等同物被配制为药物或无菌的。

[0074] 在替代性实施方案中：所述AC6mut或所述表达AC6mut的核酸或基因或所述递送运载体、载体、表达载体、重组病毒或等同物与制品、人工器官或植入物被配制或递送，在制品、人工器官或植入物上被配制或递送，或结合制品、人工器官或植入物被配制或递送。

[0075] 在替代性实施方案中：所述AC6mut或所述表达AC6mut的核酸或基因或所述递送运载体、载体、表达载体、重组病毒或等同物在体外或离体表达AC6mut多肽。

[0076] 在替代性实施方案中，本发明提供用于针对AC6mut响应性病理、感染、疾病、病患或病况治疗、改善、逆转、保护或预防个体或患者的方法，其包括实施本发明的方法。

[0077] 在替代性实施方案中，本发明提供用于在需要其的个体或患者中治疗、改善、逆转、保护或预防心脏病变或心血管疾病的方法，其包括实施本发明的方法。在替代性实施方案中，所述心脏病变或心血管疾病包括：冠状动脉疾病(CAD)；动脉粥样硬化；血栓形成；再狭窄；血管炎、自身免疫性血管炎或病毒性血管炎；结节性多动脉炎；变应性肉芽肿血管炎(Churg-Strass syndrome)；高安动脉炎(Takayasu's arteritis)；川崎病(Kawasaki Disease)；立克次体血管炎(Rickettsial vasculitis)；动脉粥样硬化性动脉瘤；心肌肥大；先天性心脏疾病(CHD)；缺血性心脏疾病；绞痛；获得性瓣膜疾病或心内膜疾病；原发性心肌病；心肌炎；心律不齐；移植排斥；代谢性心肌病；心肌病变(myocardiomyopathy)；充血性心肌病变、肥大性心肌病变或限制性心肌病变；和/或心脏移植。

[0078] 在替代性实施方案中，本发明提供包括以下：

[0079] 如权利要求1-16中任一项所述的AC6mut；表达AC6mut的核酸或基因；递送运载体、载体、表达载体、重组病毒或等同物；腺相关病毒(AAV)；重组AAV病毒或载体；或腺病毒载体或其任何假型、杂合体或衍生物，

[0080] 其中任选地，所述AAV或重组AAV病毒或载体包括AAV血清型AAV5、AAV6、AAV7、AAV8或AAV9；恒河猴AAV(AAVrh)或AAVrh10；或其任何杂合体或衍生物、或表达AC6mut的细胞或心肌细胞，

[0081] 在制备用于以下目的的药物中的用途：

[0082] (1)治疗患有心脏疾病或心力衰竭或具有患心脏疾病或心力衰竭的风险的受试者；

[0083] (2)针对以下来治疗、改善、逆转其效应、保护或预防个体或患者：

[0084] 心脏疾病、

[0085] 心力衰竭、

[0086] 心脏功能或心输出量的降低、

[0087] 由于心脏感染或心脏病况所致的心脏功能或心输出量的降低，

[0088] (3)通过增加完整心肌细胞中肌浆网(SR)Ca^{2+}摄取和/或具有缩短的松弛时间的增加的Ca^{2+}瞬变来增强完整心肌细胞中的钙处置，

[0089] (4)抑制心肌细胞中细胞内cAMP水平的生成，

[0090] (5)保护心肌细胞免受程序化细胞死亡(凋亡)信号影响，或降低在凋亡信号之后被信号传导至程序化细胞死亡(凋亡)的心肌细胞数量，

[0091] (6)在心力衰竭患者中或在患有导致心脏功能或心输出量降低的心脏感染或心脏病况的个体中：增加心脏功能或心输出量，降低症状和/或降低死亡率；或降低心力衰竭的住院频率；

[0092] (7)心脏病变或心血管疾病；或

[0093] (8)冠状动脉疾病(CAD)；动脉粥样硬化；血栓形成；再狭窄；血管炎、自身免疫性血管炎或病毒性血管炎；结节性多动脉炎；变应性肉芽肿血管炎；高安动脉炎；川崎病；立克次体血管炎；动脉粥样硬化性动脉瘤；心肌肥大；先天性心脏疾病(CHD)；缺血性心脏疾病；绞痛；获得性瓣膜疾病或心内膜疾病；原发性心肌病；心肌炎；心律不齐；移植排斥；代谢性心肌病；心肌病变；充血性心肌病变、肥大性心肌病变或限制性心肌病变；和/或心脏移植。

[0094] 在替代性实施方案中，如本文所用的或所述的或如本发明的任何方法中的治疗制

剂,其用于治疗以下疾病或用于以下目的:

[0095] (1)心脏疾病、心力衰竭、心脏功能或心输出量的降低、由于心脏感染或心脏病况所致的心脏功能或心输出量的降低,

[0096] (2)通过增加完整心肌细胞中肌浆网(SR)Ca^{2+}摄取和/或具有缩短的松弛时间的增加的Ca^{2+}瞬变来增强完整心肌细胞中的钙处置,

[0097] (3)抑制心肌细胞中细胞内cAMP水平的生成,

[0098] (4)保护心肌细胞免受程序化细胞死亡(凋亡)信号影响,或降低在凋亡信号之后被信号传导至程序化细胞死亡(凋亡)的心肌细胞数量,

[0099] (5)在心力衰竭患者中或在患有导致心脏功能或心输出量降低的心脏感染或心脏病况的个体中:增加心脏功能或心输出量,降低症状和/或降低死亡率;或降低心力衰竭的住院频率;

[0100] (6)心脏病变或心血管疾病;或

[0101] (7)冠状动脉疾病(CAD);动脉粥样硬化;血栓形成;再狭窄;血管炎、自身免疫性血管炎或病毒性血管炎;结节性多动脉炎;变应性肉芽肿血管炎;高安动脉炎;川崎病;立克次体血管炎;动脉粥样硬化性动脉瘤;心肌肥大;先天性心脏疾病(CHD);缺血性心脏疾病;绞痛;获得性瓣膜疾病或心内膜疾病;原发性心肌病;心肌炎;心律不齐;移植排斥;代谢性心肌病;心肌病变;充血性心肌病变、肥大性心肌病变或限制性心肌病变;和/或心脏移植。

[0102] 附图和下面的说明书中陈述了本发明的一个或多个实施方案的细节。根据说明书和附图并且根据权利要求书将显而易见本发明的其它特征、目标和优点。

[0103] 本文所引用的所有出版物/公开、专利、专利申请在此出于所有目的均以引用方式明确地并入。

附图说明

[0104] 图1说明本发明的示例性AC6mut的设计、表达、活性和细胞分布:

[0105] 图1A示意性地说明描绘在本发明的示例性鼠AC6mut的构造中在C1结构域(细胞内环)中在第426位(位置编号基于SEQ ID NO:17,其中SEQ ID NO:16是D=>A取代之前的序列)丙氨酸(ala)对天冬氨酸(asp)的取代(D=>A取代)位点的图解;

[0106] 图1B图解说明如通过qRT-PCR使用内源性AC6和转基因AC6mut共同的引物所评估的AC6mut mRNA表达;

[0107] 图1C说明使用抗AC5/6抗体检测AC6mut蛋白质并且使用抗AU1标签抗体确认的免疫印迹;

[0108] 图1D图解说明,用异丙肾上腺素刺激之前(基线)和刺激之后,如通过cAMP酶免疫测定所测量的AC6mut小鼠和对照小鼠分离的心肌细胞中的环AMP产生;

[0109] 图1E说明使用抗AU1抗体(红色);抗小窝蛋白3(Cav-3)抗体(绿色,对于细胞质膜微囊来说);抗蛋白质二硫键异构酶(PDI)抗体(绿色,对于肌浆网来说);抗核纤层蛋白A抗体(绿色,对于核膜来说)和抗电压依赖性阴离子选择性通道蛋白质(VDAC)抗体(绿色,对于线粒体来说)对从AC6mut小鼠和对照小鼠分离的心肌细胞中的AC6mut蛋白质的双重免疫荧光染色;细胞核是蓝色;

[0110] 如下文的实施例1中所详细论述。

[0111] 图2说明PKA、PKS和PDE的活性和表达：

[0112] 图2A的上图图解说明没有刺激(基线)或用异丙肾上腺素或NKH477刺激的分离的心肌细胞中的PKA活性水平；并且图2A的下图说明显示左心室(LV)均质物中的PKA蛋白质的凝胶免疫印迹；

[0113] 图2B说明使用来自AC6mut小鼠和对照小鼠的左心室均质物显示关键信号传导蛋白质的磷酸化的免疫印迹；显示了磷酸化(P)PKA和总(T)PKA调控亚单位II-α和II-β、PKCα、磷酸-二酯酶3A型(PDE3A)、磷酸化肌钙蛋白I(P22/23-TnI)和总TnI；

[0114] 图2C说明显示在从各组分离的培养心肌细胞中评估异丙肾上腺素刺激之前和之后的RyR2、PLB和TnI的磷酸化的免疫印迹；

[0115] 图2D图解说明来自图2C的数据，其表明AC6mut小鼠中的异丙肾上腺素刺激与心肌细胞中的RyR2、PLB和TnI的增加的磷酸化相关；数据针对载荷(GAPDH)被归一化；

[0116] 如下文的实施例1中所详细论述。

[0117] 图3图解说明左心室收缩功能：从AC6mut TG小鼠(实心圆)分离的心脏显示响应通过宽范围的异丙肾上腺素剂量进行的异丙肾上腺素刺激的保持的LV dP/dt；空心圆表示转基因阴性对照小鼠；如下文的实施例1中所详细论述。

[0118] 图4说明SR Ca^{2+}摄取、Ca^{2+}信号传导蛋白质和转录因子：

[0119] 图4A的上图图解说明来自AC6mut小鼠和TG阴性同胞对照小鼠的混合LV样品中的Ca^{2+}摄取活性；并且图4A的下图图解说明AC6mut的表达降低了SERCA2a对Ca^{2+}的亲和力；

[0120] 图4B的上图图解说明AC6mut表达与降低的LV受磷蛋白(PLB)表达相关；并且图4B的下图图解说明AC6mut表达与增加的LV CREM-1蛋白质表达相关；并且图4B的下图说明显示蛋白质水平的凝胶的免疫印迹；数据针对载荷(GAPDH)被归一化；

[0121] 图4C的上图图解说明AC6mut表达与增加的LV S100A1蛋白质表达相关；并且图4C的下图图解说明AC6mut表达与增加的LV P133-CREB蛋白质表达相关；并且图4C的下图说明显示蛋白质水平的凝胶的免疫印迹；数据针对载荷(GAPDH)被归一化；

[0122] 图4D说明显示AC6mut表达不影响SERCA2a、钙网织蛋白、肌集钙蛋白或磷酸化-S16-PLB蛋白质的LV表达的凝胶的免疫印迹；

[0123] 图4E说明使用抗AU1抗体(红色)和抗CREM-1抗体(绿色)或抗AU1和抗磷酸化CREB(S133，绿色)对从AC6mut小鼠和对照小鼠分离的心肌细胞中的AC6mut蛋白质的双重免疫荧光染色；细胞核以蓝色显示；

[0124] 如下文的实施例1中所详细论述。

[0125] 图5说明从AC6mut小鼠和对照小鼠分离的心肌细胞中的细胞溶质Ca^{2+}瞬变：

[0126] 图5A图解说明显示释放的基线Ca^{2+}(心脏收缩-心脏舒张Ca^{2+})未显示AC6mut与对照之间的组差异的数据；

[0127] 图5B图解说明显示用异丙肾上腺素刺激的心肌细胞中的代表性Indo-1Ca^{2+}瞬变记录在来自AC6mut小鼠的心肌细胞中更高的数据；总结数据展示于图5C中；

[0128] 图5C图解说明显示在异丙肾上腺素存在下释放的Ca^{2+}在来自AC6mut小鼠的心肌细胞中增加的数据；

[0129] 图5D图解说明显示在异丙肾上腺素存在下达到峰值Ca^{2+}瞬变的时间在来自AC6mut小鼠的心肌细胞中减少的数据；

[0130] 图5E图解说明显示在异丙肾上腺素存在下达到50％松弛的时间(τ)在来自AC6mut小鼠的心肌细胞中减少的数据;如下文的实施例1中所详细论述。

[0131] 各图中相同的附图标记指示相同的元件。

具体实施方式

[0132] 本发明提供组合物以及体内和离体方法,其包括施用不能产生环腺苷酸的(不能产生cAMP的)腺苷酸环化酶6型(AC6)蛋白质或多肽(也被称为“AC6mut”)或编码AC6mut的核酸或基因以治疗、改善或保护(作为预防(preventative或prophylaxis))患有心脏疾病、降低的心脏功能或输出量或心脏感染或病况的个体,所述心脏感染或病况响应降低的cAMP、增加的肌浆网(SR)Ca^{2+}摄取和/或体内完整心肌细胞中具有缩短的松弛时间的增加的Ca^{2+}瞬变。

[0133] 在替代性实施方案中,本发明提供抑制细胞内cAMP或显著降低细胞内cAMP的量或不催化产生细胞内cAMP的AC6mut。在替代性实施方案中,本发明的AC6mut以如下方式改变细胞内信号传导:1)增强完整心肌细胞中的钙处置、2)抑制心肌细胞中细胞内cAMP水平的生成和3)保护心肌细胞免于程序化细胞死亡(凋亡)。在替代性实施方案中,当AC6mut在患者的衰竭心脏中被表达或被递送到患者的衰竭心脏中时,心脏功能增加,症状减少并且死亡率降低。因此,本发明的AC6mut递送到心脏中增加心脏功能,而没有由于cAMP生成所致的有害效应。因此,在替代性实施方案中,本发明提供用于心力衰竭和降低的心脏功能的理想疗法。

[0134] 在替代性实施方案中,本发明提供用于编码AC6mut的核酸或基因、或包含(其中含有)编码AC6mut的核酸或基因的表达运载体(例如,载体、重组病毒等)的递送和表达(例如,受控表达)的组合物和方法,所述递送和表达使得AC6mut蛋白质在心肌细胞中被选择性地表达或仅被递送到心肌细胞中,或供选择地,被释放到血流或全身循环中,其中所述AC6mut蛋白质可以对身体中的例如如在治疗心血管疾病的情况下的心脏具有有益作用。

[0135] 在替代性实施方案中,本发明提供用于编码AC6mut的核酸或基因的体内表达的递送运载体、载体、表达载体、重组病毒等以实施本发明的方法。在替代性实施方案中,表达AC6mut的递送运载体、载体、表达载体、重组病毒等或所述AC6mut核酸或基因可以例如在门诊患者在例如诊所就诊期间通过肌内(IM)注射、直接注射到心脏中、通过静脉内(IV)注射、通过皮下注射、通过吸入、通过生物弹射击粒子递送系统(例如,被称为的“基因枪”)等被递送。

[0136] 在替代性实施方案中,编码AC6mut的核酸或基因(例如,包括带有它们作为“有效载荷”的递送运载体(例如,脂质体)、载体、表达载体、重组病毒等)被靶向肌细胞、心肌细胞或直接递送到心肌细胞中用于不能产生cAMP的AC的定向表达或在靶心脏器官中直接表达。

[0137] 在替代性实施方案中,这种“外周”递送模式,例如,递送运载体、载体、表达载体、重组病毒等被IM或IV注射,可以避免当基因或核酸在器官(例如,心脏、肺或肾脏)自身中被直接表达时所遇到的问题。期望的AC6mut蛋白质或递送运载体、载体、表达载体、重组病毒等在血流或全身循环中的持续分泌也避免了通过输注施用蛋白质、递送运载体、载体、表达载体、重组病毒等的困难和代价,所述施用对于在身体内展现非常短的半衰期的许多蛋白质、递送运载体、载体、表达载体、重组病毒等来说可能特别成问题。

[0138] 在替代性实施方案中，本发明提供能够针对定制治疗和保证最佳安全性而容易且有效地开启和关闭表达AC6mut的核酸或基因表达的方法。

[0139] 在替代性实施方案中，即使与它们的一个或多个作用位点相隔一定距离(例如，解剖学上的远端)而被分泌到血液或全身循环中，AC6mut蛋白质或由表达AC6mut的核酸或基因表达的蛋白质也对组织或器官(例如，心脏、血管、肺、肾脏或其它靶标)具有有益的或有利的作用(例如，治疗作用或预防作用)。

[0140] 在本发明的示例性实施方案中，使用表达AC6mut的核酸或编码不能产生cAMP的AC的基因来实施本发明的方法，包括但不限于例如治疗心脏疾病、心力衰竭、充血性心力衰竭(CHF)、心输出量或功能的任何降低或其任何组合。

[0141] 例如，在替代性实施方案中，递送运载体、载体、表达载体、重组病毒等(例如，长期病毒或病毒载体)可以在例如门诊患者在例如医生的办公室中例如在全身性静脉(例如，IV)中或通过肌内(IM)注射、通过吸入或通过生物弹射击粒子递送系统(例如，被称为的“基因枪”)被注射。在替代性实施方案中，数天或数周后(例如，4周后)，向所述个体、患者或受试者施用(例如，吸入、被注射或吞咽)诱导表达AC6mut的核酸或基因的表达的化学剂或药物；例如，每天一次(或更加频繁或更不频繁)施用口服抗生素(例如，强力霉素或雷帕霉素)，这将激活所述基因的表达。在替代性实施方案中，在所述核酸或基因的“激活”或诱导表达(例如，通过可诱导启动子)之后，AC6mut蛋白质被合成并释放到受试者的循环中(例如，进入血液中)，且随后具有有益于个体或患者(例如，有益于心脏功能)的有利生理作用，例如，治疗作用或预防作用。当医生或受试者期望终止治疗时，受试者仅仅停止服用激活的化学剂或药物，例如，抗生素。

[0142] 在替代性实施方案中，本发明的应用包括：治疗重度低射血分数心力衰竭；治疗肺动脉高压；治疗射血分数正常性心力衰竭；替代需要住院和持续静脉内输注用于治疗肺动脉高压和心力衰竭的血管活性肽的现行疗法；和治疗其它病况，其中AC6mut或AC6mut核酸或基因的受控表达促进在身体内的有利作用。

[0143] 生成和操作核酸

[0144] 在替代性实施方案中，为了实施本发明的方法，本发明提供编码AC6mut多肽的被分离的核酸或基因、合成的核酸或基因和/或重组的核酸或基因。在替代性实施方案中，为了实施本发明的方法，本发明以重组形式在(例如，被剪接成的)用于体内表达的(例如，被剪接成的)表达运载体中，例如，在载体(例如，AAV或其任何假型、杂合体或衍生物)或重组病毒中提供表达AC6mut的核酸或基因。

[0145] 在替代性实施方案中，哺乳动物(例如，人或鼠)AC6mut可以被用于实施本发明，其中所述AC6mut包含在腺苷酸环化酶(AC)多肽的催化核心中具有不带电荷的氨基酸或非极性氨基酸对带电荷的氨基酸或酸性氨基酸的取代的AC多肽。人AC6多肽(SEQ ID NO:10)的催化核心(也被称为催化区域1(C1))是氨基酸残基307到675。鼠AC6多肽(SEQ ID NO:11)的催化核心是氨基酸残基315到683。

[0146] 在替代性实施方案中，所述不带电荷的氨基酸或非极性氨基酸是丙氨酸(Ala)，并且任选地，所述酸性氨基酸是天冬氨酸(Asp)，或任选地，所述不带电荷的氨基酸或非极性氨基酸是Ala并且所述酸性氨基酸是Asp。

[0147] 在替代性实施方案中，本发明提供(鼠)AC6mut多肽(SEQ ID NO:12)，其包含在鼠

腺苷酸环化酶(AC)多肽的催化核心中的第436位具有丙氨酸或Ala(或“A”)对天冬氨酸或Asp(或“D”)的取代的鼠AC多肽;即,在该实施方案中,所述鼠腺苷酸环化酶(AC)多肽在所述鼠AC多肽的催化核心中的第436位具有取代D＝>A或Ala对Asp的取代(SEQ ID NO:11是D＝>A取代之前的氨基酸序列)。

[0148] 在替代性实施方案中,本发明提供(鼠)AC6mut多肽(SEQ ID NO:17),其包含在鼠腺苷酸环化酶(AC)多肽的催化核心中的第426位具有丙氨酸或Ala(或“A”)对天冬氨酸或Asp(或“D”)的取代(即,D＝>A取代)的鼠AC多肽。SEQ ID NO:17多肽与SEQ ID NO:12多肽的不同之处在于SEQ ID NO:17多肽缺少SEQ ID NO:12多肽的前10个氨基酸;所述多肽在其它方面是相同的。SEQ ID NO:16是D＝>A取代之前的鼠氨基酸序列。缺乏氨基末端的该异型体被认为是SEQ ID NO:11和SEQ ID NO:12的前10个氨基酸未翻译的野生型鼠多肽。

[0149] 在替代性实施方案中,本发明提供(人)AC6mut多肽(SEQ ID NO:13),其包含在人腺苷酸环化酶(AC)多肽的催化核心中的第428位具有丙氨酸或Ala(或“A”)对天冬氨酸或Asp(或“D”)的取代的人AC多肽;即,在该实施方案中,所述鼠腺苷酸环化酶(AC)多肽在鼠AC多肽的催化核心中的第428位具有取代D＝>A或Ala对Asp的取代。

[0150] 人AC6核酸编码序列(SEQ ID NO:14)相对于鼠编码序列:86％同源性(SEQ ID NO:15)。人AC6多肽(SEQ ID NO:10)相对于鼠AC6多肽(SEQ ID NO:11)在氨基酸水平下:94％同源性。

[0151] AC6mut D＝>A取代在人AC6mut的催化核心中的相对结构位置与在鼠AC6mut中完全相同,如下面所说明的(显示野生型仍然具有如下面加下划线的天冬氨酸或“D”残基:

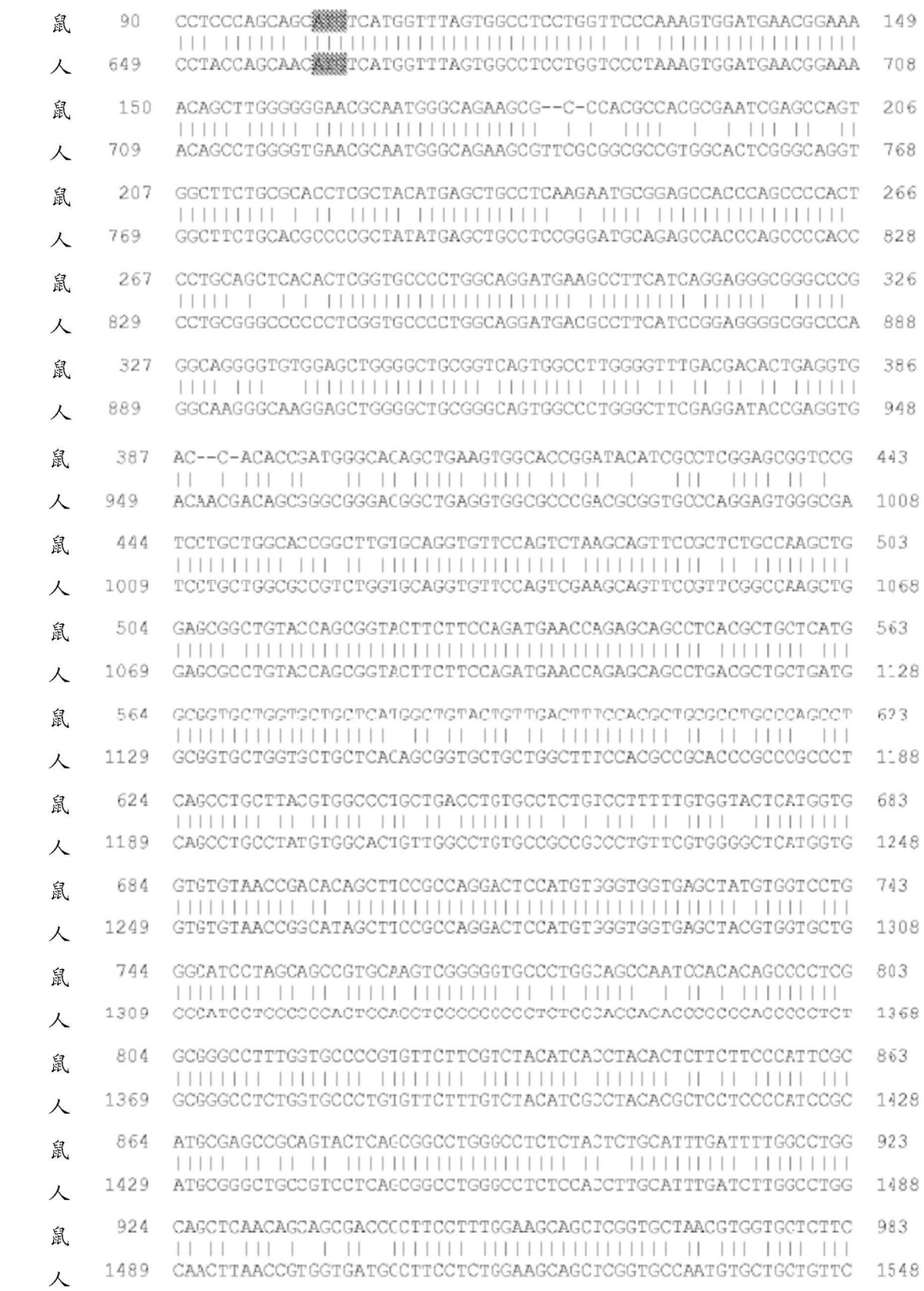
[0152]

人	1	MSWFSGLLVPKVDERKTAWGERNGQKRSRRRGTRAGGFCTPRYMSCLRDAEPPSPTPAGP	60
鼠	11	MSWFSGLLVPKVDERKTAWGERNGQKRPRH-ANRASGFCAPRYMSCLKNAEPPSPTPAAH	69
人	61	PRCPWQDDAFIRRGGPGKGKELGLRAVALGFEDTEVTTTAGGTAEVAPDAVPRSGRSCWR	120
鼠	70	TRCPWQDEAFIRRAGPGRGVELGLRSVALGFDDTEVTTPMG-TAEVAPDTSPRSGPSCWH	128
人	121	RLVQVFQSKQFRSAKLERLYQRYFFQMNQSSLTLLMAVLVLLTAVLLAFHAAPARPQPAY	180
鼠	129	RLVQVFQSKQFRSAKLERLYQRYFFQMNQSSLTLLMAVLVLLMAVLLTFHAAPAQPQPAY	188
人	181	VALLACAAALFVGLMVVCNRHSFRQDSMWVVSYVVLGILAAVQVGGALAADPRSPSAGLW	240
鼠	189	VALLTCASVLFVVLMVVCNRHSFRQDSMWVVSYVVLGILAAVQVGGALAANPHSPSAGLW	248
人	241	CPVFFVYIAYTLLPIRMRAAVLSGLGLSTLHLILAWQLNRGDAFLWRQLGANVLLFLCTN	300
鼠	249	CPVFFVYITYTLLPIRMRAAVLSGLGLSTLHLILAWQLNSSDPFLWKQLGANVVLFLCTN	308
人	301	VIGICT HYPAEVSQRQAFQETRGYIQARLHLQHENRQQERLLLSVLPQHVAMEMKEDINT	360
鼠	309	AIGVCT HYPAEVSQRQAFQETRGYIQARLHLQHENRQQERLLLSVLPQHVAMEMKEDINT	368
人	361	KKEDMMFHKIYIQKHDNVSILFADIEGFTSLASQCTAQELVMTLNELFARFDKLAAENHC	420
鼠	369	KKEDMMFHKIYIQKHDNVSILFADIEGFTSLASQCTAQELVMTLNELFARFDKLAAENHC	428
人	421	LRIKILGDCYYCVSGLPEARADHAHCCVEMGVDMIEAISLVREVTGVNVNMRVGIHSGRV	480
鼠	429	LRIKILGDCYYCVSGLPEARADHAHCCVEMGVDMIEAISLVREVTGVNVNMRVGIHSGRV	488
人	481	HCGVLGLRKWQFDVWSNDVTLANHMEAGGRAGRIHITRATLQYLNGDYEVEPGRGGERNA	540
鼠	489	HCGVLGLRKWQFDVWSNDVTLANHMEAGGRAGRIHITRATLQYLNGDYEVEPGRGGERNA	548
人	541	YLKEQHIETFLILGASQKRKEEKAMLAKLQRTRANSMEGLMPRWVPDRAFSRTKDSKAFR	600
鼠	549	YLKEQCIETFLILGASQKRKEEKAMLAKLQRTRANSMEGLMPRWVPDRAFSRTKDSKAFR	608
人	601	QMGIDDSSKDNRGTQDALNPEDEVDEFLSRAIDARSIDQLRKDHVRRFLLTFQREDLEKK	660
鼠	609	QMGIDDSSKDNRGAQDALNPEDEVDEFLGRAIDARSIDQLRKDHVRRFLLTFQREDLEKK	668
人	661	YSRKVDPRFGAYVACALLVFCFICFIQLLIFPHSTLMLGIYASIFLLLLITVLICAVYSC	720
鼠	669	YSRKVDPRFGAYVACALLVFCFICFIQLLVFPYSTLILGIYAAIFLLLLVTVLICAVCSC	728

[0153]

[0154] 在替代性实施方案中，人ACmut核酸编码序列(SEQ ID NO:13)和鼠ACmut核酸编码序列(SEQ ID NO:12)两者是通过将腺苷(或“A”)变为胞嘧啶(或“C”)而制得，如下文所示，其中“A”残基在其变为“C”之前在下面被加下线；即，下面说明的野生型人AC6(SEQ ID NO:10)和野生型鼠AC6(SEQ ID NO:11)：

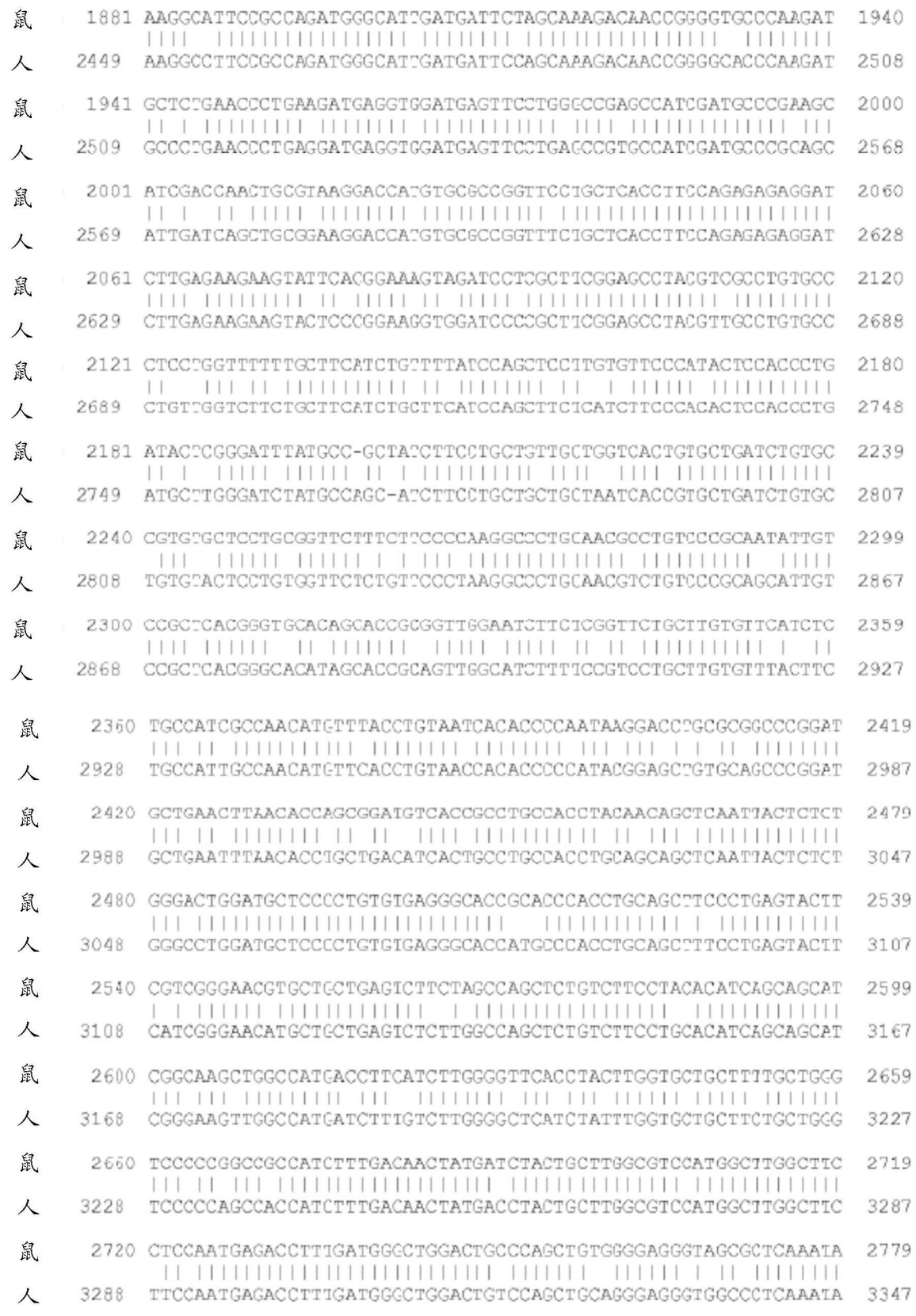
[0155]


鼠 90 CCTCCCAGCAGC[redacted]TCATGGTTTAGTGGCCTCCTGGTTCCCAAAGTGGATGAACGGAAA 149
人 649 CCTACCAGCAAC[redacted]TCATGGTTTAGTGGCCTCCTGGTCCCTAAAGTGGATGAACGGAAA 708
鼠 150 ACAGCTTGGGGGGAACGCAATGGGCAGAAGCG--C-CCACGCCACGCGAATCGAGCCAGT 206
人 709 ACAGCCTGGGGTGAACGCAATGGGCAGAAGCGTTCGCGGCGCCGTGGCACTCGGGCAGGT 768
鼠 207 GGCTTCTGCGCACCTCGCTACATGAGCTGCCTCAAGAATGCGGAGCCACCCAGCCCCACT 266
人 769 GGCTTCTGCACGCCCCGCTATATGAGCTGCCTCCGGGATGCAGAGCCACCCAGCCCCACC 828
鼠 267 CCTGCAGCTCACACTCGGTGCCCCTGGCAGGATGAAGCCTTCATCAGGAGGGCGGGCCCG 326
人 829 CCTGCGGGCCCCCCTCGGTGCCCCTGGCAGGATGACGCCTTCATCCGGAGGGGCGGCCCA 888
鼠 327 GGCAGGGGTGTGGAGCTGGGGCTGCGGTCAGTGGCCTTGGGGTTTGACGACACTGAGGTG 386
人 889 GGCAAGGGCAAGGAGCTGGGGCTGCGGGCAGTGGCCCTGGGCTTCGAGGATACCGAGGTG 948
鼠 387 AC--C-ACACCGATGGGCACAGCTGAAGTGGCACCGGATACATCGCCTCGGAGCGGTCCG 443
人 949 ACAACGACAGCGGGCGGGACGGCTGAGGTGGCGCCCGACGCGGTGCCCAGGAGTGGGCGA 1008
鼠 444 TCCTGCTGGCACCGGCTTGTGCAGGTGTTCCAGTCTAAGCAGTTCCGCTCTGCCAAGCTG 503
人 1009 TCCTGCTGGCGCCGTCTGGTGCAGGTGTTCCAGTCGAAGCAGTTCCGTTCGGCCAAGCTG 1068
鼠 504 GAGCGGCTGTACCAGCGGTACTTCTTCCAGATGAACCAGAGCAGCCTCACGCTGCTCATG 563
人 1069 GAGCGCCTGTACCAGCGGTACTTCTTCCAGATGAACCAGAGCAGCCTGACGCTGCTGATG 1128
鼠 564 GCGGTGCTGGTGCTGCTCATGGCTGTACTGTTGACTTTCCACGCTGCGCCTGCCCAGCCT 623
人 1129 GCGGTGCTGGTGCTGCTCACAGCGGTGCTGCTGGCTTTCCACGCCGCACCCGCCCGCCCT 1188
鼠 624 CAGCCTGCTTACGTGGCCCTGCTGACCTGTGCCTCTGTCCTTTTTGTGGTACTCATGGTG 683
人 1189 CAGCCTGCCTATGTGGCACTGTTGGCCTGTGCCGCCGCCCTGTTCGTGGGGCTCATGGTG 1248
鼠 684 GTGTGTAACCGACACAGCTTCCGCCAGGACTCCATGTGGGTGGTGAGCTATGTGGTCCTG 743
人 1249 GTGTGTAACCGGCATAGCTTCCGCCAGGACTCCATGTGGGTGGTGAGCTACGTGGTGCTG 1308
鼠 744 GGCATCCTAGCAGCCGTGCAAGTCGGGGGGTGCCCTGGCAGCCAATCCACACAGCCCCTCG 803
人 1309 CCCATCCTCCCCCCACTCCACCTCCCCCCCCCTCTCCCACCACACCCCCCCACCCCCTCT 1368
鼠 804 GCGGGCCTTTGGTGCCCCGTGTTCTTCGTCTACATCACCTACACTCTTCTTCCCATTCGC 863
人 1369 GCGGGCCTCTGGTGCCCTGTGTTCTTTGTCTACATCGCCTACACGCTCCTCCCCATCCGC 1428
鼠 864 ATGCGAGCCGCAGTACTCACCGGCCTGGGCCTCTCTACTCTGCATTTGATTTTGGCCTGG 923
人 1429 ATGCGGGCTGCCGTCCTCACCGGCCTGGGCCTCTCCACCTTGCATTTGATCTTGGCCTGG 1488
鼠 924 CAGCTCAACAGCAGCGACCCCTTCCTTTGGAAGCAGCTCGGTGCTAACGTGGTGCTCTTC 983
人 1489 CAACTTAACCGTGGTGATGCCTTCCTCTGGAAGCAGCTCGGTGCCAATGTGCTGCTGTTC 1548

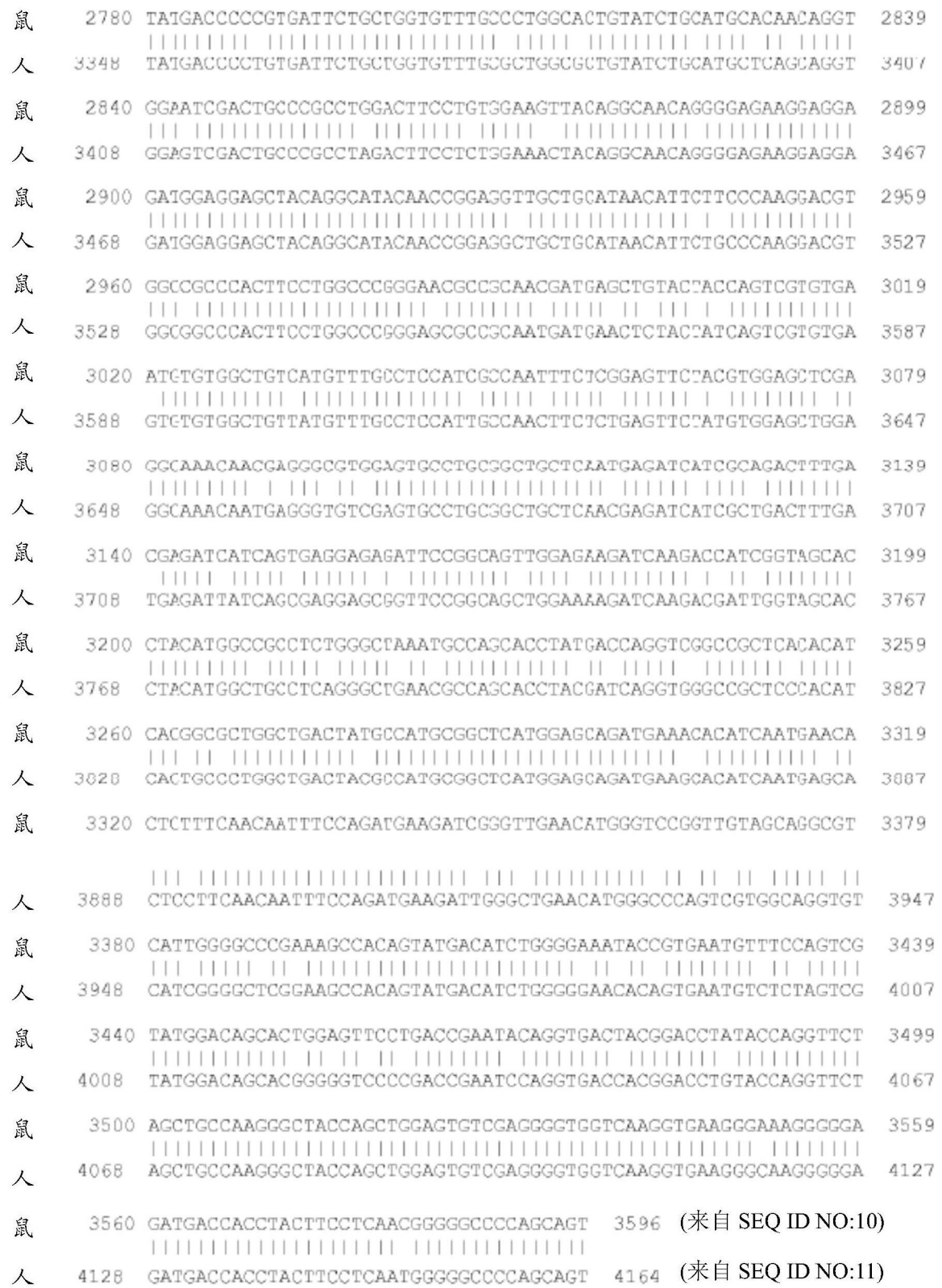
[0156]

```
鼠   984  CTCTGCACCAATGCCATCGGTGTCTGCACACACTACCCTGCTGAAGTGTCTCAGCGCCAA  1043
          ||||||||||| | ||| ||  |||||||||||||| || || || ||||||||||||||
人  1549  CTCTGCACCAACGTCATTGGCATCTGCACACACTATCCAGCAGAGGTGTCTCAGCGCCAG  1608

鼠  1044  GCTTTTCAGGAGACCCGAGGTTACATCCAGGCGCGGCTGCACCTGCAGCATGAGAACCGT  1103
          || ||||||||||||||| |||||||||||||| ||||| |||||||||||||||||| ||
人  1609  GCCTTTCAGGAGACCCGCGGTTACATCCAGGCCCGGCTCCACCTGCAGCATGAGAATCGG  1668

鼠  1104  CAGCAGGAACGGCTGCTGCTATCGGTGTTGCCCCAGCACGTTGCCATGGAGATGAAAGAA  1163
          |||||||| |||||||||||| ||||| ||||||||||||||||||||||||||||||||
人  1669  CAGCAGGAGCGGCTGCTGCTGTCGGTATTGCCCCAGCACGTTGCCATGGAGATGAAAGAA  1728

鼠  1164  GACATCAACACaaaaaaaaGAGGACATGATGTTCCATAAGATCTACATCCAGAAGCATGAT  1223
          |||||||||||||||||||| |||||||||||||| |||||||||| ||||||||||
人  1729  GACATCAACACAAAAAAAGAAGACATGATGTTCCACAAGATCTACATACAGAAGCATGAC  1788

鼠  1224  AATGTCAGCATCCTGTTTGCGGACATTGAGGGCTTCACCAGCCTGGCCTCCCAGTGCACT  1283
          |||||||||||||||||||| |||||||||||||||||||||||| ||||||||||||
人  1789  AATGTCAGCATCCTGTTTGCAGACATTGAGGGCTTCACCAGCCTGGCATCCCAGTGCACT  1848

鼠  1284  GCACAGGAACTGGTCATGACCTTGAATGAGCTCTTTGCCCGGTTTGACAAGCTGGCTGCG  1343
          || ||||| |||||||||||| |||||||||||||||||||||||||||||||||||||
人  1849  GCGCAGGAGCTGGTCATGACCCTGAATGAGCTCTTTGCCCGGTTTGACAAGCTGGCTGCG  1908

鼠  1344  GAGAATCACTGTCTGAGGATCAAGATCTTAGGAGACTGTTACTACTGCGTGTCAGGGCTG  1403

          |||||||||||| |||||||||||||||||| || |||||||||||||| ||||||||||||
人  1909  GAGAATCACTGCCTGAGGATCAAGATCTTGGGGGACTGTTACTACTGTGTGTCAGGGCTG  1968

鼠  1404  CCCGAGGCCCGGGCAGATCACGCCCACTGCTGTGTGGAGATGGGGGTAGACATGATCGAA  1463
          || |||||||||||| || || |||||||||||||||||||||||||||||||||||| ||
人  1969  CCGGAGGCCCGGGCCGACCATGCCCACTGCTGTGTGGAGATGGGGGTAGACATGATTGAG  2028

鼠  1464  GCCATCTCGCTGGTGCGTGAGGTAACAGGTGTGAACGTGAACATGCGTGTGGGCATCCAC  1523
          ||||||||||||||| |||||||| |||||||||||| |||||||||| ||||||||||||
人  2029  GCCATCTCGCTGGTACGTGAGGTGACAGGTGTGAATGTGAACATGCGCGTGGGCATCCAC  2088

鼠  1524  AGCGGACGTGTGCATTGCGGCGTCCTTGGCCTACGGAAATGGCAGTTTGATGTCTGGTCA  1583
          |||||  || |||||  |||||||||||||||  | ||||||||||||||  |||||  |||||
人  2089  AGCGGGCGCGTGCACTGCGGCGTCCTTGGCTTGCGGAAATGGCAGTTCGATGTGTGGTCC  2148

鼠  1584   AACGATGTGACCCTGGCTAACCACATGGAGGCCGG-GGGC-GGCCGG-CGCATCCACATC  1640
          || |||||||||||||| |||||||||||||| || || | ||| || ||||||||||||
人  2149  AATGATGTGACCCTGGCCAACCACATGGAGGCAGGAGGCCGGGCTGGCCGCATCCACATC  2208

鼠  1641  ACTCGGGCTACACTGCAGTACTTGAACGGGGACTATGAGGTGGAGCCAGGCCGTGGTGGT  1700
          |||||||| |||||||||||| |||||||||||||| |||||||||||||||||||||||
人  2209  ACTCGGGCAACACTGCAGTACCTGAACGGGGACTACGAGGTGGAGCCAGGCCGTGGTGGC  2268

鼠  1701   GAACGCAATGCGTACCTCAAGGAGCAGTGCATTGAGACCTTCCTCATACTTGGCGCCAGC  1760
          || ||||| |||||||||||||||||  |||||||| |||||||| || |||||||||
人  2269  GAGCGCAACGCGTACCTCAAGGAGCAGCACATTGAGACTTTCCTCATCCTGGGCGCCAGC  2328

鼠  1761  CAAAAACGGAAAGAGGAGAAAGCCATGCTGGCCAAGCTTCAGCGGACACGGGCCAACTCC  1820
          || |||||||||||||||| |||||||||||||||| |||||||| ||||||||||||
人  2329  CAGAAACGGAAAGAGGAGAAGGCCATGCTGGCCAAGCTGCAGCGGACTCGGGCCAACTCC  2388

鼠  1821  ATGGAAGGACTGATGCCCCGCTGGGTTCCTGACCGTGCCTTCTCCCGGACCAAGGACTCT  1880
          |||||||| |||||||| |||||||||||||| |||||||||||||||||||||||||||
人  2389  ATGGAAGGGCTGATGCCGCGCTGGGTTCCTGATCGTGCCTTCTCCCGGACCAAGGACTCC  2448
```

[0157]


鼠 1881 AAGGCATTCCGCCAGATGGGCATTGATGATTCTAGCAAAGACAACCGGGGTGCCCAAGAT 1940
人 2449 AAGGCCTTCCGCCAGATGGGCATTGATGATTCCAGCAAAGACAACCGGGGCACCCAAGAT 2508
鼠 1941 GCTCTGAACCCTGAAGATGAGGTGGATGAGTTCCTGGGCCGAGCCATCGATGCCCGAAGC 2000
人 2509 GCCCTGAACCCTGAGGATGAGGTGGATGAGTTCCTGACCCGTGCCATCGATGCCCGCAGC 2568
鼠 2001 ATCGACCAACTGCGTAAGGACCATGTGCGCCGGTTCCTGCTCACCTTCCAGAGAGAGGAT 2060
人 2569 ATTGATCAGCTGCGGAAGGACCATGTGCGCCGGTTTCTGCTCACCTTCCAGAGAGAGGAT 2628
鼠 2061 CTTGAGAAGAAGTATTCACGGAAAGTAGATCCTCGCTTCGGAGCCTACGTCGCCTGTGCC 2120
人 2629 CTTGAGAAGAAGTACTCCCGGAAGGTGGATCCCCGCTTCGGAGCCTACGTTGCCTGTGCC 2688
鼠 2121 CTCCTGGTTTTTTGCTTCATCTGTTTTATCCAGCTCCTTGTGTTCCCATACTCCACCCTG 2180
人 2689 CTGTTGGTCTTCTGCTTCATCTGCTTCATCCAGCTTCTCATCTTCCCACACTCCACCCTG 2748
鼠 2181 ATACTCGGGATTTATGCC-GCTACTTCCTGCTGTTGCTGGTCACTGTGCTGATCTGTGC 2239
人 2749 ATGCTTGGGATCTATGCCAGC-ACTTCCTGCTGCTGCTAATCACCGTGCTGATCTGTGC 2807
鼠 2240 CGTGTGCTCCTGCGGTTCTTTCTTCCCCAAGGCCCTGCAACGCCTGTCCCGCAATATTGT 2299
人 2808 TGTGTACTCCTGTGGTTCTCTGTTCCCTAAGGCCCTGCAACGTCTGTCCCGCAGCATTGT 2867
鼠 2300 CCGCTCACGGGTGCACAGCACCGCGGTTGGAATGTTCTCGGTTCTGCTTGTGTTCATCTC 2359
人 2868 CCGCTCACGGGCACATAGCACCGCAGTTGGCATCTTTTCCGTCCTGCTTGTGTTTACTTC 2927
鼠 2360 TGCCATCGCCAACATGTTTACCTGTAATCACACCCCAATAAGGACCTGCGCGGCCCGGAT 2419
人 2928 TGCCATTGCCAACATGTTCACCTGTAACCACACCCCCATACGGAGCTGTGCAGCCCGGAT 2987
鼠 2420 GCTGAACTTAACACCAGCGGATGTCACCGCCTGCCACCTACAACAGCTCAATTACTCTCT 2479
人 2988 GCTGAATTTAACACCTGCTGACATCACTGCCTGCCACCTGCAGCAGCTCAATTACTCTCT 3047
鼠 2480 GGGACTGGATGCTCCCCTGTGTGAGGGCACCGCACCCACCTGCAGCTTCCCTGAGTACTT 2539
人 3048 GGGCCTGGATGCTCCCCTGTGTGAGGGCACCATGCCCACCTGCAGCTTTCCTGAGTACTT 3107
鼠 2540 CGTCGGGAACGTGCTGCTGAGICTTCTAGCCAGCTCTGTCTTCCTACACATCAGCAGCAT 2599
人 3108 CATCGGGAACATGCTGCTGAGICTCTTGGCCAGCTCTGTCTTCCTGCACATCAGCAGCAT 3167
鼠 2600 CGGCAAGCTGGCCATGACCTTCATCTTGGGGTTCACCTACTTGGTGCTGCTTTTGCTGGG 2659
人 3168 CGGGAAGTTGGCCATGATCTTTGTCTTGGGGCTCATCTATTTGGTGCTGCTTCTGCTGGG 3227
鼠 2660 TCCCCCGGCCGCCATCTTTGACAACTATGATCTACTGCTTGGCGTCCATGGCTTGGCTTC 2719
人 3228 TCCCCCAGCCACCATCTTTGACAACTATGACCTACTGCTTGGCGTCCATGGCTTGGCTTC 3287
鼠 2720 CTCCAATGAGACCTTTGATGGCCTGGACTGCCCAGCTGTGGGGAGGGTAGCGCTCAAATA 2779
人 3288 TTCCAATGAGACCTTTGATGGCCTGGACTGTCCAGCTGCAGGGAGGGTGGCCCTCAAATA 3347

[0158]



[0159] 在替代性实施方案中，通过例如克隆和表达cDNA文库、通过PCR扩增信使DNA或基因组DNA等来制备、分离和/或操作本发明的核酸。用于实施本发明的核酸和基因(包括DNA、RNA、iRNA、反义核酸、cDNA、基因组DNA、载体、病毒或其杂合体)可以从各种来源分离、经遗传改造、扩增和/或重组表达/生成。从这些核酸生成的重组多肽(例如，用于实施本发明的不能产生cAMP的AC嵌合蛋白质)可以被各自分离或克隆，并且测试其期望活性。可以使用任

何重组表达系统，包括例如细菌、真菌、哺乳动物、酵母、昆虫或植物细胞表达系统或表达运载体。

[0160] 或者，用于实施本发明的核酸可以通过熟知的化学合成技术体外合成，如在例如Adams(1983)J.Am.Chem.Soc.105:661;Belousov(1997)Nucleic Acids Res.25:3440-3444;Frenkel(1995)Free Radic.Biol.Med.19:373-380;Blommers(1994)Biochemistry 33:7886-7896;Narang(1979)Meth.Enzymol.68:90;Brown(1979)Meth.Enzymol.68:109;Beaucage(1981)Tetra.Lett.22:1859;美国专利号4,458,066中所述。

[0161] 用于操作用于实施本发明的核酸的技术，如例如亚克隆、标记探针(例如，使用Klenow聚合酶的随机引物标记、切口平移、扩增)、测序、杂交等在科学和专利文献中有充分的描述，参见例如Sambrook编辑，MOLECULAR CLONING:A LABORATORY MANUAL(第2版)，1-3卷，Cold Spring Harbor Laboratory,(1989);CURRENT PROTOCOLS IN MOLECULAR BIOLOGY,Ausubel编辑，John Wiley&Sons,Inc.,New York(1997);LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY:HYBRIDIZATION WITH NUCLEIC ACID PROBES,Part I.Theory and Nucleic Acid Preparation,Tijssen编辑，Elsevier,N.Y.(1993)。

[0162] 获得和操作用于实施本发明的方法的核酸的另一有用手段是从基因组样品中克隆，并且如果期望的话，筛选和再克隆从例如基因组克隆或cDNA克隆分离或扩增的插入物。用于本发明的方法中的核酸的来源包括基因组或cDNA文库，所述文库包含在例如哺乳动物人工染色体(MAC)，参见例如美国专利号5,721,118;6,025,155;人类人工染色体，参见例如Rosenfeld(1997)Nat.Genet.15:333-335;酵母人工染色体(YAC);细菌人工染色体(BAC);P1人工染色体，参见例如Woon(1998)Genomics50:306-316;源于P1的载体(PAC)，参见例如Kern(1997)Biotechniques23:120-124;粘粒、重组病毒、噬菌体或质粒中。

[0163] 在替代性实施方案中，为了实施本发明的方法，使用AC6mut融合蛋白质和编码它们的核酸。任何AC6mut多肽均可用于实施本发明。在替代性实施方案中，AC6mut蛋白质可以被融合到异源肽或多肽，如用于将所述多肽靶向期望的细胞类型如心肌细胞的肽。

[0164] 在替代性实施方案中，接合或融合到用于实施本发明的蛋白质的异源肽或多肽可以是赋予期望特性如荧光检测、增加的稳定性和/或简化的纯化的N-末端鉴别肽。用于实施本发明的肽和多肽还可以作为融合蛋白质被合成和表达，所述融合蛋白质中连接有一个或多个额外的结构域，用于例如用于产生免疫原性更强的肽、以便更易于分离重组合成的肽、以便鉴别和分离抗体和表达抗体的B细胞等。促进检测和纯化的结构域包括例如金属螯合肽，如允许在固定的金属上纯化的多组氨酸标签(polyhistidine tract)和组氨酸-色氨酸模块；允许在固定的免疫球蛋白上纯化的蛋白A结构域；和用于FLAGS延伸/亲和纯化系统(Immunex Corp,Seattle WA)中的结构域。在纯化结构域与包含基序的肽或多肽之间包含可切割的连接体序列如Xa因子或肠激酶(Invitrogen,San Diego CA)以促进纯化。例如，表达载体可以包括编码表位的核酸序列，该核酸序列连接到六组氨酸残基，之后是硫氧还蛋白和肠激酶切割位点(参见例如Williams(1995)Biochemistry 34:1787-1797;Dobeli(1998)Protein Expr.Purif.12:404-414)。组氨酸残基促进检测和纯化，而肠激酶切割位点提供了从融合蛋白的剩余部分纯化表位的手段。关于编码融合蛋白质的载体的技术和融合蛋白质的应用在科学和专利文献中有充分的描述，参见例如Kroll(1993)DNA Cell.Biol.,12:441-53。

[0165] 用于实施本发明的核酸或核酸序列(例如,编码AC6mut的核酸)可以是寡核苷酸、核苷酸、聚核苷酸;或者是指这些中的任何一种的片段;是指基因组的或合成来源的DNA或RNA,它们可以是单链或双链,并且可以代表有义链或反义链;是指肽核酸(PNA);或者是指天然或合成来源的任何DNA样或RNA样物质。用于实施本发明的化合物包括“核酸”或“核酸序列”,包括寡核苷酸、核苷酸、聚核苷酸或这些中的任一个的任何片段;并且包括基因组的或合成来源的DNA或RNA(例如,mRNA、rRNA、tRNA、iRNA),它们可以是单链或双链;并且可以是有义链或反义链、或肽核酸(PNA)、或天然或合成来源的任何DNA样或RNA样物质,包括例如iRNA、核糖核蛋白质(例如,例如,双链iRNA,例如,iRNP)。用于实施本发明的化合物包括含有天然核苷酸的已知类似物的核酸,即寡核苷酸。用于实施本发明的化合物包括具有合成骨架的核酸样结构,参见例如Mata(1997)Toxicol.Appl.Pharmacol.144:189-197;Strauss-Soukup(1997)Biochemistry 36:8692-8698;Samstag(1996)Antisense Nucleic Acid Drug Dev 6:153-156。用于实施本发明的化合物包括“寡核苷酸”,其包括可以化学合成的单链聚脱氧核苷酸或两条互补的聚脱氧核苷酸链。用于实施本发明的化合物包括不具有5’磷酸盐的合成寡核苷酸,因此不是在激酶存在下用ATP添加磷酸的情况下,所述合成寡核苷酸将不连接到另一寡核苷酸。合成寡核苷酸可以连接到未被去磷酸化的片段。

[0166] 在替代性方面,用于实施本发明的化合物包括参与产生AC6mut多肽的基因或任何DNA区段;它可以包括在编码区之前和之后的区域(前导区和非转录尾区)以及(适用时)各编码区段(外显子)之间的插入序列(内含子)。“可操作地连接”可以指两个或更多个核酸(例如,DNA)区段之间的功能关系。在替代性方面,它可以指转录调控序列与被转录序列的功能关系。例如,如果启动子刺激或调节编码序列在适当的宿主细胞或其它表达系统中的转录,则其可以可操作地连接到该编码序列,如用于实施本发明的核酸。在替代性方面,当启动子转录调控序列可以与被转录序列物理地邻接,即它们可以顺式作用时,则它们可以可操作地连接到所述被转录序列。在替代性方面,转录调控序列如增强子无需物理地邻接被它们增强的编码序列或与所述编码序列极为接近地定位。

[0167] 在替代性方面,本发明包括使用包含用于实施本发明的核苷酸序列的“表达盒”,其能够影响核酸(例如,结构基因或转录物(例如,编码AC6mut蛋白质))在与此类序列相容的宿主中的表达。表达盒可以包括至少一个启动子,该启动子与多肽编码序列或抑制序列可操作地连接;且一个方面,与其它序列(例如,转录终止信号)可操作地连接。还可以使用在影响表达方面有必要的或有帮助的额外因子,例如,增强子。

[0168] 在替代性方面,用于实施本发明的表达盒还包括质粒、表达载体、重组病毒、任何形式的重组“裸DNA”载体等。在替代性方面,用于实施本发明的“载体”可以包含可以感染、转染、短暂地或永久地转导细胞的核酸。在替代性方面,用于实施本发明的载体可以是裸核酸或与蛋白质或脂质复合的核酸。在替代性方面,用于本发明的载体可以包含病毒或细菌核酸和/或蛋白质和/或膜(例如,细胞膜、病毒脂质包膜等)。在替代性方面,用于实施本发明的载体可以包括但不限于复制子(例如,RNA复制子、噬菌体),DNA片段可以与该复制子连接并被复制。载体因此包括但不限于RNA、自主自我复制的环状或线性DNA或RNA(例如,质粒、病毒等,参见例如美国专利号5,217,879),并且可以包括表达质粒和非表达质粒两者。在替代性方面,用于实施本发明的载体可以在细胞有丝分裂期间作为自主结构被细胞稳定地复制,或可以被并入宿主基因组内。

[0169] 在替代性方面，用于实施本发明的“启动子”包括能够驱动编码序列在细胞(例如哺乳动物细胞，如心脏、肺、肌肉、神经或脑细胞)中转录的所有序列。因此，用于本发明的构建体中的启动子包括参与调控或调节基因转录的时机和/或速率的顺式作用转录控制元件和调控序列。例如，用于实施本发明的启动子可以是顺式作用转录控制元件，包括增强子、启动子、转录终止子、复制起点、染色体整合序列、5’和3’非翻译区或内含子序列，它们参与转录的调控。这些顺式作用序列通常与蛋白质或其它生物分子相互作用来实施(打开/关闭、调控、调节等)转录。

[0170] 在替代性实施方案中，用于实施本发明的“组成型”启动子可以是在多数环境条件和发育状态或细胞分化状态下连续地驱动表达的那些。在替代性实施方案中，用于实施本发明的“诱导型”或“可调控型”启动子可以在环境条件、所施用的化学剂或发育条件的影响下指导本发明的核酸的表达。

[0171] 腺病毒载体和腺相关病毒(AAV)的递送

[0172] 在替代性实施方案中，递送运载体、载体、表达载体、重组病毒或等同物是或包括：腺相关病毒(AAV)；重组AAV病毒、载体或病毒粒子；或腺病毒载体。在替代性实施方案中，AAV、重组AAV病毒或载体或腺病毒载体是或包括AAV血清型AAV5、AAV6、AAV7、AAV8或AAV9；恒河猴AAV(AAVrh)或AAVrh10；或其任何假型、杂合体或衍生物。

[0173] 在替代性实施方案中，这些载体中的任何一种(或本发明的任何递送运载体)趋向于特定的细胞、组织或器官，或被设计用于特异性递送到特定的细胞、组织或器官。例如，在替代性实施方案中，用于实施本发明的AAV(或用于实施本发明的任何载体或递送运载体)趋向于心脏(或对心脏具有趋向性)。在其它实施方案中，用于实施本发明的AAV(或任何载体或递送运载体)趋向于另一组织或器官，例如，肝脏，或被设计用于特异性递送到另一组织或器官，例如，肝脏。在替代性实施方案中，这种“外周”递送模式，例如，递送运载体、载体、重组病毒等被IM或IV注射，可以避免当基因或核酸在器官(例如，心脏、肺或肾脏)自身中被直接表达时所遇到的问题。例如，已发现AAV5、AAV6和AAV9趋向于心脏，参见例如Fang等人，Hum Gene Ther Methods 2012年10月17日；Zincarelli等人，Clin Transl Sci.2010年6月；3(3):81-9。

[0174] 用于实施本发明的腺相关病毒(AAV)可以是细小病毒科(Parvoviridae)的小的无包膜单链DNA动物病毒的任何非病原性成员。AAV需要辅助病毒(例如，腺病毒)用于复制，因此用于实施本发明的AAV在被向受试者施用时不复制。AAV可以感染相对宽范围的细胞类型并且仅刺激轻微的免疫应答，特别是相较于许多其它病毒如腺病毒来说。用于实施本发明的AAV血清型包括例如AAV1、AAV2、AAV3、AAV4、AAV5、AAV6、AAV7、AAV8、AAV9、AAV10、AAV11和AAV12。用于实施本发明的AAV可以来自其它动物，包括：例如鸟类(例如，禽AAV或AAAV)、牛科动物(例如，牛科动物AAV或BAAV)、犬科动物、马科动物、绵羊和猪。

[0175] 在替代性实施方案中，用于实施本发明的AAV载体是其中至少一部分AAV基因组被异源核酸分子置换的重组核酸分子；可以例如通过去除病毒复制和衣壳基因来置换约4.7千碱基(kb)的AAV基因组DNA。在替代性实施方案中，异源核酸分子仅仅在每个末端上侧翼有AAV反向末端重复(ITR)。ITR用作复制起点并且含有拯救、整合、从克隆载体切除和封装所需的顺式作用元件。在替代性实施方案中，用于实施本发明的AAV包含可操作地连接到异源核酸分子的启动子以控制表达。

[0176] AAV载体可借助于在细胞中表达的辅助病毒或辅助功能体(helper function)被体外包装到AAV衣壳中以获得AAV病毒粒子。AAV病毒粒子的血清型和细胞趋向性由病毒衣壳蛋白质的性质被赋予。AAV载体和AAV病毒粒子可以有效地转导细胞,包括分裂细胞和非分裂细胞。AAV载体和病毒粒子已被证明是安全的并且导致在各种细胞类型中的长期体内存留和表达。

[0177] 在替代性实施方案中,一种ITR接合到编码AC6mut的核酸分子的5'末端,并且一种ITR接合到编码AC6mut的核酸分子的3'末端。ITR的示例包括但不限于AAV1、AAV2、AAV3、AAV4、AAV5、AAV6、AAV7、AAV8、AAV9、AAV10、AAV11、AAV12、AAAV、BAAV和本领域技术人员已知的其它AAV ITR。在一个实施方案中,AAV ITR选自AAV2ITR、AAV5ITR、AAV6ITR和BAAV ITR。在一个实施方案中,AAV ITR是AAV2ITR。在一个实施方案中,AAV ITR是AAV5ITR。在一个实施方案中,AAV ITR是AAV6ITR。在一个实施方案中,AAV ITR是BAAV ITR。

[0178] 在替代性实施方案中,用于实施本发明的AAV载体(和其它载体、重组病毒等)包含其它序列,如表达控制序列,例如,启动子、增强子、阻遏物(represssor)、核糖体结合位点、RNA剪接位点、聚腺苷酸化位点、转录终止子序列和微小RNA结合位点。启动子的示例包括但不限于AAV启动子(如p5、p19或p40启动子)、腺病毒启动子(如腺病毒主要晚期启动子)、巨细胞病毒(CMV)启动子、乳头瘤病毒启动子、多瘤病毒启动子、呼吸道合胞病毒(RSV)启动子、肉瘤病毒启动子、SV40启动子、其它病毒启动子、肌动蛋白启动子、淀粉酶启动子、免疫球蛋白启动子、激肽释放酶启动子、金属硫蛋白启动子、热休克启动子、内源性启动子、由雷帕霉素或其它小分子调控的启动子、其它细胞启动子和本领域技术人员已知的其它启动子。在一个实施方案中,所述启动子是AAV启动子。在一个实施方案中,所述启动子是CMV启动子。本领域技术人员可以实现对于待包含的表达控制序列的选择。

[0179] 在替代性实施方案中,使用不同血清型(如通过此类载体内的ITR的血清型所确定)的AAV载体,例如:AAV1载体、AAV2载体、AAV3载体、AAV4载体、AAV5载体、AAV6载体、AAV7载体、AAV8载体、AAV9载体、AAV10载体、AAV11载体、AAV 12载体、AAAV载体和BAAV载体。在替代性实施方案中,AAV载体是AAV2载体、AAV5载体、AAV6载体或BAAV载体。

[0180] 在替代性实施方案中,使用嵌合的、穿梭的或衣壳改性的AAV衍生物向病毒载体提供一种或多种期望的功能。在替代性实施方案中,相较于包含天然存在的AAV基因组的AAV病毒载体,这些衍生物可以展示增加的基因递送效率、降低的免疫原性(体液免疫原性或细胞免疫原性)、改变的趋向性范围和/或对特定细胞类型的改进的靶向。在替代性实施方案中,通过以下方式实现增加的基因递送效率:细胞表面处的改进的受体或共受体结合、改进的内在化、在细胞内和向细胞核中的改进的运输、病毒粒子的改进的脱壳和/或单链基因组向双链形式的改进的转化。在替代性实施方案中,对特定细胞群体的改变的趋向性范围或靶向使效率增加,使得所述载体的剂量不被施用至不需要载体的组织而稀释。

[0181] 在替代性实施方案中,使用如例如美国专利申请号20140107186中所述的无衣壳的AAV载体。在替代性实施方案中,使用如例如美国专利申请号20140056854中所述的趋向于心脏或肝脏的AAV9载体。在替代性实施方案中,使用描述于例如美国专利申请号20130310443;20130136729中的AAV载体来实施本发明。

[0182] 在替代性实施方案中,针对例如改进或改变的性能将AAV载体假型化,例如,以改进或改变病毒的趋向性或其它特征,如例如美国专利申请号20120220492中所述。例如,特

异性的靶向或改进的靶向允许递送运载体(例如,AAV病毒粒子)仅感染欲被感染的那些细胞并且仅将治疗性核酸(例如,AC6mut)递送至欲被感染的那些细胞,由此降低基因疗法的不需要的副作用的风险并增加基因疗法的功效。

[0183] 在替代性实施方案中,病毒载体的剂量由诸如所治疗的病况、患者的年龄、体重和健康的因素来确定,因此可以随患者而变化。例如,病毒载体的治疗有效的人剂量通常在约0.1ml到约100ml的含有浓度为约$1x10^9$到$1x\ 10^{16}$个基因组的病毒载体的溶液的范围。用于递送到大器官(例如,肝脏、肌肉、心脏和肺)的示例性人剂量可以是每1kg约$5x\ 10^{10}$到$5x\ 10^{13}$个AAV基因组,体积为约1mL到100mL。调节所述剂量以使治疗益处与任何副作用保持平衡并且此类剂量可以根据采用所述重组载体的治疗应用而变化。可以监测转基因的表达水平以确定剂量频率,从而得到病毒载体,例如,AAV载体。

[0184] 制剂

[0185] 在替代性实施方案中,本发明提供用于在心肌细胞中体内递送和表达AC6mut的组合物和方法。在替代性实施方案中,这些组合物包含被配制用于这些目的的编码AC6mut的核酸,例如,在缓冲液、盐水溶液、粉末、乳液、囊泡、脂质体、纳米粒子、纳米脂质体粒子(nanolipoparticle)等中配制的表达运载体或编码AC6mut的核酸。

[0186] 在替代性实施方案中,所述组合物可以任何方式被配制并且可以各种浓度和形式被应用,这取决于期望的体内或离体条件,包括期望的体内或离体施用方法等。关于用于体内或离体制剂和施用的技术的细节在科学和专利文献中有充分描述。

[0187] 用于实施本发明的编码AC6mut的核酸的制剂和/或运送体(carrier)在本领域内众所周知。用于实施本发明的制剂和/或运送体可以呈适于体内或离体应用的诸如片剂、丸剂、粉末、胶囊、液体、凝胶、糖浆、浆液、悬浮液等的形式。

[0188] 在替代性实施方案中,用于实施本发明的编码AC6mut的核酸可以与水溶液和/或缓冲溶液混合,或作为水性悬浮液和/或缓冲悬浮液,例如,包含悬浮剂,如羧甲基纤维素钠、甲基纤维素、羟丙基甲基纤维素、海藻酸钠、聚乙烯吡咯烷酮、黄蓍胶和阿拉伯胶;和分散剂或润湿剂,如天然存在的磷脂(例如,卵磷脂)、烯基氧化物与脂肪酸的缩合产物(例如,聚氧乙烯硬脂酸酯)、氧化乙烯与长链脂肪醇的缩合产物(例如,十七氧乙烯鲸蜡醇(heptadecaethylene oxycetanol))、氧化乙烯与衍生自脂肪酸与己糖醇的偏酯的缩合产物(例如,聚氧乙烯山梨糖醇单油酸酯)或氧化乙烯与衍生自脂肪酸和己糖醇酐的偏酯的缩合产物(例如,聚氧乙烯脱水山梨糖醇单油酸酯)。水性悬浮液还可以含有一种或多种防腐剂,如对羟基苯甲酸乙酯或对羟基苯甲酸正丙酯。可以通过例如使用适当的缓冲剂来调节制剂的渗透性(osmolarity)。

[0189] 在实施本发明时,本发明的化合物(例如,制剂)可以包含溶解于药学上可接受的运送中的编码AC6mut的核酸或基因的溶液,例如可以采用的可接受的运载体和溶剂包括水和林格氏溶液(Ringer's solution)、等渗氯化钠。另外,可以采用无菌的固定油作为溶剂或悬浮介质。为此目的,可以采用任何固定油,包括合成的甘油单酯或甘油二酯或脂肪酸如油酸。在一个实施方案中,用于实施本发明的溶液和制剂是无菌的并且可以被制造成通常不含不期望的物质。在一个实施方案中,这些溶液和制剂通过常规的熟知灭菌技术灭菌。

[0190] 用于实施本发明的溶液和制剂可以包含按要求接近生理条件的辅助物质,如pH调节剂和缓冲剂、毒性调节剂,例如,乙酸钠、氯化钠、氯化钾、氯化钙、乳酸钠等。活性剂(例

如，编码AC6mut的核酸或基因)在这些制剂中的浓度可以广泛地变化，并且可以主要基于流体体积、粘度等，根据所选的特定的体内或离体施用模式和期望的结果(例如，增加体内AC6mut表达)来选择。

[0191] 用于实施本发明的溶液和制剂可以被冻干；例如，本发明提供包含编码AC6mut的核酸或基因的稳定冻干制剂。在一个方面，通过将包含编码AC6mut的核酸或基因和增量剂(例如，甘露醇、海藻糖、棉子糖和蔗糖或其混合物)的溶液冻干制得该制剂。用于制备稳定的冻干制剂的方法可以包括将约2.5mg/mL蛋白质、约15mg/mL蔗糖、约19mg/mL NaCl和pH大于5.5但小于6.5的柠檬酸钠缓冲液的溶液冻干。参见例如美国专利申请号20040028670。

[0192] 本发明的组合物和制剂可以通过使用脂质体来递送(还参见下文的论述)。通过使用脂质体，特别是在脂质体表面带有对靶细胞(例如，心肌细胞)具有特异性或以其他方式优先针对特定组织或器官类型(例如，心脏)的配体时，可以在体内或离体施加中集中将活性剂递送到靶细胞(例如，心肌细胞)中。

[0193] 纳米粒子、纳米脂质体粒子和脂质体

[0194] 本发明还提供包含用于实施本发明方法的化合物(例如，AC6mut或编码AC6mut的核酸或基因)的纳米粒子、纳米脂质体粒子、囊泡和脂质体膜，例如，以在体内或离体将AC6mut或编码AC6mut的核酸或基因递送到心肌细胞。在替代性实施方案中，这些组合物被设计成靶向特定的分子，包括生物分子，如多肽，包括细胞表面多肽，例如，用于靶向期望的细胞类型，例如，哺乳动物心细胞、心肌细胞等。

[0195] 本发明提供包含用于实施本发明的化合物的多层脂质体，例如，如Park等人，美国专利公开号20070082042中所述。多层脂质体可以使用包含角鲨烷、固醇、神经酰胺、中性脂质或中性油、脂肪酸和卵磷脂的油相组分的混合物制备成约200nm到5000nm的粒度，例如，以捕获编码不能产生cAMP的AC的核酸或基因。

[0196] 脂质体可以使用例如如Park等人，美国专利公开号20070042031中所述的任何方法制得，包括通过包封活性剂(例如，编码AC6mut的核酸或基因)来产生脂质体的方法，所述方法包括在第一储器中提供水溶液；在第二储器中提供有机脂质溶液，然后在第一混合区中混合所述水溶液与所述有机脂质溶液以产生脂质体溶液，其中所述有机脂质溶液与所述水溶液混合以基本上瞬时地产生包封所述活性剂的脂质体；并且然后立即混合所述脂质体溶液与缓冲溶液以产生稀释的脂质体溶液。

[0197] 在一个实施方案中，用于实施本发明的脂质体组合物包含被取代的铵和/或聚阴离子，例如，用于将用于实施本发明的化合物(例如，编码AC6mut的核酸或基因)靶向递送到期望的细胞类型，如例如美国专利公开号20070110798中所述。

[0198] 本发明还提供包含用于实施本发明的化合物(例如，编码AC6mut的核酸或基因)的纳米粒子，其呈含活性剂的纳米粒子(例如，二级纳米粒子)的形式，如例如美国专利公开号20070077286中所述。在一个实施方案中，本发明提供包含本发明的脂溶性活性剂或脂溶解的水溶性活性剂的纳米粒子以与二价或三价金属盐作用。

[0199] 在一个实施方案中，固体脂质悬浮液可以用于配制用于实施本发明的编码AC6mut的核酸或基因和将用于实施本发明的编码AC6mut的核酸或基因递送到体内或离体的哺乳动物细胞，如例如美国专利公开号20050136121中所述。

[0200] 递送运载体

[0201] 在替代性实施方案中，任何递送运载体均可以用于实施本发明的方法或组合物，例如，以递送AC6mut或编码AC6mut的核酸或基因以在体内或离体实施本发明的方法。例如，可以使用包含聚阳离子、阳离子型聚合物和/或阳离子型肽(如聚乙烯亚胺衍生物)的递送运载体，例如，如例如美国专利公开号20060083737中所述。

[0202] 在一个实施方案中，干燥的多肽-表面活性剂复合体被用于配制本发明的组合物，其中表面活性剂通过非共价键与核酸缔合，例如如例如美国专利公开号20040151766中所述。

[0203] 在一个实施方案中，用于实施本发明的核酸可以作为聚合水凝胶或水溶性共聚物被施加到细胞，例如，如美国专利号7,413,739中所述；例如，核酸可以通过亲核加成通过强亲核体和共轭不饱和键或共轭不饱和基团之间的反应来聚合，其中每种前体组分包含至少两种强亲核体或至少两个共轭不饱和键或共轭不饱和基团。

[0204] 在一个实施方案中，使用带有细胞膜渗透性肽缀合物的运载体将核酸施加到细胞，例如，如美国专利号7,306,783；6,589,503中所述。在一个方面，核酸本身缀合到细胞膜渗透性肽。在一个实施方案中，核酸和/或递送运载体缀合到介导转运的肽，例如，如美国专利号5,846,743中所述，该美国专利描述了高度碱性且结合聚磷酸肌醇的介导转运的肽。

[0205] 在一个实施方案中，电透化被用作主要手段或辅助手段以将编码AC6mut的核酸或基因递送到细胞，例如，使用如例如美国专利号7,109,034；6,261,815；5,874,268中所述的任何电穿孔系统进行。

[0206] 体内植入细胞

[0207] 在替代性实施方案中，本发明的方法还包括植入或移入细胞，例如，心细胞或心肌细胞，所述细胞包含或表达用于实施本发明的编码AC6mut的核酸或基因；并且在一个方面，本发明的方法包括在血管、组织或器官(例如，心脏或心肌细胞)中离体或体内植入或移入编码AC6mut的核酸或基因(或表达它们的细胞)，或在有此需要的个体中植入或移入再程序化的分化细胞。

[0208] 可以从个体中移出细胞，使用本发明的组合物和/或方法对所述细胞进行处理，并且使用任何已知的技术或方案将其再插入(例如，注入或移入)到组织、器官或该个体中。例如，可以使用微球体再植入(例如，注入或移入)去分化的再程序化细胞、干细胞或再程序化的分化细胞，例如如美国专利号7,442,389中所述；例如，在一个方面，细胞运送体包括增量剂以及包含这些细胞的自体运送体，所述增量剂包含圆的光滑的聚甲基丙烯酸甲酯微粒(其被预加载在混合和递送系统内)。在另一实施方案中，向组织、器官(例如，心脏)和/或需要其的个体再施用于生物相容性交联基质中的所述细胞，如例如美国专利申请公开号20050027070中所述。

[0209] 在另一实施方案中，向组织、器官和/或需要其的个体再施用(例如，注入或移入)于生物相容性非免疫原性涂层内或由生物相容性非免疫原性涂层所保护的本发明的细胞(例如，通过实施本发明方法制得的细胞)，所述涂层例如在合成植入体的表面上，例如如美国专利号6,969,400中所述，该美国专利描述了例如以下方案：其中AC6mut可以缀合到聚乙二醇，该聚乙二醇已被修饰而含有多个亲核基团，如伯氨基或硫醇基。

[0210] 在一个实施方案中，使用如例如美国专利号7,442,390；5,733,542中所述的移植方法向组织、器官和/或需要其的个体再施用(例如，注入或移入)本发明的细胞(例如，通过

实施本发明方法制得的细胞)。

[0211] 可以使用用于向组织或器官(例如,心肌细胞、心脏)递送多肽、核酸和/或细胞的任何方法,并且这些方案在本领域内众所周知,例如,如美国专利号(USPN)7,514,401中所述,该美国专利描述了例如使用向心脏原位冠状动脉内(IC)、静脉内(IV)和/或局部递送(直接心肌注射)多肽、核酸和/或细胞。例如,在替代性实施方案中,气溶胶药物粒子进入肺以及进入血流中、基因疗法、连续输注、重复注射和/或缓释聚合物可以被用于向组织或器官(例如,肺、肾脏、心脏)递送多肽、核酸和/或细胞。在替代性实施方案中,核酸和/或细胞可以通过导管被给予冠状动脉中或通过有限的胸廓切开术进行直接注射被给予左心房或心室心肌中;或通过在心导管插入术期间通过的导管被递送到心肌中;或被递送到心包腔中。

[0212] 在替代性实施方案中,用于实施本发明的核酸或包含用于实施本发明的核酸的载体(例如,腺病毒相关病毒或载体(AAV)、或腺病毒基因疗法载体)、或本发明的囊泡、脂质体、纳米粒子或纳米脂质粒子(NLP)等,例如如USPN 7,501,486中所述至组织或器官(例如,肺、肾脏、心脏)。

[0213] 用于实施本发明的组合物可以与其它治疗剂组合使用,所述其它治疗剂例如血管生成剂、抗血栓剂、抗炎剂、免疫抑制剂、抗心律不齐剂、肿瘤坏死因子抑制剂、内皮素抑制剂、血管紧张素转化酶抑制剂、钙拮抗剂、抗生素剂、抗病毒剂和病毒载体。

[0214] 用于实施本发明的组合物可以用于改善或治疗各种心脏病变和心血管疾病中的任何一种,例如,心脏病变和心血管疾病,例如,冠状动脉疾病(CAD);动脉粥样硬化;血栓形成;再狭窄;血管炎,包括自身免疫性血管炎和病毒性血管炎,如结节性多动脉炎、变应性肉芽肿血管炎、高安动脉炎、川崎病和立克次体血管炎;动脉粥样硬化性动脉瘤;心肌肥大;先天性心脏疾病(CHD);缺血性心脏疾病和绞痛;获得性瓣膜疾病/心内膜疾病;原发性心肌病,包括心肌炎;心律不齐;和移植排斥;代谢性心肌病和心肌病变,如充血性心肌病变、肥大性心肌病变或限制性心肌病变;和/或心脏移植。

[0215] 试剂盒和说明书

[0216] 本发明提供包含本发明组合物和方法的试剂盒,包括其使用说明书。因此,还可提供本发明的细胞、递送运载体、载体、表达载体、重组病毒等。

[0217] 例如,在替代性实施方案中,本发明提供包含组合物的试剂盒,所述组合物包含(a)编码AC6mut的核酸、(b)本发明的递送运载体、载体、表达载体、重组病毒等、(c)本发明的液体或水性制剂、或(d)本发明的囊泡、脂质体、纳米粒子或纳米脂质粒子。在一个方面,所述试剂盒进一步包含用于实施本发明的任何方法的说明书,所述方法例如用于向心肌细胞递送期望的AC6mut或表达AC6mut的核酸、载体等的体外或离体方法。

[0218] 将参考以下实施例来进一步描述本发明;然而,应当理解,本发明并不限于此类实施例。

[0219] 实施例

[0220] 实施例1:不能产生cAMP的AC的递送增加心脏功能

[0221] 本实施例证实本发明的示例性实施方案的有效性:向心肌细胞递送不能产生cAMP的AC用于治疗心力衰竭。在本研究中,我们探问减少LV cAMP产生的AC突变分子是否会通过其单独对Ca2+处置的作用而对左心室(LV)功能具有有利的作用。

[0222] 正性肌肉强缩剂(positive inotrope)的如此多的临床试验已经失败,因此现在不言自明的是,增加cAMP的药剂对衰竭的心脏有害。一种替代策略是使用不影响cAMP的药剂改变心肌Ca^{2+}处置或肌丝对Ca^{2+}的响应。虽然左心室(LV)功能与腺苷酸环化酶(AC)活性紧密关联,但AC的有益作用可能与cAMP无关,而是源于对Ca^{2+}处置的作用。

[0223] 在本研究中,我们生成了具有不能产生环腺苷酸的(不能产生cAMP的)腺苷酸环化酶6型(AC6)多肽(被称为的“AC6突变”或“AC6mut”)的心脏定向表达的转基因小鼠。这些AC6mut转基因小鼠的心肌细胞显示响应异丙肾上腺素的受损的cAMP产生(74%降低;$p<0.001$),但LV大小和功能正常。经分离的心脏显示响应异丙肾上腺素刺激的保持的LV功能。AC6mut表达与增加的肌浆网Ca^{2+}摄取相关并且用于SERCA2a激活的EC50被降低。从AC6mut小鼠分离的心肌细胞显示响应异丙肾上腺素的Ca^{2+}瞬变的增加的幅度($p=0.0001$)。AC6mut表达还与LV S100A1的增加的表达($p=0.03$)和受磷蛋白蛋白质的降低的表达($p=0.01$)相关。本研究确定LV AC突变表达与正常的心脏功能相关,尽管cAMP生成受损。所述机制似乎通过对Ca^{2+}处置的作用—尽管cAMP降低但仍然发生的作用进行。

[0224] 来自先前研究的数据表明增加的心AC 6型(AC6)(在哺乳动物心肌细胞中表达的优势AC异型体[6])对衰竭的左心室(LV)具有多种有益作用[7],[8],[9],[10],[11],[12]。这些出乎意料的有益作用必须与对心脏的贝塔(beta)(β)肾上腺素能受体(βAR)刺激和细胞内cAMP的升高的可怕后果协调[13],[14],[15],[16],[17],[18]。实际上,衰竭的心脏中的AC6表达的明显益处是矛盾的。使用药理学抑制剂,来自先前研究的数据表明增加的心脏AC6表达的一些有益作用不取决于增加的cAMP生成[2],[3]。由于在被培养的心肌细胞中使用药理学抑制的研究的固有限制,因此我们通过在催化核心中的第426位(426位:位置编号基于SEQ ID NO:16)进行Ala对Asp的取代生成了催化无活性的鼠AC6突变(AC6mut)分子,预测该变化改变Mg^{2+}结合但不影响G-蛋白质动力学[4]。当被体外研究时,该鼠AC6mut分子显著地损害cAMP生成,但保持与AC6相关的细胞分布模式[4]。此类体外研究远未确定此类分子如何可能影响体内心脏功能。

[0225] 因此,我们生成了具有AC6mut的心脏定向表达的转基因鼠系。我们希望这样的系会提供关于cAMP相对于Ca^{2+}处置对完整正常心脏的功能的作用区别的额外了解。此外,此类研究可以表明AC6mut是否提供无增加的cAMP的潜在有害作用的影响肌肉收缩的刺激。我们假设,尽管cAMP生成能力显著降低,但通过AC6对Ca^{2+}处置赋予的有益的平衡作用,LV功能仍会保持正常。

[0226] 方法

[0227] AC6mut转基因小鼠的生成(图1A)。动物的使用依照实验室动物护理评估和认证协会(Association for Assessment and Accreditation of Laboratory Animal Care)准则并且被VA圣地亚哥健康保健系统的公共机构动物护理和使用委员会(Institutional Animal Care and Use Committee of VA San Diego Healthcare System)批准。为了生成具有AC6mut的心脏定向表达的小鼠,在α-肌球蛋白重链启动子与SV40polyA之间亚克隆在C-末端具有AU1标签的鼠AC6mut cDNA[4]。含有表达盒的9.2-kb片段被用于在加利福尼亚大学圣地亚哥分校(University of California,San Diego)的转基因小鼠设施(近交C57BL/6)中实施的原核注射。通过从尾部尖端制备的基因组DNA的聚合酶链式反应(PCR)鉴别首建者(Founder)小鼠。

[0228] 使用与α-MHC启动子同源的引物(正向:5'CACATAGAAGCCTAGCCCACACC)(SEQ ID NO:1)来检测AC6mut基因;反向引物用于AC6区(5'CAGGAGGCCACTAAACCATGAC)(SEQ ID NO:2)。

[0229] 使用以下引物来检测AC6mut mRNA:(正向:5'TGGGCCTCTCTACTCTGCAT(SEQ ID NO:3);反向:5'TGGATGTAACCTCGGGTCTC)(SEQ ID NO:4),使得能够对AC6mut mRNA相对于内源性AC6mRNA的增加倍数进行定量。

[0230] 使用与其3'-非翻译区同源的引物(正向:5'GGCATTGAGTGGGACTTTGT(SEQ ID NO:5);反向:5'TCTGCATCCAAACAAACGAA)(SEQ ID NO:6)来检测内源性AC6mRNA。该3'非翻译区不存在于AC6mut cDNA中,使得能够对内源性AC6进行定量。

[0231] 将首建者动物与相同品系的野生型小鼠杂交,并且将所选动物用于心脏转基因表达的分析。我们记录了独立系中可变转基因表达并且选择了AC6mut蛋白质表达17倍增加(相对于内源性AC6)的系用于我们的研究。如先前所述[5]使用定量RT-PCR来确定AC 2-9型的LV表达水平。

[0232] 超声心动描记术。利用5%异氟烷(以1L/min氧的流动速率)诱导麻醉并且利用氧中1%异氟烷维持。如先前所报道的[7],使用16L MHz线性探针和Sonos 5500®超声心动描记器系统(Philips Medical Systems,Bothell,WA)获得图像。在不了解群组特性的情况下获得数据并分析。

[0233] 被分离的灌注心脏:LV收缩功能。如先前所报道的[7],以不受反射激活或麻醉影响的方式在被分离的灌注心脏中评估心脏功能以评估LV收缩功能。采用心室内气囊导管来测量等容LV压力(LV舒张末期压10mmHg;1.7mM离子化Ca^{2+})。当记录LV压力时,以推注剂量(0.1nM到300nM)以5分钟间隔递送异丙肾上腺素。随后,推导出LV压力的一阶导数(LV dP/dt)并且将其用作LV收缩功能的替代物。在不了解群组特性的情况下获得数据并分析。

[0234] 钙摄取。如先前所述[11],通过经修改的Millipore过滤技术测量LV均质物中的ATP-依赖性肌浆网(SR)Ca^{2+}摄取的初始速率。

[0235] 钙瞬变。如先前所述[19],使用Indo-1测量细胞溶质钙瞬变。将心肌细胞平铺到经层粘连蛋白涂覆的玻璃盖片上并且用indo-1/AM(3μM,Calbiochem,La Jolla CA)和分散剂pluronic F-127(0.02mg/ml,Calbiochem,La Jolla CA)加载30min。在加载染料后,将盖片安装在表面灌流室(superfusion chamber)上,冲洗以去除过量的indo-1/AM,并且安装在装配有40x物镜的Nikon DIAPHOT™落射荧光显微镜上,该物镜介接到通过单色仪将激发波长设定为365nm的Photon Technologies测光系统(Birmingham NJ)。通过分别以405nm和485nm为中心的20-nm带通滤波器将荧光发射分离并且引导到两个光电倍增管。比率F405/F485代表$[Ca^{2+}]i$的量度。在这些测量期间,用含有2mM $CaCl_2$的25mM HEPES(pH7.3)对心肌细胞进行表面灌流。在0.3Hz下对肌细胞进行场刺激。通过向缓冲液中添加异丙肾上腺素(10μM)来确定异丙肾上腺素刺激的Ca^{2+}瞬变。从每个心脏至少20个细胞记录钙瞬变,并且每组至少3个心脏。每次循环分别从基线F405/F485比率和最大F405/F485比率获得心脏舒张细胞内Ca^{2+}水平和心脏收缩细胞内Ca^{2+}水平。

[0236] 心肌细胞分离。如先前所述[4]进行心肌细胞分离。

[0237] 环AMP测量。用异丙肾上腺素(10μM,10min)或水溶性福司柯林(forskolin)类似物NKH477(10μM,10min)刺激被分离的心肌细胞,然后将其溶解(2.5%十二烷基三甲基溴化

铵、0.05M乙酸钠(pH 5.8)和0.02％牛血清白蛋白)。如先前所报道的[4],使用cAMP BIOTRAK™酶免疫测定系统(GE Healthcare,Pittsburgh,PA)测量环AMP。

[0238] PKA活性测定。用异丙肾上腺素(10μM,10min)或NKH477(10μM,10min)刺激被分离的心肌细胞。将心肌细胞在缓冲液A(20mM Tris-HCl(pH 7.4)、0.5mM EGTA,0.5mM EDTA和蛋白酶抑制剂混合剂,来自Invitrogen)中均质化并且离心(14,000x g,5min,4℃)。将上清液与PKA生物素化的肽底物(SignaTECT® (SIGNATECT®)cAMP-依赖性蛋白质激酶测定系统(Promega,Madison WI))在[γ-^{32}P]ATP存在下孵育。利用抗生蛋白链菌素基质回收^{32}P-标记的生物素化的底物,并且确定PKA的比活性。

[0239] 兰尼碱(Ryanodine)受体-2、PLB和肌钙蛋白I在心肌细胞中的被异丙肾上腺素刺激的磷酸化。为了确定关键Ca^{2+}调控蛋白质的动态磷酸化,我们在从各组分离的被培养的心肌细胞中进行了RyR2、PLB和TnI的基线磷酸化和异丙肾上腺素刺激的磷酸化的研究(图2C)。在这些研究中使用被培养的心肌细胞(每孔100,000个细胞)并且在与异丙肾上腺素(10μM,10min)孵育之前和之后进行免疫印迹。将细胞在溶解缓冲液中(20mM Tris-HCl(pH 7.5)、150mM NaCl、1mM Na2EDTA、1mM EGTA、1％Triton、2.5mM焦磷酸钠、1mMβ-甘油磷酸盐、1mM Na3VO4、1μg/ml亮抑酶肽)溶解。使用Bradford方法测量蛋白质浓度。将免疫印迹归一化到GAPDH并且进行比较(图2D)。

[0240] PDE活性测定。使用环核苷酸磷酸二酯酶测定试剂盒(Enzo)测定磷酸二酯酶活性。将LV组织在含有10mM Tris-HCl(pH 7.4)、1mM PMSF、10mM激活的原钒酸盐、1x蛋白酶抑制剂混合剂(Life Sciences)的缓冲液中均质化并且在微量离心机中以10,000rpm(10min)离心。通过凝胶过滤使用脱盐柱树脂(Enzo)将组织均质物脱盐。向各孔中添加20μg蛋白质(Bradford)并且测量PDE活性。

[0241] 免疫荧光。将被分离的心肌细胞附着到经层粘连蛋白涂覆的2孔室载玻片达1hr,进行洗涤,固定(10％福尔马林,15min,23℃),用正常的山羊血清封闭(1hr)并且与以下孵育(4℃,过夜):抗AU1抗体(Fitzgerald,1:300;用于检测AC6mut转基因蛋白质);抗Cav3抗体(BD Pharmagen,1:100;用于检测细胞质膜微囊);抗PDI抗体(Invitrogen,1:1000;用于检测SR);抗核纤层蛋白A(Abcam,1:200;用于检测核包膜);抗CREM-1抗体(Santa Cruz,1:50);或抗磷酸化CREB抗体(Upstate,1:100)。将心肌细胞用PBS洗涤,然后与二级抗体(Alexia Fluo 488或594缀合的,1:1000稀释)孵育1hr。为了鉴别细胞核,将细胞与Hoechst染料(1:1000稀释,20min)孵育。然后如先前所述[2]使心肌细胞成像。

[0242] mRNA和免疫印迹的检测。使用定量反转录聚合酶链式反应(RT-qPCR)对mRNA进行定量并且使用免疫印迹对蛋白质含量进行定量[4]。RyR2的引物包括(正向:5'AACCTACCAGGCTGTGGATG)(SEQ ID NO:7);和(反向:5'GACTCGATGGGCAAGTCAAT)(SEQ ID NO:8)。

[0243] 我们使用抗AC5/6抗体来鉴别内源性AC6和AC6mut(Santa Cruz,1:200稀释)。AC5/6抗体的表位在AC6和AC6mut的C-末端(序列:KGYQLECRGVVKVKGKGEMTTYFLNGGPSS(SEQ ID NO:9);蛋白质登录号为O43306和Q01234)。我们使用AU1抗体(Fitzgerald,1:2,000)来检测AC6mut蛋白质。所用的额外抗体包括:钙网织蛋白(ABR Affinity,1:1,000);肌集钙蛋白(Novus Biologicals,1:1,000);GAPDH(Fitzgerald,1:20,000);PDE3A(Advam);PKA催化亚单位(BD Transduction,1:1,000);p-PKA催化亚单位(Cell Signaling,1:1,000);PKA-RII

α和PKA-RIIβ(BD Transduction,1:1,000);磷酸化PKA-RIIα(S96)和磷酸-PKA-RIIβ(S114)(Santa Cruz,1:200);PKCα催化亚单位(Santa Cruz,1:200);PLB(Affinity Bioreagents,1:5,000);磷酸化S16-PLB(Badrilla,1:3,000稀释);磷酸化RyR2(S2808)(Abcam,1:1,000);S100A1(Epiyomics,1:1,000);SERCA2a(Enzo,1:1,000);肌钙蛋白I和磷酸化TnI(S22/23)(Cell Signaling,各1:1,000)

[0244] 统计学分析。数据表示平均值±SE;使用ANOVA,之后使用邦弗朗尼(Bonferroni)t检验,或在适当时使用学生t检验(不成对的,双尾)测试群组差异的统计学显著性。当$p<0.05$时,拒绝零假设。

[0245] 结果

[0246] AC6mut转基因小鼠。AC6mut小鼠与它们的转基因阴性同胞在身体上不可区分。对成年小鼠的尸体剖检显示体重、胫骨长度、LV重量和肺重量未显示群组差异。(表1)。

[0247] AC6mut的LV表达。相对于内源性AC6的水平,AC6mut mRNA增加了62倍并且蛋白质增加了17倍,它们是使用针对内源性AC6和转基因AC6mut两者上的共同区域的引物和抗体在RT-PCR和免疫印迹中检测的(图1B和图1C)。

[0248] 内源性AC类型的LV表达。内源性AC 2-9型的mRNA未显示群组差异(数据未显示)。

[0249] LV cAMP产生。来自AC6mut小鼠的LV样品显示当用异丙肾上腺素(74%降低;$p<0.001$)或NKH477(一种水溶性福司柯林类似物)(52%降低;$p=0.05$)刺激时的降低的cAMP产生(图1D);基线cAMP产生不变。因此,转基因系适于测试在增加的AC6mut表达存在下降低的被βAR刺激的cAMP产生对LV功能的总体作用。

[0250] PKA活性和表达。从AC6mut小鼠分离的心肌细胞显示基线PKA活性的48%降低($p=0.01$)。另外,存在通过异丙肾上腺素(38%降低;$p=0.006$);和NKH477(38%降低;$p=0.001$)刺激的PKA活性的降低(图2A,上部)。AC6mut表达未改变PKA催化亚单位的LV表达(图2A,下部)或PKA-RII-α和PKA-RII-β的表达或磷酸化(磷酸化PKA-RIIα:AC6mut,0.32±0.04du;对照,0.30±0.03du,$p=0.7$;磷酸化PKA-RIIβ:AC6mut,7.1±1.1du;对照,10.6±01.4du;$p=0.09$;图2B)。PKC催化亚单位表达也未显示群组差异(PKCα:AC6mut,0.8±0.1du;对照,0.7±0.1du,$p=0.4$;图2B)

[0251] 兰尼碱受体-2、PLB和肌钙蛋白I在心肌细胞中的被异丙肾上腺素刺激的磷酸化。RyR2、PLB和TnI的基线磷酸化未显示群组差异(P-RyR2:AC6mut,4.4±0.6,相对于对照,2.4±0.5du,$p=0.06$;P-PLB:AC6mut,0.3±0.03,相对于对照,0.2±0.1du,$p=0.8$;P-TnI:AC6mut,0.8±0.2,相对于对照,1.0±0.01du,$p=0.24$,图2C)。异丙肾上腺素刺激与两组中RyR2、PLB和TnI的增加的磷酸化(相对未被刺激的)相关,但来自AC6mut小鼠的LV中的磷酸化程度通常更大(P-RyR2:AC6mut,30.0±1.1,相对于对照,7.4±1.1du,$p=0.001$,;P-PLB:AC6mut,16.8±2.4,相对于对照,5.3±0.1du,$p=0.01$;P-TnI:AC6mut,5.8±1.4,相对于对照,2.2±0.7du,$p=0.07$;图2C)。组间的TnI蛋白质表达并无不同(AC6mut,0.9±0.1,相对于对照,0.7±0.2du;$p=0.5$;图2B。RyR2mRNA表达未显示群组差异。

[0252] PDE活性和PDE3A表达。LV样品中的PDE活性没有群组差异(AC6mut:1252±23单位/mg,$n=7$;对照:1293±39单位/mg,$n=6$;$p=0.38$)。LV PDE3A蛋白质表达未显示群组差异(AC6mut:0.3±0.1,相对于对照,0.4±0.1du,$p=0.6$.图2B)。

[0253] AC6mut的细胞内分布。鉴别AC6mut蛋白质结合细胞质膜微囊(主要结合质膜)、SR

和核包膜(图1E)。

[0254] 超声心动描记术。超声心动描记术显示AC6mut的心脏定向表达未改变基本心脏结构和功能。组间的LV尺寸并无不同,且基线LV射血分数和周缘纤维缩短速度是相似的(表2)。因此,尽管AC6mut小鼠中的LV cAMP生成能力显著降低,但LV结构和基本功能不变。

[0255] 响应异丙肾上腺素的LV收缩功能。为了以独立于自主神经影响、内源性儿茶酚胺和麻醉的方式评估心脏收缩性,在被分离的灌注心脏中测量LV压力发展。尽管LV cAMP生成能力显著降低,但基线LV dP/dt和异丙肾上腺素刺激的LV dP/dt未显示群组差异(图3)。

[0256] Ca^{2+}摄取和Ca^{2+}相关蛋白质。确定从AC6mut和转基因-阴性同胞对照小鼠汇集的LV均质物中的ATP依赖性SR Ca^{2+}摄取速率。增加的AC6mut表达与增加的SR Ca^{2+}摄取相关(图4A,上部图形)。另外,SERCA2a对Ca^{2+}的增加的亲和力反映在实现一半最大效应所需的降低的Ca^{2+}浓度上(EC50:AC6mut 1.1μmol/L;对照3.7μmol/L,n=6,图4A,下部图形)。

[0257] 与Ca^{2+}处置的这些生理变化相关的是调控SR Ca^{2+}摄取的蛋白质的改变的LV表达。例如,AC6mut表达与LV PLB蛋白质表达的43%降低(p=0.01)和LV S100A1蛋白质含量的73%增加(p=0.03)相关(图4B和图4C)。LV SERCA2a、钙网织蛋白和肌集钙蛋白的含量不变,并且Ser16处的PLB磷酸化不变(图4D)。

[0258] 转录因子。AC6mut表达与CREM-1的LV表达的1倍增加(p=0.03,图4B)和CREB在Ser133处的磷酸化的0.7倍增加(p=0.01,图4C)相关;总CREB蛋白质含量不变。为了确定增加的CREM-1和磷酸化CREB是否存在于细胞核中,使用抗CREM-1和抗磷酸化CREB(S133)抗体进行被分离的心肌细胞的免疫荧光染色。我们检测到CREM-1和磷酸化CREB在AC6mut小鼠中的增加的核定位(图4E)。

[0259] 钙瞬变:为了确定与AC6mut表达相关的增加的SR Ca^{2+}摄取是否会影响细胞溶质$[Ca^{2+}]i$,使用参比(ratiometric)染料Indo-1评估心肌细胞实时$[Ca^{2+}]i$。收缩期间的基线Ca^{2+}释放不变(图5A)。然而,AC6mut表达与在异丙肾上腺素刺激后的增加的峰值心脏收缩Ca^{2+}瞬变幅度相关(p=0.0001,图5B和图5C),并且达到峰值幅度的时间缩短(p=0.03,图5D)。另外,来自AC6mut小鼠的心肌细胞中达到50%松驰的时间(τ)缩短(p=0.04)(图5E)。因此,SERCA2a活性、PLB和S100A1的表达以及被异丙肾上腺素刺激的Ca^{2+}瞬变全部通过AC6mut表达以会有利地影响LV功能的方式被改变。

[0260] 讨论

[0261] 本研究的最令人惊讶且重要的发现是显著地损害被βAR刺激的cAMP产生的突变AC6分子的心脏定向表达与响应异丙肾上腺素刺激的保持的LV功能相关。这是通过被分离的灌注心脏中的超声心动描记术和收缩功能研究确认的。在其它背景下心脏cAMP生成的显著降低与LV收缩功能的成比例降低相关。例如,在其中cAMP损害通常被降低50%的多数心力衰竭模型中,存在LV dP/dt和βAR-响应性的类似降低[10],[11],[12],[13],[14]。此外,与cAMP生成能力的60%降低相关的AC6缺失也与LV收缩功能的类似降低相关[5]。那么是什么解释被异丙肾上腺素刺激的LV收缩功能的保持?

[0262] AC6mut系中尽管显著地损害cAMP生成,但LV功能得以保持的最接近的机制是关于Ca^{2+}处置的有利变化。我们先前报道AC6的心脏定向表达增加衰竭的心脏的功能,但由于AC6对βAR信号传导的显著作用,因此不可能确定这些有益作用所反映的增强的βAR信号传导自身相对于Ca^{2+}处置的程度[10],[11]。以下观察支持AC6与Ca^{2+}处置的联系:AC6缺失对Ca^{2+}处

置具有显著不利的作用[5],但由于cAMP-生成能力在AC6缺失后降低,因此AC6对Ca^{2+}处置的独立作用难以确定。然而,本研究中的新颖之处在于在TG小鼠中证实AC6突变分子似乎模拟了母体分子对Ca^{2+}处置的有利作用,由此保持LV功能,即使在cAMP生成能力显著降低时。似乎AC6对Ca^{2+}处置的作用无需cAMP生成,因此必定通过替代机制进行。

[0263] 我们发现AC6mut表达与LV均质物中增加的SR Ca^{2+}摄取和完整心肌细胞中具有缩短的松驰时间的增加的Ca^{2+}瞬变相关。与AC6mut表达的这些生理有利的作用相关的是降低的PLB表达,抑制SERCA2a活性的Ca^{2+}调控因子。降低的PLB含量或Ser16处增加的PLB磷酸化与其抑制作用的降低相关,这增加了SERCA2a活性[20],[21],[22]。我们先前发现PLB表达在表达AC6或AC6mut的被培养的心肌细胞中降低[4],但现行研究首次证实该作用也在体内见到(图4B)。从AC6mut小鼠分离的心肌细胞中RyR2、PLB和(在更小的程度上)TnI的被异丙肾上腺素刺激的磷酸化的程度的增加(图2C)也被预测会增加LV收缩功能。

[0264] AC6mut表达与CREM-1(图3B和图3E)的增加的表达和核转位相关,CREM-1是CREB/ATF家族中的转录抑制物[23]。我们先前鉴别,在AC6表达的背景下,PLB启动子在新生大鼠心肌细胞中由增加的ATF3通过PLB启动子中的CRE位点被负调控[2]。在本研究中,我们未见到与AC6mut表达相关的增加的ATF3表达。然而,ATF3和CREM-1两者均识别同一CRE位点,因此似乎合理的是,与AC6mut有关的增加的CREM-1可能在降低的PLB表达方面在机制上是重要的。这将需要额外的研究。

[0265] AC6mut表达与Ca^{2+}敏化蛋白质S100A1的LV表达的未曾预料到的增加相关,该蛋白质通过调节RyR2和SERCA2a来增加收缩功能[24]。AC6mut表达如何可能与增加的LV S100A1表达关联?AC6mut表达与CREB的增加的磷酸化和核转位相关(图4C和图4E),CREB的磷酸化和核转位是CREB激活所需的过程。CREB是通过基因的启动子中的CRE位点来调控许多基因的转录激活子[25]。S100A1启动子具有CRE位点[26],表明S100A1表达似乎可能已被AC6mut表达激活。另外,PKA和cAMP的区室化也可能是因素[27],[28]。

[0266] 尽管cAMP生成显著降低,但Ca^{2+}处置的实质性改进似乎保持了LV功能。增加量的AC6mut借以影响转录调控并且最终影响心肌细胞的生理行为和LV功能的精确途径将需要额外的研究。组织学研究(图1E)确认大量的转基因AC6mut存在于多个细胞内区室中,而不是仅仅存在于质膜中。这使得AC6mut蛋白质能够与影响细胞内信号传导且因此影响生理功能的重要的细胞内蛋白质相互作用。

[0267] 最近通过AC6缺失强调了AC6对于Ca^{2+}处置的重要性[5]。在这一背景下,cAMP生成能力降低,虽然未达到与本研究中一样多的程度,但Ca^{2+}处置显著受损。在本研究中,我们见到cAMP生成的更显著损害,但Ca^{2+}处置增加,而非降低。这是因为,不同于在AC6缺失中,AC6分子虽然cAMP生成能力不足,但存在于胞质中,在此AC6分子可能影响Ca^{2+}处置。

[0268] 我们未检查表达降低量的AC6mut的转基因系以确定生理作用是否与AC6mut表达的水平成比例。有人可能认为AC6mut蛋白质的17倍增加(相对于内源性AC6)可能以非特异性方式影响信号传导。虽然我们的数据不能排除这种可能性,但重要的是认识到内源性AC6是超低丰度的蛋白质—丰度是例如Gsα的大约1/100[29]。因此,即使以内源性AC617倍高的水平表达,其丰度仍然比Gsα低。此外,催化活性(正常)AC6的类似增加与可恢复的cAMP产生的显著增加相关[30]。这些观察表明该发现是特异的。

[0269] 结论。尽管cAMP生成显著降低,但Ca^{2+}处置的实质性改进似乎保持了LV功能。免疫

荧光表明AC6mut位于核膜上，为AC6mut影响转录因子的表达和功能提供机会。增加的CREM-1(转录抑制子)和增加的磷酸-CREB(图4E)可能分别参与PLB和S100A1的改变的表达。我们推断AC6mut通过增加的Ca^{2+}处置和改变的蛋白质表达来保持心脏功能，尽管cAMP生成降低。这些结果提供了关于对于LV功能在Ca^{2+}处置与βAR信号传导之间的相互作用的了解，并且表明AC6mut可以提供没有增加的cAMP的潜在有害作用的肌肉收缩刺激。数据表明与衰竭的心脏中的AC6mut表达相关的降低的心肌细胞凋亡，这是我们实验室中正在进行的研究的焦点。

附图说明

[0270]

[0271] 图1.AC6mut的设计、表达、活性和细胞分布

[0272] A.该图描绘了在AC6mut的构造中在C1结构域(细胞内环)中的第426位(位置编号基于SEQ ID NO:16)丙氨酸(ala)对天冬氨酸(asp)的取代位点。该取代抑制Mg^{2+}结合并且改变催化核心的由Gsα介导的激活的效率，这损害了AC6的酶促活性，从而导致降低的cAMP产生。M1和M2，AC6的跨膜结构域；C1和C2，AC6的胞质结构域，其形成催化核心；βAR，β-肾上腺素能受体；βϒ和α，鸟苷5'-三磷酸(GTP)-结合蛋白质Gs的组分

[0273] B.AC6mut mRNA表达是通过qRT-PCR使用内源性AC6和转基因AC6mut共同的引物评估的。用于检测GAPDH mRNA的引物用于qRT-PCR反应的内部对照。相对于内源性AC6，AC6mut mRNA增加了62倍。条形中的动物数+SE；学生t检验(不成对的，双尾)

[0274] C.以免疫印迹法使用抗AC5/6抗体检测AC6mut蛋白质并且使用抗AU1标签抗体确认。相对于内源性AC6，AC6mut蛋白质增加了17倍。

[0275] D.用异丙肾上腺素(Iso；10μM，10min)或NKH477(NKH；10μM，10min)刺激之前(基线)和刺激之后，从AC6mut小鼠和对照小鼠分离的心肌细胞中的环AMP产生；cAMP酶免疫测定。来自AC6mut小鼠的心肌细胞(M相对于C，对照)显示响应Iso和NKH477(福司柯林类似物)的受损的cAMP产生。条形表示平均值+SE；p值源于单因素ANOVA和之后的邦弗朗尼后检验(n＝6，各组)。

[0276] E.使用抗AU1抗体(红色)、抗小窝蛋白3(Cav-3)抗体(绿色，对于细胞质膜微囊来说)；抗蛋白质二硫键异构酶(PDI)抗体(绿色，对于肌浆网来说)；抗核纤层蛋白A抗体(绿色，对于核膜来说)和抗电压依赖性阴离子选择性通道蛋白质(VDAC)抗体(绿色，对于线粒体来说)对从AC6mut小鼠和对照小鼠分离的心肌细胞中的AC6mut蛋白质的双重免疫荧光染色。细胞核是蓝色。在细胞质膜微囊、SR和核膜中检测AC6mut转基因，但与线粒体无关。

[0277] 图2.PKA、PKS和PDE的活性和表达

[0278] A.上图：无刺激(基线)或用异丙肾上腺素(Iso；10μM，10min)或NKH477(NKH；10μM，10min)刺激的被分离的心肌细胞中的PKA活性。AC6mut表达降低了基线PKA活性(p＝0.01)并且Iso(p＝0.001)和NKH(p＝0.001)二者活性也降低(n＝3，各组)。下部凝胶：LV均质物中的PKA蛋白质。AC6mut表达未改变LV PKA催化亚单位蛋白质表达。

[0279] B.使用来自AC6mut小鼠和对照小鼠的左心室均质物在免疫印迹中显示关键信号传导蛋白质的表达和它们的磷酸化。未观察到群组差异。显示了磷酸化(P)PKA和总(T)PKA的调控亚单位II-α和II-β、PKCα、磷酸二酯酶3A型(PDE3A)、磷酸-肌钙蛋白I(P22/23-TnI)

和总TnI。

[0280] C.在从各组分离的被培养心肌细胞中评估异丙肾上腺素刺激之前和之后的RyR2、PLB和TnI的磷酸化。RyR2、PLB和TnI的基线磷酸化未显示群组差异。异丙肾上腺素刺激与两组中的RyR2、PLB和TnI的增加的磷酸化相关，但在来自AC6mut小鼠的心肌细胞中更加大量(图2C)。

[0281] D.来自图2C的数据以针对载荷(GAPDH)被归一化的图表形式显示，所述数据表明异丙肾上腺素刺激与来自AC6mut小鼠的心肌细胞中的RyR2、PLB和TnI的增加的磷酸化相关。TnI磷酸化的增加不是统计学显著的(p＝0.07)。

[0282] 图3.左心室收缩功能

[0283] 从AC6mut TG小鼠(实心圆;n＝11)分离的心脏显示响应通过宽范围的异丙肾上腺素剂量进行的异丙肾上腺素刺激的保持的LV dP/dt。在不了解群组特性的情况下获得数据并分析。空心圆，转基因阴性对照小鼠(n＝12)。没有群组差异(双因素ANOVA)。数据点表示平均值±SE。

[0284] 图4.SR Ca^{2+}摄取、Ca^{2+}信号传导蛋白质和转录因子

[0285] A.上部:来自AC6mut小鼠的混合LV样品和来自TG阴性同胞对照小鼠(n＝6，两组)混合LV样品中的Ca^{2+}摄取活性

[0286] 下部:AC6mut的表达降低了SERCA2a对Ca^{2+}的亲和力。从不同的游离Ca^{2+}浓度下的初始ATP依赖性Ca^{2+}摄取速率计算Ca^{2+}实现50％最大效应的有效浓度(EC_{50})。

[0287] B.上部:AC6mut表达与降低的LV受磷蛋白(PLB)表达相关。

[0288] 下部:AC6mut表达与增加的LV CREM-1蛋白质表达相关。

[0289] C.上部:AC6mut表达与增加的LV S100A1蛋白质表达相关。

[0290] 下部:AC6mut表达与增加的LV P133-CREB蛋白质表达相关。两组中的总CREB表达类似。

[0291] D.AC6mut表达不影响SERCA2a、钙网织蛋白、肌集钙蛋白或磷酸化S16-PLB蛋白质的LV表达;(n＝4，两组)。

[0292] E.使用抗AU1抗体(红色)和抗CREM-1抗体(绿色)或抗AU1和抗磷酸化CREB(S133，绿色)对从AC6mut小鼠和对照小鼠分离的心肌细胞中的AC6mut蛋白质的双重免疫荧光染色。细胞核以蓝色显示。AC6mut表达增加了CREM-1和磷酸化CREB的核定位。

[0293] 在图(A、B、C)中，条形表示平均值+SE;条形中的数字指示群组大小;条形上方的数字表示来自学生t检验(不成对的，双尾)的p值

[0294] 图5.从AC6mut小鼠和对照小鼠分离的心肌细胞中的细胞溶质Ca^{2+}瞬变

[0295] A.基线Ca^{2+}释放(心脏收缩-心脏舒张Ca^{2+})未显示组差异。

[0296] B.用异丙肾上腺素(Iso;10μM)刺激的心肌细胞中的代表性Indo-1Ca^{2+}瞬变记录在来自AC6mut小鼠的心肌细胞中更高。总结数据展示于图C中。

[0297] C.在异丙肾上腺素存在下释放的Ca^{2+}在来自AC6mut小鼠的心肌细胞中增加。

[0298] D.在异丙肾上腺素存在下达到峰值Ca^{2+}瞬变的时间在来自AC6mut小鼠的心肌细胞中减少。

[0299] E.在异丙肾上腺素存在下达到50％松弛的时间(τ)在来自AC6mut小鼠的心肌细胞中减少。

[0300] 将实验重复4次。条形表示平均值+SE;条形中的数字指示心肌细胞数;条形上方的数字指示来自学生t检验(不成对的,双尾)的p值。

[0301] 参考文献

[0302] 1.Cleland JG,Teerlink JR,Senior R,Nifontov EM,Mc Murray JJ等人,(2011)The effects of the cardiac myosin activator,omecamtiv mecarbil,on cardiac function in systolic heart failure:a double-blind,placebo-controlled,crossover,dose-ranging phase 2trial.Lancet 378:676-683.

[0303] 2.Gao,MH,Tang T,Guo T,Sun SQ,Feramisco JR等人,(2004)Adenylyl cyclase type VI gene transfer reduces phospholamban expression in cardiac myocytes via activating transcription factor 3.J Biol Chem279:38797-3802.

[0304] 3.Gao MH,Tang T,Guo T,Yajima T,Pestonjamasp K等人,(2008)Adenylyl cyclase type VI increases Akt activity and phospholamban phosphorylation in cardiac myocytes.J Biol Chem 283:33527-33535.

[0305] 4.Gao MH,Tang T,Lai NC,Yajima T,Miyanohara A等人,(2011)Beneficial effects of adenylyl cyclase type 6(AC6)expression persist using a catalytically inactive AC6mutant.Mol Pharmacol 79:381-388.

[0306] 5.Tang T,Gao MH,Lai NC,Firth AL,Takahashi T等人,(2008)Adenylyl cyclase type 6deletion decreases left ventricular function via impaired calcium handling.Circulation.117:61-69.

[0307] 6.Ping P,Anzai T,Gao MH,Hammond HK.(1996)Adenylyl cyclase and G protein receptor kinase expression during development of heart failure.Am J Physiol Heart Circ Physiol 273:H707-717.

[0308] 7.Roth DM,Bayat H,Drumm JD,Gao MH,Swaney JS,Ander A等人,(2002)Adenylyl cyclase increases survival in cardiomyopathy.Circulation105:1989-1994.

[0309] 8.Takahashi T,Tang T,Lai NC,Roth DM,Rebolledo B,Saito M等人,(2006)Increased cardiac adenylyl cyclase expression is associated with increased survival after myocardial infarction.Circulation 114:388-396.

[0310] 9.Lai NC,Roth DM,Gao MH,Tang T,Dalton N等人,(2004)Intracoronary adenovirus encoding adenylyl cyclase VI increases left ventricular function in heart failure.Circulation 110:330-336.

[0311] 10.Roth DM,Gao MH,Lai NC,Drumm J,Dalton N等人,(1999)Cardiac-directed adenylyl cyclase expression improves heart function in murine cardiomyopathy.Circulation 99:3099-3102.

[0312] 11.Lai NC,Tang T,Gao MH,Saito M,Takahashi T等人,(2008)Activation of cardiac adenylyl cyclase expression increases function of the failing ischemic heart in mice.J Am Coll Cardiol 51:1490-1497.

[0313] 12.Tang T,Gao MH,Roth DM,Guo T,Hammond HK.(2004)Adenylyl cyclase type VI corrects cardiac sarcoplasmic reticulum calcium uptake defects in

cardiomyopathy.Am J Physiol 287:H1906-1912.

[0314] 13.Gaudin C,Ishikawa Y,Wight DC,Mahdavi V,Nadal-Ginard B等人,(1995)Overexpression of Gsαprotein in the hearts of transgenic mice.J Clin Invest 95:1676-1683.

[0315] 14.Engelhardt S,Hein L,Wiesmann F,Lohse MJ.(1999)Progressive hypertrophy and heart failure inβ1-adrenergic receptor transgenic mice.Proc Natl Acad Sci 96:7059-7064.

[0316] 15.Liggett SB,Tepe NM,Lorenz JN,Canning AM,Jantz TD等人,(2000)Early and delayed consequences ofβ2-adrenergic receptor overexpression in mouse hearts:critical role for expression level.Circulation101:1707-1714.

[0317] 16.Antos CL,Frey N,Marx SO,Reiken S,Gaburjakova M等人,(2001)Dilated cardiomyopathy and sudden death resulting from constitutive activation of protein kinase A.Circ Res 89:997-1004.

[0318] 17.Packer M,Carver JR,Rodeheffer RJ,Ivanhoe RJ,Dibianco R等人,(1991)Effect of oral milrinone on mortality in severe chronic heart failure.The PROMISE Study Research Group.N Engl J Med 325:1468-1475.

[0319] 18.Bristow MR,Ginsburg R,Minobe W,Cubicciotti RS,Sageman WS等人,(1982)Decreased catecholamine sensitivity and beta-adrenergic-receptor density in failing human hearts.N Engl J Med307:205-211.

[0320] 19.Suarez J,Scott B,Dillmann WH.(2008)Conditional increase in SERCA2a protein is able to reverse contractile dysfunction and abnormal calcium flux in established diabetic cardiomyopathy.Am J Physiol295:R1439-1445.

[0321] 20.Brittsan AG,Kranias EG.(2000)Phospholamban and cardiac contractile function.J Mol Cell Cardiol 32:2131-2139.

[0322] 21.Chu G,Lester JW,Young KB,Luo W,Zhai J等人,(2000)A single site (Ser16)phosphorylation in phospholamban is sufficient in mediating its maximal cardiac responses to beta-agonists.J Biol Chem 275:38938-38943.

[0323] 22.Luo W,Chu G,Sato Y,Zhou Z,Kadambi VJ等人,(1998)Transgenic approaches to define the functional role of dual site phospholamban phosphorylation.J Biol Chem 273:4734-4739.

[0324] 23.Foulkes NS,Borrelli E,Sassone-Corsi P.(1991)CREM gene:use of alternative DNA-binding domains generates multiple antagonists of cAMP-induced transcription.Cell 64:739-749.

[0325] 24.Most P,Remppis A,Pleger ST,Löffler E,Ehlermann P等人,(2003)Transgenic overexpression of the Ca2+-binding protein S100A1in the heart leads to increase in vivo myocardial contractile performance.J Biol Chem278:33809-33817.

[0326] 25.Mayr B,Montminy M.(2001)Transcriptional regulation by the phosphorylation-dependent factor CREB.Nat Rev Mol Cell Biol 2:599-609.

[0327] 26.Song W,Zimmer DB.(1996)Expression of the rat S100A1gene in neurons,glia,and skeletal muscle.Brain Res 721:204-216.

[0328] 27.Iancu RV,Ramamurthy G,Harvey RD.(2008)Spatial and temporal aspects of cAMP signaling in cardiac myocytes.Clin Exp Pharmacol Physiol35:1343-1348.

[0329] 28.Efendiev R,Samelson BK,Nguyen BT,Phatarpekar PV,Baameur F等人,(2010)AKAP79interacts with multiple adenylyl cyclase(AC)isoforms and scaffolds AC5and AC6toα-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate(AMPA)receptors.J Biol Chem 285:14450-14458.

[0330] 29.Ostrom RS,Post SR,Insel PA.(2000).Stoichiometry and compartmentation in G protein-coupled receptor signaling:implications for therapeutic interventions involving Gs.J Pharmacol Exp Ther 294:407-412.

[0331] 30.Gao MH,Lai NC,Roth DM,Zhou J,Zhu J,Anzai T,Dalton N,Hammond HK.(1999)Adenylylcyclase increases responsiveness to catecholamine stimulation in transgenic mice.Circulation 99:1618-1622.

[0332]

表 1. 体重、LV 重量和肺重量

	AC6mut (23)	TG-对照(16)	p
体重(g)	25.5±0.7	25.0±1.2	0.7
LV (mg)	91±2.7	89±3.4	0.6
胫骨长度(mm)	17±0.1	16.7±0.2	0.3
LV/身体(mg/g)	3.6±0.1	3.6±0.1	0.9
LV/TL (mg/mm)	5.4±0.1	5.3±0.2	0.7
肺(mg)	150±4.9	149±6.7	0.9
肺/身体(mg/g)	6.0±0.2	6.0±0.2	0.9

[0333] LV,左心室;TL,胫骨长度。值表示平均值±SE;学生t检验(不成对的,双尾)。

[0334]

表 2. 超声心动描记术(基线)

	AC6mut (8)	TG-对照(12)	p
HR (bpm)	501±26	506±17	0.9
EDD (mm)	4.2±0.2	4.3±0.1	0.7
ESD (mm)	2.9±0.2	3.0±0.1	0.4
PW 厚度(mm)	0.6±0.1	0.6±0.1	0.5
室中隔厚度(mm)	0.6±0.1	0.6±0.1	0.4
EDV (μL)	76±7	78±4	0.8
ESV (μL)	25±4	27±2	0.6
EF (%)	69±3	65±2	0.2
CO (μL/min)	26±2	26±2	0.8
Vcf (circ/sec)	7.0±0.7	6.2±0.3	0.2

[0335] 已经描述了本发明的多个实施方案。然而，应当理解，可在不脱离本发明的精神和范围的前提下作出各种修改。因此，其它实施方案在所附权利要求书的范围内。

序列表

<110> 加利福利亚大学董事会

<120> 用于治疗心力衰竭和增加心脏功能的不能产生环腺苷酸的腺苷酸环化酶和组合物和方法

<130> 00015-253W01/SD-2013-027-1 PCT

<140> 待指定
<141> 2014-05-26

<150> 61/832,759
<151> 2013-06-07

<160> 17

<170> PatentIn version 3.5

<210> 1
<211> 23
<212> DNA
<213> 人工序列

<220>
<223> 合成寡核苷酸

<400> 1
cacatagaag cctagcccac acc 23

<210> 2
<211> 22
<212> DNA
<213> 人工序列

<220>
<223> 合成寡核苷酸

[0001]

<400> 2
caggaggcca ctaaaccatg ac 22

<210> 3
<211> 20
<212> DNA
<213> 人工序列

<220>
<223> 合成寡核苷酸

<400> 3
tgggcctctc tactctgcat 20

<210> 4
<211> 20
<212> DNA
<213> 人工序列

<220>
<223> 合成寡核苷酸

<400> 4
tggatgtaac ctcgggtctc 20

<210> 5
<211> 20
<212> DNA
<213> 人工序列

<220>
<223> 合成寡核苷酸

<400> 5
ggcattgagt gggactttgt 20

<210> 6
<211> 20
<212> DNA
<213> 人工序列

<220>
<223> 合成寡核苷酸

<400> 6
tctgcatcca aacaaacgaa 20

<210> 7
<211> 20
<212> DNA
<213> 人工序列

<220>
<223> 合成寡核苷酸

<400> 7
aacctaccag gctgtggatg 20

<210> 8
<211> 20
<212> DNA
<213> 人工序列

<220>
<223> 合成寡核苷酸

<400> 8
gactcgatgg gcaagtcaat 20

[0002]

<210> 9
<211> 30
<212> PRT
<213> 人工序列

<220>
<223> 合成肽

<400> 9

Lys Gly Tyr Gln Leu Glu Cys Arg Gly Val Val Lys Val Lys Gly Lys
1 5 10 15

Gly Glu Met Thr Thr Tyr Phe Leu Asn Gly Gly Pro Ser Ser
20 25 30

<210> 10
<211> 1168
<212> PRT
<213> 智人 (homo sapiien)

<400> 10

Met Ser Trp Phe Ser Gly Leu Leu Val Pro Lys Val Asp Glu Arg Lys
1 5 10 15

Thr Ala Trp Gly Glu Arg Asn Gly Gln Lys Arg Ser Arg Arg Arg Gly
20 25 30

Thr Arg Ala Gly Gly Phe Cys Thr Pro Arg Tyr Met Ser Cys Leu Arg
35 40 45

Asp Ala Glu Pro Pro Ser Pro Thr Pro Ala Gly Pro Pro Arg Cys Pro
50 55 60

```
Trp Gln Asp Asp Ala Phe Ile Arg Arg Gly Gly Pro Gly Lys Gly Lys
65                  70                  75                  80

Glu Leu Gly Leu Arg Ala Val Ala Leu Gly Phe Glu Asp Thr Glu Val
                85                  90                  95

Thr Thr Thr Ala Gly Gly Thr Ala Glu Val Ala Pro Asp Ala Val Pro
            100                 105                 110

Arg Ser Gly Arg Ser Cys Trp Arg Arg Leu Val Gln Val Phe Gln Ser
        115                 120                 125

Lys Gln Phe Arg Ser Ala Lys Leu Glu Arg Leu Tyr Gln Arg Tyr Phe
    130                 135                 140

Phe Gln Met Asn Gln Ser Ser Leu Thr Leu Leu Met Ala Val Leu Val
145                 150                 155                 160

Leu Leu Thr Ala Val Leu Leu Ala Phe His Ala Ala Pro Ala Arg Pro
                165                 170                 175

Gln Pro Ala Tyr Val Ala Leu Leu Ala Cys Ala Ala Ala Leu Phe Val
            180                 185                 190

Gly Leu Met Val Val Cys Asn Arg His Ser Phe Arg Gln Asp Ser Met
        195                 200                 205
```

[0003]

```
Trp Val Val Ser Tyr Val Val Leu Gly Ile Leu Ala Ala Val Gln Val
    210                 215                 220

Gly Gly Ala Leu Ala Ala Asp Pro Arg Ser Pro Ser Ala Gly Leu Trp
225                 230                 235                 240

Cys Pro Val Phe Phe Val Tyr Ile Ala Tyr Thr Leu Leu Pro Ile Arg
                245                 250                 255

Met Arg Ala Ala Val Leu Ser Gly Leu Gly Leu Ser Thr Leu His Leu
            260                 265                 270

Ile Leu Ala Trp Gln Leu Asn Arg Gly Asp Ala Phe Leu Trp Arg Gln
        275                 280                 285

Leu Gly Ala Asn Val Leu Leu Phe Leu Cys Thr Asn Val Ile Gly Ile
    290                 295                 300

Cys Thr His Tyr Pro Ala Glu Val Ser Gln Arg Gln Ala Phe Gln Glu
305                 310                 315                 320

Thr Arg Gly Tyr Ile Gln Ala Arg Leu His Leu Gln His Glu Asn Arg
                325                 330                 335

Gln Gln Glu Arg Leu Leu Leu Ser Val Leu Pro Gln His Val Ala Met
            340                 345                 350

Glu Met Lys Glu Asp Ile Asn Thr Lys Lys Glu Asp Met Met Phe His
        355                 360                 365
```

```
Lys Ile Tyr Ile Gln Lys His Asp Asn Val Ser Ile Leu Phe Ala Asp
    370                 375                 380

Ile Glu Gly Phe Thr Ser Leu Ala Ser Gln Cys Thr Ala Gln Glu Leu
385                 390                 395                 400

Val Met Thr Leu Asn Glu Leu Phe Ala Arg Phe Asp Lys Leu Ala Ala
                405                 410                 415

Glu Asn His Cys Leu Arg Ile Lys Ile Leu Gly Asp Cys Tyr Tyr Cys
            420                 425                 430

Val Ser Gly Leu Pro Glu Ala Arg Ala Asp His Ala His Cys Cys Val
        435                 440                 445

Glu Met Gly Val Asp Met Ile Glu Ala Ile Ser Leu Val Arg Glu Val
    450                 455                 460

Thr Gly Val Asn Val Asn Met Arg Val Gly Ile His Ser Gly Arg Val
465                 470                 475                 480

His Cys Gly Val Leu Gly Leu Arg Lys Trp Gln Phe Asp Val Trp Ser
                485                 490                 495

Asn Asp Val Thr Leu Ala Asn His Met Glu Ala Gly Gly Arg Ala Gly
            500                 505                 510

Arg Ile His Ile Thr Arg Ala Thr Leu Gln Tyr Leu Asn Gly Asp Tyr
        515                 520                 525
```

[0004]

```
Glu Val Glu Pro Gly Arg Gly Gly Glu Arg Asn Ala Tyr Leu Lys Glu
    530                 535                 540

Gln His Ile Glu Thr Phe Leu Ile Leu Gly Ala Ser Gln Lys Arg Lys
545                 550                 555                 560

Glu Glu Lys Ala Met Leu Ala Lys Leu Gln Arg Thr Arg Ala Asn Ser
                565                 570                 575

Met Glu Gly Leu Met Pro Arg Trp Val Pro Asp Arg Ala Phe Ser Arg
            580                 585                 590

Thr Lys Asp Ser Lys Ala Phe Arg Gln Met Gly Ile Asp Asp Ser Ser
        595                 600                 605

Lys Asp Asn Arg Gly Thr Gln Asp Ala Leu Asn Pro Glu Asp Glu Val
    610                 615                 620

Asp Glu Phe Leu Ser Arg Ala Ile Asp Ala Arg Ser Ile Asp Gln Leu
625                 630                 635                 640

Arg Lys Asp His Val Arg Arg Phe Leu Leu Thr Phe Gln Arg Glu Asp
                645                 650                 655

Leu Glu Lys Lys Tyr Ser Arg Lys Val Asp Pro Arg Phe Gly Ala Tyr
            660                 665                 670

Val Ala Cys Ala Leu Leu Val Phe Cys Phe Ile Cys Phe Ile Gln Leu
```

675 680 685

Leu Ile Phe Pro His Ser Thr Leu Met Leu Gly Ile Tyr Ala Ser Ile
690 695 700

Phe Leu Leu Leu Leu Ile Thr Val Leu Ile Cys Ala Val Tyr Ser Cys
705 710 715 720

Gly Ser Leu Phe Pro Lys Ala Leu Gln Arg Leu Ser Arg Ser Ile Val
725 730 735

Arg Ser Arg Ala His Ser Thr Ala Val Gly Ile Phe Ser Val Leu Leu
740 745 750

Val Phe Thr Ser Ala Ile Ala Asn Met Phe Thr Cys Asn His Thr Pro
755 760 765

Ile Arg Ser Cys Ala Ala Arg Met Leu Asn Leu Thr Pro Ala Asp Ile
770 775 780

Thr Ala Cys His Leu Gln Gln Leu Asn Tyr Ser Leu Gly Leu Asp Ala
785 790 795 800

Pro Leu Cys Glu Gly Thr Met Pro Thr Cys Ser Phe Pro Glu Tyr Phe
805 810 815

Ile Gly Asn Met Leu Leu Ser Leu Leu Ala Ser Ser Val Phe Leu His
820 825 830

[0005]

Ile Ser Ser Ile Gly Lys Leu Ala Met Ile Phe Val Leu Gly Leu Ile
835 840 845

Tyr Leu Val Leu Leu Leu Leu Gly Pro Pro Ala Thr Ile Phe Asp Asn
850 855 860

Tyr Asp Leu Leu Leu Gly Val His Gly Leu Ala Ser Ser Asn Glu Thr
865 870 875 880

Phe Asp Gly Leu Asp Cys Pro Ala Ala Gly Arg Val Ala Leu Lys Tyr
885 890 895

Met Thr Pro Val Ile Leu Leu Val Phe Ala Leu Ala Leu Tyr Leu His
900 905 910

Ala Gln Gln Val Glu Ser Thr Ala Arg Leu Asp Phe Leu Trp Lys Leu
915 920 925

Gln Ala Thr Gly Glu Lys Glu Glu Met Glu Glu Leu Gln Ala Tyr Asn
930 935 940

Arg Arg Leu Leu His Asn Ile Leu Pro Lys Asp Val Ala Ala His Phe
945 950 955 960

Leu Ala Arg Glu Arg Arg Asn Asp Glu Leu Tyr Tyr Gln Ser Cys Glu
965 970 975

Cys Val Ala Val Met Phe Ala Ser Ile Ala Asn Phe Ser Glu Phe Tyr
980 985 990

Val Glu Leu Glu Ala Asn Asn Glu Gly Val Glu Cys Leu Arg Leu Leu
995 1000 1005

Asn Glu Ile Ile Ala Asp Phe Asp Glu Ile Ile Ser Glu Glu Arg
1010 1015 1020

Phe Arg Gln Leu Glu Lys Ile Lys Thr Ile Gly Ser Thr Tyr Met
1025 1030 1035

Ala Ala Ser Gly Leu Asn Ala Ser Thr Tyr Asp Gln Val Gly Arg
1040 1045 1050

Ser His Ile Thr Ala Leu Ala Asp Tyr Ala Met Arg Leu Met Glu
1055 1060 1065

Gln Met Lys His Ile Asn Glu His Ser Phe Asn Asn Phe Gln Met
1070 1075 1080

Lys Ile Gly Leu Asn Met Gly Pro Val Val Ala Gly Val Ile Gly
1085 1090 1095

Ala Arg Lys Pro Gln Tyr Asp Ile Trp Gly Asn Thr Val Asn Val
1100 1105 1110

Ser Ser Arg Met Asp Ser Thr Gly Val Pro Asp Arg Ile Gln Val
1115 1120 1125

[0006] Thr Thr Asp Leu Tyr Gln Val Leu Ala Ala Lys Gly Tyr Gln Leu
1130 1135 1140

Glu Cys Arg Gly Val Val Lys Val Lys Gly Lys Gly Glu Met Thr
1145 1150 1155

Thr Tyr Phe Leu Asn Gly Gly Pro Ser Ser
1160 1165

<210> 11
<211> 1176
<212> PRT
<213> 小家鼠 (mus musculus)

<400> 11

Met Pro Thr Ser Pro Arg Pro Val Ala Ser Met Ser Trp Phe Ser Gly
1 5 10 15

Leu Leu Val Pro Lys Val Asp Glu Arg Lys Thr Ala Trp Gly Glu Arg
20 25 30

Asn Gly Gln Lys Arg Pro Arg His Ala Asn Arg Ala Ser Gly Phe Cys
35 40 45

Ala Pro Arg Tyr Met Ser Cys Leu Lys Asn Ala Glu Pro Pro Ser Pro
50 55 60

Thr Pro Ala Ala His Thr Arg Cys Pro Trp Gln Asp Glu Ala Phe Ile
65 70 75 80

```
Arg Arg Ala Gly Pro Gly Arg Gly Val Glu Leu Gly Leu Arg Ser Val
                85                  90                  95

Ala Leu Gly Phe Asp Asp Thr Glu Val Thr Thr Pro Met Gly Thr Ala
            100                 105                 110

Glu Val Ala Pro Asp Thr Ser Pro Arg Ser Gly Pro Ser Cys Trp His
        115                 120                 125

Arg Leu Val Gln Val Phe Gln Ser Lys Gln Phe Arg Ser Ala Lys Leu
    130                 135                 140

Glu Arg Leu Tyr Gln Arg Tyr Phe Phe Gln Met Asn Gln Ser Ser Leu
145                 150                 155                 160

Thr Leu Leu Met Ala Val Leu Val Leu Leu Met Ala Val Leu Leu Thr
                165                 170                 175

Phe His Ala Ala Pro Ala Gln Pro Gln Pro Ala Tyr Val Ala Leu Leu
            180                 185                 190

Thr Cys Ala Ser Val Leu Phe Val Val Leu Met Val Val Cys Asn Arg
        195                 200                 205

His Ser Phe Arg Gln Asp Ser Met Trp Val Val Ser Tyr Val Val Leu
    210                 215                 220

Gly Ile Leu Ala Ala Val Gln Val Gly Gly Ala Leu Ala Ala Asn Pro
225                 230                 235                 240
```

[0007]

```
His Ser Pro Ser Ala Gly Leu Trp Cys Pro Val Phe Phe Val Tyr Ile
                245                 250                 255

Thr Tyr Thr Leu Leu Pro Ile Arg Met Arg Ala Ala Val Leu Ser Gly
            260                 265                 270

Leu Gly Leu Ser Thr Leu His Leu Ile Leu Ala Trp Gln Leu Asn Ser
        275                 280                 285

Ser Asp Pro Phe Leu Trp Lys Gln Leu Gly Ala Asn Val Val Leu Phe
    290                 295                 300

Leu Cys Thr Asn Ala Ile Gly Val Cys Thr His Tyr Pro Ala Glu Val
305                 310                 315                 320

Ser Gln Arg Gln Ala Phe Gln Glu Thr Arg Gly Tyr Ile Gln Ala Arg
                325                 330                 335

Leu His Leu Gln His Glu Asn Arg Gln Gln Glu Arg Leu Leu Leu Ser
            340                 345                 350

Val Leu Pro Gln His Val Ala Met Glu Met Lys Glu Asp Ile Asn Thr
        355                 360                 365

Lys Lys Glu Asp Met Met Phe His Lys Ile Tyr Ile Gln Lys His Asp
    370                 375                 380

Asn Val Ser Ile Leu Phe Ala Asp Ile Glu Gly Phe Thr Ser Leu Ala
```

385 390 395 400

Ser Gln Cys Thr Ala Gln Glu Leu Val Met Thr Leu Asn Glu Leu Phe
405 410 415

Ala Arg Phe Asp Lys Leu Ala Ala Glu Asn His Cys Leu Arg Ile Lys
420 425 430

Ile Leu Gly Asp Cys Tyr Tyr Cys Val Ser Gly Leu Pro Glu Ala Arg
435 440 445

Ala Asp His Ala His Cys Cys Val Glu Met Gly Val Asp Met Ile Glu
450 455 460

Ala Ile Ser Leu Val Arg Glu Val Thr Gly Val Asn Val Asn Met Arg
465 470 475 480

Val Gly Ile His Ser Gly Arg Val His Cys Gly Val Leu Gly Leu Arg
485 490 495

Lys Trp Gln Phe Asp Val Trp Ser Asn Asp Val Thr Leu Ala Asn His
500 505 510

Met Glu Ala Gly Gly Arg Ala Gly Arg Ile His Ile Thr Arg Ala Thr
515 520 525

Leu Gln Tyr Leu Asn Gly Asp Tyr Glu Val Glu Pro Gly Arg Gly Gly
530 535 540

[0008]

Glu Arg Asn Ala Tyr Leu Lys Glu Gln Cys Ile Glu Thr Phe Leu Ile
545 550 555 560

Leu Gly Ala Ser Gln Lys Arg Lys Glu Glu Lys Ala Met Leu Ala Lys
565 570 575

Leu Gln Arg Thr Arg Ala Asn Ser Met Glu Gly Leu Met Pro Arg Trp
580 585 590

Val Pro Asp Arg Ala Phe Ser Arg Thr Lys Asp Ser Lys Ala Phe Arg
595 600 605

Gln Met Gly Ile Asp Asp Ser Ser Lys Asp Asn Arg Gly Ala Gln Asp
610 615 620

Ala Leu Asn Pro Glu Asp Glu Val Asp Glu Phe Leu Gly Arg Ala Ile
625 630 635 640

Asp Ala Arg Ser Ile Asp Gln Leu Arg Lys Asp His Val Arg Arg Phe
645 650 655

Leu Leu Thr Phe Gln Arg Glu Asp Leu Glu Lys Lys Tyr Ser Arg Lys
660 665 670

Val Asp Pro Arg Phe Gly Ala Tyr Val Ala Cys Ala Leu Leu Val Phe
675 680 685

Cys Phe Ile Cys Phe Ile Gln Leu Leu Val Phe Pro Tyr Ser Thr Leu
690 695 700

Ile Leu Gly Ile Tyr Ala Ala Ile Phe Leu Leu Leu Val Thr Val
705 710 715 720

Leu Ile Cys Ala Val Cys Ser Cys Gly Ser Phe Phe Pro Lys Ala Leu
725 730 735

Gln Arg Leu Ser Arg Asn Ile Val Arg Ser Arg Val His Ser Thr Ala
740 745 750

Val Gly Ile Phe Ser Val Leu Leu Val Phe Ile Ser Ala Ile Ala Asn
755 760 765

Met Phe Thr Cys Asn His Thr Pro Ile Arg Thr Cys Ala Ala Arg Met
770 775 780

Leu Asn Leu Thr Pro Ala Asp Val Thr Ala Cys His Leu Gln Gln Leu
785 790 795 800

Asn Tyr Ser Leu Gly Leu Asp Ala Pro Leu Cys Glu Gly Thr Ala Pro
805 810 815

Thr Cys Ser Phe Pro Glu Tyr Phe Val Gly Asn Val Leu Leu Ser Leu
820 825 830

Leu Ala Ser Ser Val Phe Leu His Ile Ser Ser Ile Gly Lys Leu Ala
835 840 845

[0009] Met Thr Phe Ile Leu Gly Phe Thr Tyr Leu Val Leu Leu Leu Leu Gly
850 855 860

Pro Pro Ala Ala Ile Phe Asp Asn Tyr Asp Leu Leu Leu Gly Val His
865 870 875 880

Gly Leu Ala Ser Ser Asn Glu Thr Phe Asp Gly Leu Asp Cys Pro Ala
885 890 895

Val Gly Arg Val Ala Leu Lys Tyr Met Thr Pro Val Ile Leu Leu Val
900 905 910

Phe Ala Leu Ala Leu Tyr Leu His Ala Gln Gln Val Glu Ser Thr Ala
915 920 925

Arg Leu Asp Phe Leu Trp Lys Leu Gln Ala Thr Gly Glu Lys Glu Glu
930 935 940

Met Glu Glu Leu Gln Ala Tyr Asn Arg Arg Leu Leu His Asn Ile Leu
945 950 955 960

Pro Lys Asp Val Ala Ala His Phe Leu Ala Arg Glu Arg Arg Asn Asp
965 970 975

Glu Leu Tyr Tyr Gln Ser Cys Glu Cys Val Ala Val Met Phe Ala Ser
980 985 990

Ile Ala Asn Phe Ser Glu Phe Tyr Val Glu Leu Glu Ala Asn Asn Glu
995 1000 1005

Gly Val Glu Cys Leu Arg Leu Leu Asn Glu Ile Ile Ala Asp Phe
1010 1015 1020

Asp Glu Ile Ile Ser Glu Glu Arg Phe Arg Gln Leu Glu Lys Ile
1025 1030 1035

Lys Thr Ile Gly Ser Thr Tyr Met Ala Ala Ser Gly Leu Asn Ala
1040 1045 1050

Ser Thr Tyr Asp Gln Val Gly Arg Ser His Ile Thr Ala Leu Ala
1055 1060 1065

Asp Tyr Ala Met Arg Leu Met Glu Gln Met Lys His Ile Asn Glu
1070 1075 1080

His Ser Phe Asn Asn Phe Gln Met Lys Ile Gly Leu Asn Met Gly
1085 1090 1095

Pro Val Val Ala Gly Val Ile Gly Ala Arg Lys Pro Gln Tyr Asp
1100 1105 1110

Ile Trp Gly Asn Thr Val Asn Val Ser Ser Arg Met Asp Ser Thr
1115 1120 1125

Gly Val Pro Asp Arg Ile Gln Val Thr Thr Asp Leu Tyr Gln Val
1130 1135 1140

Leu Ala Ala Lys Gly Tyr Gln Leu Glu Cys Arg Gly Val Val Lys
[0010] 1145 1150 1155

Val Lys Gly Lys Gly Glu Met Thr Thr Tyr Phe Leu Asn Gly Gly
1160 1165 1170

Pro Ser Ser
1175

<210> 12
<211> 1176
<212> PRT
<213> 人工序列

<220>
<223> 合成多肽

<400> 12

Met Pro Thr Ser Pro Arg Pro Val Ala Ser Met Ser Trp Phe Ser Gly
1 5 10 15

Leu Leu Val Pro Lys Val Asp Glu Arg Lys Thr Ala Trp Gly Glu Arg
20 25 30

Asn Gly Gln Lys Arg Pro Arg His Ala Asn Arg Ala Ser Gly Phe Cys
35 40 45

Ala Pro Arg Tyr Met Ser Cys Leu Lys Asn Ala Glu Pro Pro Ser Pro
50 55 60

Thr Pro Ala Ala His Thr Arg Cys Pro Trp Gln Asp Glu Ala Phe Ile
65 70 75 80

Arg Arg Ala Gly Pro Gly Arg Gly Val Glu Leu Gly Leu Arg Ser Val
85 90 95

Ala Leu Gly Phe Asp Asp Thr Glu Val Thr Thr Pro Met Gly Thr Ala
100 105 110

Glu Val Ala Pro Asp Thr Ser Pro Arg Ser Gly Pro Ser Cys Trp His
115 120 125

Arg Leu Val Gln Val Phe Gln Ser Lys Gln Phe Arg Ser Ala Lys Leu
130 135 140

Glu Arg Leu Tyr Gln Arg Tyr Phe Phe Gln Met Asn Gln Ser Ser Leu
145 150 155 160

Thr Leu Leu Met Ala Val Leu Val Leu Leu Met Ala Val Leu Leu Thr
165 170 175

Phe His Ala Ala Pro Ala Gln Pro Gln Pro Ala Tyr Val Ala Leu Leu
180 185 190

Thr Cys Ala Ser Val Leu Phe Val Val Leu Met Val Val Cys Asn Arg
195 200 205

His Ser Phe Arg Gln Asp Ser Met Trp Val Val Ser Tyr Val Val Leu
210 215 220

[0011] Gly Ile Leu Ala Ala Val Gln Val Gly Gly Ala Leu Ala Ala Asn Pro
225 230 235 240

His Ser Pro Ser Ala Gly Leu Trp Cys Pro Val Phe Phe Val Tyr Ile
245 250 255

Thr Tyr Thr Leu Leu Pro Ile Arg Met Arg Ala Ala Val Leu Ser Gly
260 265 270

Leu Gly Leu Ser Thr Leu His Leu Ile Leu Ala Trp Gln Leu Asn Ser
275 280 285

Ser Asp Pro Phe Leu Trp Lys Gln Leu Gly Ala Asn Val Val Leu Phe
290 295 300

Leu Cys Thr Asn Ala Ile Gly Val Cys Thr His Tyr Pro Ala Glu Val
305 310 315 320

Ser Gln Arg Gln Ala Phe Gln Glu Thr Arg Gly Tyr Ile Gln Ala Arg
325 330 335

Leu His Leu Gln His Glu Asn Arg Gln Gln Glu Arg Leu Leu Leu Ser
340 345 350

Val Leu Pro Gln His Val Ala Met Glu Met Lys Glu Asp Ile Asn Thr
355 360 365

Lys Lys Glu Asp Met Met Phe His Lys Ile Tyr Ile Gln Lys His Asp
370 375 380

Asn Val Ser Ile Leu Phe Ala Asp Ile Glu Gly Phe Thr Ser Leu Ala
385 390 395 400

Ser Gln Cys Thr Ala Gln Glu Leu Val Met Thr Leu Asn Glu Leu Phe
405 410 415

Ala Arg Phe Asp Lys Leu Ala Ala Glu Asn His Cys Leu Arg Ile Lys
420 425 430

Ile Leu Gly Ala Cys Tyr Tyr Cys Val Ser Gly Leu Pro Glu Ala Arg
435 440 445

Ala Asp His Ala His Cys Cys Val Glu Met Gly Val Asp Met Ile Glu
450 455 460

Ala Ile Ser Leu Val Arg Glu Val Thr Gly Val Asn Val Asn Met Arg
465 470 475 480

Val Gly Ile His Ser Gly Arg Val His Cys Gly Val Leu Gly Leu Arg
485 490 495

Lys Trp Gln Phe Asp Val Trp Ser Asn Asp Val Thr Leu Ala Asn His
500 505 510

Met Glu Ala Gly Gly Arg Ala Gly Arg Ile His Ile Thr Arg Ala Thr
515 520 525

[0012] Leu Gln Tyr Leu Asn Gly Asp Tyr Glu Val Glu Pro Gly Arg Gly Gly
530 535 540

Glu Arg Asn Ala Tyr Leu Lys Glu Gln Cys Ile Glu Thr Phe Leu Ile
545 550 555 560

Leu Gly Ala Ser Gln Lys Arg Lys Glu Glu Lys Ala Met Leu Ala Lys
565 570 575

Leu Gln Arg Thr Arg Ala Asn Ser Met Glu Gly Leu Met Pro Arg Trp
580 585 590

Val Pro Asp Arg Ala Phe Ser Arg Thr Lys Asp Ser Lys Ala Phe Arg
595 600 605

Gln Met Gly Ile Asp Asp Ser Ser Lys Asp Asn Arg Gly Ala Gln Asp
610 615 620

Ala Leu Asn Pro Glu Asp Glu Val Asp Glu Phe Leu Gly Arg Ala Ile
625 630 635 640

Asp Ala Arg Ser Ile Asp Gln Leu Arg Lys Asp His Val Arg Arg Phe
645 650 655

Leu Leu Thr Phe Gln Arg Glu Asp Leu Glu Lys Lys Tyr Ser Arg Lys
660 665 670

Val Asp Pro Arg Phe Gly Ala Tyr Val Ala Cys Ala Leu Leu Val Phe
675 680 685

Cys Phe Ile Cys Phe Ile Gln Leu Leu Val Phe Pro Tyr Ser Thr Leu
690 695 700

Ile Leu Gly Ile Tyr Ala Ala Ile Phe Leu Leu Leu Leu Val Thr Val
705 710 715 720

Leu Ile Cys Ala Val Cys Ser Cys Gly Ser Phe Phe Pro Lys Ala Leu
725 730 735

Gln Arg Leu Ser Arg Asn Ile Val Arg Ser Arg Val His Ser Thr Ala
740 745 750

Val Gly Ile Phe Ser Val Leu Leu Val Phe Ile Ser Ala Ile Ala Asn
755 760 765

Met Phe Thr Cys Asn His Thr Pro Ile Arg Thr Cys Ala Ala Arg Met
770 775 780

Leu Asn Leu Thr Pro Ala Asp Val Thr Ala Cys His Leu Gln Gln Leu
785 790 795 800

Asn Tyr Ser Leu Gly Leu Asp Ala Pro Leu Cys Glu Gly Thr Ala Pro
805 810 815

Thr Cys Ser Phe Pro Glu Tyr Phe Val Gly Asn Val Leu Leu Ser Leu
820 825 830

Leu Ala Ser Ser Val Phe Leu His Ile Ser Ser Ile Gly Lys Leu Ala
835 840 845

[0013]

Met Thr Phe Ile Leu Gly Phe Thr Tyr Leu Val Leu Leu Leu Leu Gly
850 855 860

Pro Pro Ala Ala Ile Phe Asp Asn Tyr Asp Leu Leu Leu Gly Val His
865 870 875 880

Gly Leu Ala Ser Ser Asn Glu Thr Phe Asp Gly Leu Asp Cys Pro Ala
885 890 895

Val Gly Arg Val Ala Leu Lys Tyr Met Thr Pro Val Ile Leu Leu Val
900 905 910

Phe Ala Leu Ala Leu Tyr Leu His Ala Gln Gln Val Glu Ser Thr Ala
915 920 925

Arg Leu Asp Phe Leu Trp Lys Leu Gln Ala Thr Gly Glu Lys Glu Glu
930 935 940

Met Glu Glu Leu Gln Ala Tyr Asn Arg Arg Leu Leu His Asn Ile Leu
945 950 955 960

Pro Lys Asp Val Ala Ala His Phe Leu Ala Arg Glu Arg Arg Asn Asp
965 970 975

Glu Leu Tyr Tyr Gln Ser Cys Glu Cys Val Ala Val Met Phe Ala Ser
980 985 990

Ile Ala Asn Phe Ser Glu Phe Tyr Val Glu Leu Glu Ala Asn Asn Glu

995 1000 1005

Gly Val Glu Cys Leu Arg Leu Leu Asn Glu Ile Ile Ala Asp Phe
1010 1015 1020

Asp Glu Ile Ile Ser Glu Glu Arg Phe Arg Gln Leu Glu Lys Ile
1025 1030 1035

Lys Thr Ile Gly Ser Thr Tyr Met Ala Ala Ser Gly Leu Asn Ala
1040 1045 1050

Ser Thr Tyr Asp Gln Val Gly Arg Ser His Ile Thr Ala Leu Ala
1055 1060 1065

Asp Tyr Ala Met Arg Leu Met Glu Gln Met Lys His Ile Asn Glu
1070 1075 1080

His Ser Phe Asn Asn Phe Gln Met Lys Ile Gly Leu Asn Met Gly
1085 1090 1095

Pro Val Val Ala Gly Val Ile Gly Ala Arg Lys Pro Gln Tyr Asp
1100 1105 1110

Ile Trp Gly Asn Thr Val Asn Val Ser Ser Arg Met Asp Ser Thr
1115 1120 1125

Gly Val Pro Asp Arg Ile Gln Val Thr Thr Asp Leu Tyr Gln Val
1130 1135 1140

[0014]

Leu Ala Ala Lys Gly Tyr Gln Leu Glu Cys Arg Gly Val Val Lys
1145 1150 1155

Val Lys Gly Lys Gly Glu Met Thr Thr Tyr Phe Leu Asn Gly Gly
1160 1165 1170

Pro Ser Ser
1175

<210> 13
<211> 1168
<212> PRT
<213> 人工序列

<220>
<223> 合成多肽

<400> 13

Met Ser Trp Phe Ser Gly Leu Leu Val Pro Lys Val Asp Glu Arg Lys
1 5 10 15

Thr Ala Trp Gly Glu Arg Asn Gly Gln Lys Arg Ser Arg Arg Arg Gly
20 25 30

Thr Arg Ala Gly Gly Phe Cys Thr Pro Arg Tyr Met Ser Cys Leu Arg
35 40 45

Asp Ala Glu Pro Pro Ser Pro Thr Pro Ala Gly Pro Pro Arg Cys Pro
50 55 60

```
Trp Gln Asp Asp Ala Phe Ile Arg Arg Gly Gly Pro Gly Lys Gly Lys
65                  70                  75                  80

Glu Leu Gly Leu Arg Ala Val Ala Leu Gly Phe Glu Asp Thr Glu Val
                85                  90                  95

Thr Thr Thr Ala Gly Gly Thr Ala Glu Val Ala Pro Asp Ala Val Pro
            100                 105                 110

Arg Ser Gly Arg Ser Cys Trp Arg Arg Leu Val Gln Val Phe Gln Ser
        115                 120                 125

Lys Gln Phe Arg Ser Ala Lys Leu Glu Arg Leu Tyr Gln Arg Tyr Phe
    130                 135                 140

Phe Gln Met Asn Gln Ser Ser Leu Thr Leu Leu Met Ala Val Leu Val
145                 150                 155                 160

Leu Leu Thr Ala Val Leu Leu Ala Phe His Ala Ala Pro Ala Arg Pro
                165                 170                 175

Gln Pro Ala Tyr Val Ala Leu Leu Ala Cys Ala Ala Ala Leu Phe Val
            180                 185                 190

Gly Leu Met Val Val Cys Asn Arg His Ser Phe Arg Gln Asp Ser Met
        195                 200                 205

Trp Val Val Ser Tyr Val Val Leu Gly Ile Leu Ala Ala Val Gln Val
    210                 215                 220
```

[0015]

```
Gly Gly Ala Leu Ala Ala Asp Pro Arg Ser Pro Ser Ala Gly Leu Trp
225                 230                 235                 240

Cys Pro Val Phe Phe Val Tyr Ile Ala Tyr Thr Leu Leu Pro Ile Arg
                245                 250                 255

Met Arg Ala Ala Val Leu Ser Gly Leu Gly Leu Ser Thr Leu His Leu
            260                 265                 270

Ile Leu Ala Trp Gln Leu Asn Arg Gly Asp Ala Phe Leu Trp Arg Gln
        275                 280                 285

Leu Gly Ala Asn Val Leu Leu Phe Leu Cys Thr Asn Val Ile Gly Ile
    290                 295                 300

Cys Thr His Tyr Pro Ala Glu Val Ser Gln Arg Gln Ala Phe Gln Glu
305                 310                 315                 320

Thr Arg Gly Tyr Ile Gln Ala Arg Leu His Leu Gln His Glu Asn Arg
                325                 330                 335

Gln Gln Glu Arg Leu Leu Leu Ser Val Leu Pro Gln His Val Ala Met
            340                 345                 350

Glu Met Lys Glu Asp Ile Asn Thr Lys Lys Glu Asp Met Met Phe His
        355                 360                 365

Lys Ile Tyr Ile Gln Lys His Asp Asn Val Ser Ile Leu Phe Ala Asp
```

370 375 380

Ile Glu Gly Phe Thr Ser Leu Ala Ser Gln Cys Thr Ala Gln Glu Leu
385 390 395 400

Val Met Thr Leu Asn Glu Leu Phe Ala Arg Phe Asp Lys Leu Ala Ala
405 410 415

Glu Asn His Cys Leu Arg Ile Lys Ile Leu Gly Ala Cys Tyr Tyr Cys
420 425 430

Val Ser Gly Leu Pro Glu Ala Arg Ala Asp His Ala His Cys Cys Val
435 440 445

Glu Met Gly Val Asp Met Ile Glu Ala Ile Ser Leu Val Arg Glu Val
450 455 460

Thr Gly Val Asn Val Asn Met Arg Val Gly Ile His Ser Gly Arg Val
465 470 475 480

His Cys Gly Val Leu Gly Leu Arg Lys Trp Gln Phe Asp Val Trp Ser
485 490 495

Asn Asp Val Thr Leu Ala Asn His Met Glu Ala Gly Gly Arg Ala Gly
500 505 510

Arg Ile His Ile Thr Arg Ala Thr Leu Gln Tyr Leu Asn Gly Asp Tyr
515 520 525

[0016]

Glu Val Glu Pro Gly Arg Gly Gly Glu Arg Asn Ala Tyr Leu Lys Glu
530 535 540

Gln His Ile Glu Thr Phe Leu Ile Leu Gly Ala Ser Gln Lys Arg Lys
545 550 555 560

Glu Glu Lys Ala Met Leu Ala Lys Leu Gln Arg Thr Arg Ala Asn Ser
565 570 575

Met Glu Gly Leu Met Pro Arg Trp Val Pro Asp Arg Ala Phe Ser Arg
580 585 590

Thr Lys Asp Ser Lys Ala Phe Arg Gln Met Gly Ile Asp Asp Ser Ser
595 600 605

Lys Asp Asn Arg Gly Thr Gln Asp Ala Leu Asn Pro Glu Asp Glu Val
610 615 620

Asp Glu Phe Leu Ser Arg Ala Ile Asp Ala Arg Ser Ile Asp Gln Leu
625 630 635 640

Arg Lys Asp His Val Arg Arg Phe Leu Leu Thr Phe Gln Arg Glu Asp
645 650 655

Leu Glu Lys Lys Tyr Ser Arg Lys Val Asp Pro Arg Phe Gly Ala Tyr
660 665 670

Val Ala Cys Ala Leu Leu Val Phe Cys Phe Ile Cys Phe Ile Gln Leu
675 680 685

Leu Ile Phe Pro His Ser Thr Leu Met Leu Gly Ile Tyr Ala Ser Ile
690 695 700

Phe Leu Leu Leu Leu Ile Thr Val Leu Ile Cys Ala Val Tyr Ser Cys
705 710 715 720

Gly Ser Leu Phe Pro Lys Ala Leu Gln Arg Leu Ser Arg Ser Ile Val
725 730 735

Arg Ser Arg Ala His Ser Thr Ala Val Gly Ile Phe Ser Val Leu Leu
740 745 750

Val Phe Thr Ser Ala Ile Ala Asn Met Phe Thr Cys Asn His Thr Pro
755 760 765

Ile Arg Ser Cys Ala Ala Arg Met Leu Asn Leu Thr Pro Ala Asp Ile
770 775 780

Thr Ala Cys His Leu Gln Gln Leu Asn Tyr Ser Leu Gly Leu Asp Ala
785 790 795 800

Pro Leu Cys Glu Gly Thr Met Pro Thr Cys Ser Phe Pro Glu Tyr Phe
805 810 815

Ile Gly Asn Met Leu Leu Ser Leu Leu Ala Ser Ser Val Phe Leu His
820 825 830

[0017] Ile Ser Ser Ile Gly Lys Leu Ala Met Ile Phe Val Leu Gly Leu Ile
835 840 845

Tyr Leu Val Leu Leu Leu Leu Gly Pro Pro Ala Thr Ile Phe Asp Asn
850 855 860

Tyr Asp Leu Leu Leu Gly Val His Gly Leu Ala Ser Ser Asn Glu Thr
865 870 875 880

Phe Asp Gly Leu Asp Cys Pro Ala Ala Gly Arg Val Ala Leu Lys Tyr
885 890 895

Met Thr Pro Val Ile Leu Leu Val Phe Ala Leu Ala Leu Tyr Leu His
900 905 910

Ala Gln Gln Val Glu Ser Thr Ala Arg Leu Asp Phe Leu Trp Lys Leu
915 920 925

Gln Ala Thr Gly Glu Lys Glu Glu Met Glu Glu Leu Gln Ala Tyr Asn
930 935 940

Arg Arg Leu Leu His Asn Ile Leu Pro Lys Asp Val Ala Ala His Phe
945 950 955 960

Leu Ala Arg Glu Arg Arg Asn Asp Glu Leu Tyr Tyr Gln Ser Cys Glu
965 970 975

Cys Val Ala Val Met Phe Ala Ser Ile Ala Asn Phe Ser Glu Phe Tyr
980 985 990

Val Glu Leu Glu Ala Asn Asn Glu Gly Val Glu Cys Leu Arg Leu Leu
995 1000 1005

Asn Glu Ile Ile Ala Asp Phe Asp Glu Ile Ile Ser Glu Glu Arg
1010 1015 1020

Phe Arg Gln Leu Glu Lys Ile Lys Thr Ile Gly Ser Thr Tyr Met
1025 1030 1035

Ala Ala Ser Gly Leu Asn Ala Ser Thr Tyr Asp Gln Val Gly Arg
1040 1045 1050

Ser His Ile Thr Ala Leu Ala Asp Tyr Ala Met Arg Leu Met Glu
1055 1060 1065

Gln Met Lys His Ile Asn Glu His Ser Phe Asn Asn Phe Gln Met
1070 1075 1080

Lys Ile Gly Leu Asn Met Gly Pro Val Val Ala Gly Val Ile Gly
1085 1090 1095

Ala Arg Lys Pro Gln Tyr Asp Ile Trp Gly Asn Thr Val Asn Val
1100 1105 1110

Ser Ser Arg Met Asp Ser Thr Gly Val Pro Asp Arg Ile Gln Val
1115 1120 1125

Thr Thr Asp Leu Tyr Gln Val Leu Ala Ala Lys Gly Tyr Gln Leu
1130 1135 1140

[0018]

Glu Cys Arg Gly Val Val Lys Val Lys Gly Lys Gly Glu Met Thr
1145 1150 1155

Thr Tyr Phe Leu Asn Gly Gly Pro Ser Ser
1160 1165

<210> 14
<211> 3537
<212> DNA
<213> 智人

<220>
<221> misc_feature
<222> (163)..(163)
<223> n是a、c、g或t

<400> 14
gaattcgccc ttcggcagca tgtcatggtt tagtggcctc ctggtcccta aagtggatga 60

acggaaaaca gcctggggtg aacgcaatgg gcagaagcgt tcgcggcgcc gtggcactcg 120

ggcaggtggc ttctgcacgc cccgctatat gagctgcctc cgngatgcag agccacccag 180

ccccacccct gcgggcccca ctcggtgccc ctggcaggat gacgccttca tccggagggg 240

cggcccaggc aagggcaagg agctggggct gcgggcagtg gccctgggct tcgaggatac 300

cgaggtgaca acgacagcgg gcgggacggc tgaggtggcg cccgacgcgg tgcccaggag 360

tgggcgatcc tgctggcgcc gtctggtgca ggtgttccag tcgaagcagt tccgttcggc 420

caagctggag cgcctgtacc agcggtactt cttccagatg aaccagagca gcctgacgct 480

[0019]

gctgatggcg gtgctggtgc tgctcacagc ggtgctgctg gctttccacg ccgcacccgc 540

ccgccctcag cctgcctatg tggcactgtt ggcctgtgcc gccgccctgt tcgtggggct 600

catggtggtg tgtaaccggc atagcttccg ccaggactcc atgtgggtgg tgagttacgt 660

ggtgctgggc atcctggcgg cagtgcaggt cgggggcgct ctcgcagcag acccgcgcag 720

cccctctgcg ggcctctggt gccctgtgtt ctttgtctac atcgcctaca cgctcctccc 780

catccgcatg cgggctgccg tcctcagcgg cctgggcctc tccaccttgc atttgatctt 840

ggcctggcaa cttaaccgtg gtgatgcctt cctctggagg cagctcggtg ccaatgtgct 900

gctgttcctc tgcaccaacg tcattggcat ctgcacacac tatccagcag aggtgtctca 960

gcgccaggcc tttcaggaga cccgcggtta catccaggcc cggctccacc tgcagcatga 1020

gaatcggcag caggagcggc tgctgctgtc ggtattgccc cagcacgtcg ccatggagat 1080

gaaagaagac atcaacacaa aaaaagaaga catgatgttc cacaagatct acatacagaa 1140

gcatgacaat gtcagcatcc tgtttgcaga cattgagggc ttcaccagcc tggcatccca 1200

gtgcactgcg caggagctgg tcatgaccct gaatgagctc tttgcccggt ttgacaagct 1260

ggctgcggag aatcactgcc tgaggatcaa gatcttgggg gactgttact actgtgtgtc 1320

agggctgccg gaggcccggg ccgaccatgc ccactgctgt gtggagatgg gggtagacat 1380

gattgaggcc atctcgctgg tacgtgaggt gacaggtgtg aatgtgaaca tgcgcgtggg 1440

catccacagc gggcgcgtgc actgcggcgt ccttggcttg cggaaatggc agttcgatgt 1500

gtggtccaat gatgtgaccc tggccaacca catggaggca ggaggccggg ctggccgcat 1560

ccacatcact cgggcaacac tgcagtacct gaacggggac tacgaggtgg agccaggccg 1620

tggtggcgag cgcaacgcgt acctcaagga gcagcacatt gagactttcc tcatcctggg 1680

cgccagccag aaacggaaag aggagaaggc catgctggcc aagctgcagc ggactcgggc 1740

caactccatg gaagggctga tgccgcgctg ggttcctgat cgtgccttct cccggaccaa 1800

ggactccaag gccttccgcc agatgggcat tgatgattcc agcaaagaca accggggcac 1860

ccaagatgcc ctgaaccctg aggatgaggt ggatgagttc ctgagccgtg ccatcgatgc 1920

ccgcagcatt gatcagctgc ggaaggacca tgtgcgccgg tttctgctca ccttccagag 1980

agaggatctt gagaagaagt actcccggaa ggtggatccc cgcttcggag cctacgttgc 2040

ctgtgccctg ttggtcttct gcttcatctg cttcatccag cttctcatct tcccacactc 2100

caccctgatg cttgggatct atgccagcat cttcctgctg ctgctaatca ccgtgctgat 2160

ctgtgctgtg tactcctgtg gttctctgtt ccctaaggcc ctgcaacgtc tgtcccgcag 2220

cattgtccgc tcacgggcac atagcaccgc agttggcatc ttttccgtcc tgcttgtgtt 2280

tacttctgcc attgccaaca tgttcacctg taaccacacc cccatacgga gctgtgcagc 2340

ccggatgctg aatttaacac ctgctgacat cactgcctgc cacctgcagc agctcaatta 2400

ctctctgggc ctggatgctc ccctgtgtga gggcaccatg cccacctgca gctttcctga 2460

gtacttcatc gggaacatgc tgctgagtct cttggccagc tctgtcttcc tgcacatcag 2520

cagcatcggg aagttggcca tgatctttgt cttggggctc atctatttgg tgctgcttct 2580

gctgggtccc ccagccacca tctttgacaa ctatgaccta ctgcttggcg tccatggctt 2640

ggcttcttcc aatgagacct ttgatgggct ggactgtcca gctgcaggga gggtggccct 2700

caaatatatg acccctgtga ttctgctggt gtttgcgctg gcgctgtatc tgcatgctca 2760

gcaggtggag tcgactgccc gcctagactt cctctggaaa ctacaggcaa caggggagaa 2820

ggaggagatg gaggagctac aggcatacaa ccggaggctg ctgcataaca ttctgcccaa 2880

ggacgtggcg gcccacttcc tggcccggga gcgccgcaat gatgaactct actatcagtc 2940

gtgtgagtgt gtggctgtta tgtttgcctc cattgccaac ttctctgagt tctatgtgga 3000

gctggaggca aacaatgagg gtgtcgagtg cctgcggctg ctcaacgaga tcatcgctga 3060

ctttgatgag attatcagcg aggagcggtt ccggcagctg gaaaagatca agacgattgg 3120

tagcacctac atggctgcct cagggctgaa cgccagcacc tacgatcagg tgggccgctc 3180

ccacatcact gccctggctg actacgccat gcggctcatg gagcagatga agcacatcaa 3240

tgagcactcc ttcaacaatt tccagatgaa gattgggctg aacatgggcc cagtcgtggc 3300

aggtgtcatc ggggctcgga agccacagta tgacatctgg gggaacacag tgaatgtctc 3360

tagtcgtatg gacagcacgg gggtccccga ccgaatccag gtgaccacgg acctgtacca 3420

ggttctagct gccaagggct accagctgga gtgtcgaggg gtggtcaagg tgaagggcaa 3480

gggggagatg accacctact tcctcaatgg gggccccagc agttaacagg ggaattc 3537

<210> 15
<211> 3537
<212> DNA
<213> 小家鼠

<220>
<221> misc_feature
<222> (163)..(163)
<223> n是a、c、g或t

[0020]

<400> 15
gaattcgccc ttcggcagca tgtcatggtt tagtggcctc ctggtcccta aagtggatga 60

acggaaaaca gcctggggtg aacgcaatgg gcagaagcgt tcgcggcgcc gtggcactcg 120

ggcaggtggc ttctgcacgc cccgctatat gagctgcctc cgngatgcag agccacccag 180

ccccacccct gcgggccccc ctcggtgccc ctggcaggat gacgccttca tccggagggg 240

cggcccaggc aagggcaagg agctggggct gcgggcagtg gccctgggct tcgaggatac 300

cgaggtgaca acgacagcgg gcgggacggc tgaggtggcg cccgacgcgg tgcccaggag 360

tgggcgatcc tgctggcgcc gtctggtgca ggtgttccag tcgaagcagt tccgttcggc 420

caagctggag cgcctgtacc agcggtactt cttccagatg aaccagagca gcctgacgct 480

gctgatggcg gtgctggtgc tgctcacagc ggtgctgctg gctttccacg ccgcacccgc 540

ccgccctcag cctgcctatg tggcactgtt ggcctgtgcc gccgccctgt tcgtggggct 600

catggtggtg tgtaaccggc atagcttccg ccaggactcc atgtgggtgg tgagttacgt 660

ggtgctgggc atcctggcgg cagtgcaggt cgggggcgct ctcgcagcag acccgcgcag 720

cccctctgcg ggcctctggt gccctgtgtt ctttgtctac atcgcctaca cgctcctccc 780

catccgcatg cgggctgccg tcctcagcgg cctgggcctc tccaccttgc atttgatctt 840

ggcctggcaa cttaaccgtg gtgatgcctt cctctggagg cagctcggtg ccaatgtgct 900

gctgttcctc tgcaccaacg tcattggcat ctgcacacac tatccagcag aggtgtctca 960

gcgccaggcc tttcaggaga cccgcggtta catccaggcc cggctccacc tgcagcatga 1020

gaatcggcag caggagcggc tgctgctgtc ggtattgccc cagcacgtcg ccatggagat 1080

gaaagaagac atcaacacaa aaaaagaaga catgatgttc cacaagatct acatacagaa 1140

gcatgacaat gtcagcatcc tgtttgcaga cattgagggc ttcaccagcc tggcatccca 1200

gtgcactgcg caggagctgg tcatgaccct gaatgagctc tttgcccggt ttgacaagct 1260

ggctgcggag aatcactgcc tgaggatcaa gatcttgggg gactgttact actgtgtgtc 1320

agggctgccg gaggcccggg ccgaccatgc ccactgctgt gtggagatgg gggtagacat 1380

gattgaggcc atctcgctgg tacgtgaggt gacaggtgtg aatgtgaaca tgcgcgtggg 1440

catccacagc gggcgcgtgc actgcggcgt ccttggcttg cggaaatggc agttcgatgt 1500

gtggtccaat gatgtgaccc tggccaacca catggaggca ggaggccggg ctggccgcat 1560

ccacatcact cgggcaacac tgcagtacct gaacggggac tacgaggtgg agccaggccg 1620

tggtggcgag cgcaacgcgt acctcaagga gcagcacatt gagactttcc tcatcctggg 1680

cgccagccag aaacggaaag aggagaaggc catgctggcc aagctgcagc ggactcgggc 1740

caactccatg gaagggctga tgccgcgctg ggttcctgat cgtgccttct cccggaccaa 1800

ggactccaag gccttccgcc agatgggcat tgatgattcc agcaaagaca accggggcac 1860

ccaagatgcc ctgaaccctg aggatgaggt ggatgagttc ctgagccgtg ccatcgatgc 1920

ccgcagcatt gatcagctgc ggaaggacca tgtgcgccgg tttctgctca ccttccagag 1980

agaggatctt gagaagaagt actcccggaa ggtggatccc cgcttcggag cctacgttgc 2040

ctgtgccctg ttggtcttct gcttcatctg cttcatccag cttctcatct tcccacactc 2100

caccctgatg cttgggatct atgccagcat cttcctgctg ctgctaatca ccgtgctgat 2160

ctgtgctgtg tactcctgtg gttctctgtt ccctaaggcc ctgcaacgtc tgtcccgcag 2220

cattgtccgc tcacgggcac atagcaccgc agttggcatc ttttccgtcc tgcttgtgtt 2280

[0021] tacttctgcc attgccaaca tgttcacctg taaccacacc cccatacgga gctgtgcagc 2340

ccggatgctg aatttaacac ctgctgacat cactgcctgc cacctgcagc agctcaatta 2400

ctctctgggc ctggatgctc ccctgtgtga gggcaccatg cccacctgca gctttcctga 2460

gtacttcatc gggaacatgc tgctgagtct cttggccagc tctgtcttcc tgcacatcag 2520

cagcatcggg aagttggcca tgatctttgt cttggggctc atctatttgg tgctgcttct 2580

gctgggtccc ccagccacca tctttgacaa ctatgaccta ctgcttggcg tccatggctt 2640

ggcttcttcc aatgagacct ttgatgggct ggactgtcca gctgcaggga gggtggccct 2700

caaatatatg acccctgtga ttctgctggt gtttgcgctg gcgctgtatc tgcatgctca 2760

gcaggtggag tcgactgccc gcctagactt cctctggaaa ctacaggcaa caggggagaa 2820

ggaggagatg gaggagctac aggcatacaa ccggaggctg ctgcataaca ttctgcccaa 2880

ggacgtggcg gcccacttcc tggcccggga gcgccgcaat gatgaactct actatcagtc 2940

gtgtgagtgt gtggctgtta tgtttgcctc cattgccaac ttctctgagt tctatgtgga 3000

gctggaggca aacaatgagg gtgtcgagtg cctgcggctg ctcaacgaga tcatcgctga 3060

ctttgatgag attatcagcg aggagcggtt ccggcagctg gaaaagatca agacgattgg 3120

tagcacctac atggctgcct cagggctgaa cgccagcacc tacgatcagg tgggccgctc 3180

ccacatcact gccctggctg actacgccat gcggctcatg gagcagatga agcacatcaa 3240

tgagcactcc ttcaacaatt tccagatgaa gattgggctg aacatgggcc cagtcgtggc 3300

aggtgtcatc ggggctcgga agccacagta tgacatctgg gggaacacag tgaatgtctc 3360

tagtcgtatg gacagcacgg gggtccccga ccgaatccag gtgaccacgg acctgtacca 3420

ggttctagct gccaagggct accagctgga gtgtcgaggg gtggtcaagg tgaagggcaa 3480

ggggagatg accacctact tcctcaatgg gggccccagc agttaacagg ggaattc 3537

<210> 16
<211> 1166
<212> PRT
<213> 小家鼠

<400> 16

Met Ser Trp Phe Ser Gly Leu Leu Val Pro Lys Val Asp Glu Arg Lys
1 5 10 15

Thr Ala Trp Gly Glu Arg Asn Gly Gln Lys Arg Pro Arg His Ala Asn
20 25 30

Arg Ala Ser Gly Phe Cys Ala Pro Arg Tyr Met Ser Cys Leu Lys Asn
35 40 45

Ala Glu Pro Pro Ser Pro Thr Pro Ala Ala His Thr Arg Cys Pro Trp
50 55 60

Gln Asp Glu Ala Phe Ile Arg Arg Ala Gly Pro Gly Arg Gly Val Glu
65 70 75 80

Leu Gly Leu Arg Ser Val Ala Leu Gly Phe Asp Asp Thr Glu Val Thr
85 90 95

Thr Pro Met Gly Thr Ala Glu Val Ala Pro Asp Thr Ser Pro Arg Ser
100 105 110

[0022]

Gly Pro Ser Cys Trp His Arg Leu Val Gln Val Phe Gln Ser Lys Gln
115 120 125

Phe Arg Ser Ala Lys Leu Glu Arg Leu Tyr Gln Arg Tyr Phe Phe Gln
130 135 140

Met Asn Gln Ser Ser Leu Thr Leu Leu Met Ala Val Leu Val Leu Leu
145 150 155 160

Met Ala Val Leu Leu Thr Phe His Ala Ala Pro Ala Gln Pro Gln Pro
165 170 175

Ala Tyr Val Ala Leu Leu Thr Cys Ala Ser Val Leu Phe Val Val Leu
180 185 190

Met Val Val Cys Asn Arg His Ser Phe Arg Gln Asp Ser Met Trp Val
195 200 205

Val Ser Tyr Val Val Leu Gly Ile Leu Ala Ala Val Gln Val Gly Gly
210 215 220

Ala Leu Ala Ala Asn Pro His Ser Pro Ser Ala Gly Leu Trp Cys Pro
225 230 235 240

Val Phe Phe Val Tyr Ile Thr Tyr Thr Leu Leu Pro Ile Arg Met Arg
245 250 255

Ala Ala Val Leu Ser Gly Leu Gly Leu Ser Thr Leu His Leu Ile Leu
260 265 270

Ala Trp Gln Leu Asn Ser Ser Asp Pro Phe Leu Trp Lys Gln Leu Gly
275 280 285

Ala Asn Val Val Leu Phe Leu Cys Thr Asn Ala Ile Gly Val Cys Thr
290 295 300

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

Gly Tyr Ile Gln Ala Arg Leu His Leu Gln His Glu Asn Arg Gln Gln
325 330 335

Glu Arg Leu Leu Leu Ser Val Leu Pro Gln His Val Ala Met Glu Met
340 345 350

Lys Glu Asp Ile Asn Thr Lys Lys Glu Asp Met Met Phe His Lys Ile
355 360 365

Tyr Ile Gln Lys His Asp Asn Val Ser Ile Leu Phe Ala Asp Ile Glu
370 375 380

Gly Phe Thr Ser Leu Ala Ser Gln Cys Thr Ala Gln Glu Leu Val Met
385 390 395 400

Thr Leu Asn Glu Leu Phe Ala Arg Phe Asp Lys Leu Ala Ala Glu Asn
405 410 415

[0023] His Cys Leu Arg Ile Lys Ile Leu Gly Asp Cys Tyr Tyr Cys Val Ser
420 425 430

Gly Leu Pro Glu Ala Arg Ala Asp His Ala His Cys Cys Val Glu Met
435 440 445

Gly Val Asp Met Ile Glu Ala Ile Ser Leu Val Arg Glu Val Thr Gly
450 455 460

Val Asn Val Asn Met Arg Val Gly Ile His Ser Gly Arg Val His Cys
465 470 475 480

Gly Val Leu Gly Leu Arg Lys Trp Gln Phe Asp Val Trp Ser Asn Asp
485 490 495

Val Thr Leu Ala Asn His Met Glu Ala Gly Gly Arg Ala Gly Arg Ile
500 505 510

His Ile Thr Arg Ala Thr Leu Gln Tyr Leu Asn Gly Asp Tyr Glu Val
515 520 525

Glu Pro Gly Arg Gly Gly Glu Arg Asn Ala Tyr Leu Lys Glu Gln Cys
530 535 540

Ile Glu Thr Phe Leu Ile Leu Gly Ala Ser Gln Lys Arg Lys Glu Glu
545 550 555 560

Lys Ala Met Leu Ala Lys Leu Gln Arg Thr Arg Ala Asn Ser Met Glu
565 570 575

Gly Leu Met Pro Arg Trp Val Pro Asp Arg Ala Phe Ser Arg Thr Lys
580 585 590

Asp Ser Lys Ala Phe Arg Gln Met Gly Ile Asp Asp Ser Ser Lys Asp
595 600 605

Asn Arg Gly Ala Gln Asp Ala Leu Asn Pro Glu Asp Glu Val Asp Glu
610 615 620

Phe Leu Gly Arg Ala Ile Asp Ala Arg Ser Ile Asp Gln Leu Arg Lys
625 630 635 640

Asp His Val Arg Arg Phe Leu Leu Thr Phe Gln Arg Glu Asp Leu Glu
645 650 655

Lys Lys Tyr Ser Arg Lys Val Asp Pro Arg Phe Gly Ala Tyr Val Ala
660 665 670

Cys Ala Leu Leu Val Phe Cys Phe Ile Cys Phe Ile Gln Leu Leu Val
675 680 685

Phe Pro Tyr Ser Thr Leu Ile Leu Gly Ile Tyr Ala Ala Ile Phe Leu
690 695 700

Leu Leu Leu Val Thr Val Leu Ile Cys Ala Val Cys Ser Cys Gly Ser
705 710 715 720

[0024] Phe Phe Pro Lys Ala Leu Gln Arg Leu Ser Arg Asn Ile Val Arg Ser
725 730 735

Arg Val His Ser Thr Ala Val Gly Ile Phe Ser Val Leu Leu Val Phe
740 745 750

Ile Ser Ala Ile Ala Asn Met Phe Thr Cys Asn His Thr Pro Ile Arg
755 760 765

Thr Cys Ala Ala Arg Met Leu Asn Leu Thr Pro Ala Asp Val Thr Ala
770 775 780

Cys His Leu Gln Gln Leu Asn Tyr Ser Leu Gly Leu Asp Ala Pro Leu
785 790 795 800

Cys Glu Gly Thr Ala Pro Thr Cys Ser Phe Pro Glu Tyr Phe Val Gly
805 810 815

Asn Val Leu Leu Ser Leu Leu Ala Ser Ser Val Phe Leu His Ile Ser
820 825 830

Ser Ile Gly Lys Leu Ala Met Thr Phe Ile Leu Gly Phe Thr Tyr Leu
835 840 845

Val Leu Leu Leu Leu Gly Pro Pro Ala Ala Ile Phe Asp Asn Tyr Asp
850 855 860

Leu Leu Leu Gly Val His Gly Leu Ala Ser Ser Asn Glu Thr Phe Asp
865 870 875 880

Gly Leu Asp Cys Pro Ala Val Gly Arg Val Ala Leu Lys Tyr Met Thr
885 890 895

Pro Val Ile Leu Leu Val Phe Ala Leu Ala Leu Tyr Leu His Ala Gln
900 905 910

Gln Val Glu Ser Thr Ala Arg Leu Asp Phe Leu Trp Lys Leu Gln Ala
915 920 925

Thr Gly Glu Lys Glu Glu Met Glu Glu Leu Gln Ala Tyr Asn Arg Arg
930 935 940

Leu Leu His Asn Ile Leu Pro Lys Asp Val Ala Ala His Phe Leu Ala
945 950 955 960

Arg Glu Arg Arg Asn Asp Glu Leu Tyr Tyr Gln Ser Cys Glu Cys Val
965 970 975

Ala Val Met Phe Ala Ser Ile Ala Asn Phe Ser Glu Phe Tyr Val Glu
980 985 990

Leu Glu Ala Asn Asn Glu Gly Val Glu Cys Leu Arg Leu Leu Asn Glu
995 1000 1005

Ile Ile Ala Asp Phe Asp Glu Ile Ile Ser Glu Glu Arg Phe Arg
1010 1015 1020

Gln Leu Glu Lys Ile Lys Thr Ile Gly Ser Thr Tyr Met Ala Ala
1025 1030 1035

[0025]

Ser Gly Leu Asn Ala Ser Thr Tyr Asp Gln Val Gly Arg Ser His
1040 1045 1050

Ile Thr Ala Leu Ala Asp Tyr Ala Met Arg Leu Met Glu Gln Met
1055 1060 1065

Lys His Ile Asn Glu His Ser Phe Asn Asn Phe Gln Met Lys Ile
1070 1075 1080

Gly Leu Asn Met Gly Pro Val Val Ala Gly Val Ile Gly Ala Arg
1085 1090 1095

Lys Pro Gln Tyr Asp Ile Trp Gly Asn Thr Val Asn Val Ser Ser
1100 1105 1110

Arg Met Asp Ser Thr Gly Val Pro Asp Arg Ile Gln Val Thr Thr
1115 1120 1125

Asp Leu Tyr Gln Val Leu Ala Ala Lys Gly Tyr Gln Leu Glu Cys
1130 1135 1140

Arg Gly Val Val Lys Val Lys Gly Lys Gly Glu Met Thr Thr Tyr
1145 1150 1155

Phe Leu Asn Gly Gly Pro Ser Ser
1160 1165

<210> 17

<211> 1166
<212> PRT
<213> 小家鼠

<400> 17

Met Ser Trp Phe Ser Gly Leu Leu Val Pro Lys Val Asp Glu Arg Lys
1 5 10 15

Thr Ala Trp Gly Glu Arg Asn Gly Gln Lys Arg Pro Arg His Ala Asn
20 25 30

Arg Ala Ser Gly Phe Cys Ala Pro Arg Tyr Met Ser Cys Leu Lys Asn
35 40 45

Ala Glu Pro Pro Ser Pro Thr Pro Ala Ala His Thr Arg Cys Pro Trp
50 55 60

Gln Asp Glu Ala Phe Ile Arg Arg Ala Gly Pro Gly Arg Gly Val Glu
65 70 75 80

Leu Gly Leu Arg Ser Val Ala Leu Gly Phe Asp Asp Thr Glu Val Thr
85 90 95

Thr Pro Met Gly Thr Ala Glu Val Ala Pro Asp Thr Ser Pro Arg Ser
100 105 110

Gly Pro Ser Cys Trp His Arg Leu Val Gln Val Phe Gln Ser Lys Gln
115 120 125

[0026]

Phe Arg Ser Ala Lys Leu Glu Arg Leu Tyr Gln Arg Tyr Phe Phe Gln
130 135 140

Met Asn Gln Ser Ser Leu Thr Leu Leu Met Ala Val Leu Val Leu Leu
145 150 155 160

Met Ala Val Leu Leu Thr Phe His Ala Ala Pro Ala Gln Pro Gln Pro
165 170 175

Ala Tyr Val Ala Leu Leu Thr Cys Ala Ser Val Leu Phe Val Val Leu
180 185 190

Met Val Val Cys Asn Arg His Ser Phe Arg Gln Asp Ser Met Trp Val
195 200 205

Val Ser Tyr Val Val Leu Gly Ile Leu Ala Ala Val Gln Val Gly Gly
210 215 220

Ala Leu Ala Ala Asn Pro His Ser Pro Ser Ala Gly Leu Trp Cys Pro
225 230 235 240

Val Phe Phe Val Tyr Ile Thr Tyr Thr Leu Leu Pro Ile Arg Met Arg
245 250 255

Ala Ala Val Leu Ser Gly Leu Gly Leu Ser Thr Leu His Leu Ile Leu
260 265 270

Ala Trp Gln Leu Asn Ser Ser Asp Pro Phe Leu Trp Lys Gln Leu Gly
275 280 285

Ala Asn Val Val Leu Phe Leu Cys Thr Asn Ala Ile Gly Val Cys Thr
290 295 300

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

Gly Tyr Ile Gln Ala Arg Leu His Leu Gln His Glu Asn Arg Gln Gln
325 330 335

Glu Arg Leu Leu Leu Ser Val Leu Pro Gln His Val Ala Met Glu Met
340 345 350

Lys Glu Asp Ile Asn Thr Lys Lys Glu Asp Met Met Phe His Lys Ile
355 360 365

Tyr Ile Gln Lys His Asp Asn Val Ser Ile Leu Phe Ala Asp Ile Glu
370 375 380

Gly Phe Thr Ser Leu Ala Ser Gln Cys Thr Ala Gln Glu Leu Val Met
385 390 395 400

Thr Leu Asn Glu Leu Phe Ala Arg Phe Asp Lys Leu Ala Ala Glu Asn
405 410 415

His Cys Leu Arg Ile Lys Ile Leu Gly Ala Cys Tyr Tyr Cys Val Ser
420 425 430

[0027] Gly Leu Pro Glu Ala Arg Ala Asp His Ala His Cys Cys Val Glu Met
435 440 445

Gly Val Asp Met Ile Glu Ala Ile Ser Leu Val Arg Glu Val Thr Gly
450 455 460

Val Asn Val Asn Met Arg Val Gly Ile His Ser Gly Arg Val His Cys
465 470 475 480

Gly Val Leu Gly Leu Arg Lys Trp Gln Phe Asp Val Trp Ser Asn Asp
485 490 495

Val Thr Leu Ala Asn His Met Glu Ala Gly Gly Arg Ala Gly Arg Ile
500 505 510

His Ile Thr Arg Ala Thr Leu Gln Tyr Leu Asn Gly Asp Tyr Glu Val
515 520 525

Glu Pro Gly Arg Gly Gly Glu Arg Asn Ala Tyr Leu Lys Glu Gln Cys
530 535 540

Ile Glu Thr Phe Leu Ile Leu Gly Ala Ser Gln Lys Arg Lys Glu Glu
545 550 555 560

Lys Ala Met Leu Ala Lys Leu Gln Arg Thr Arg Ala Asn Ser Met Glu
565 570 575

Gly Leu Met Pro Arg Trp Val Pro Asp Arg Ala Phe Ser Arg Thr Lys
580 585 590

```
Asp Ser Lys Ala Phe Arg Gln Met Gly Ile Asp Asp Ser Ser Lys Asp
        595                 600                 605

Asn Arg Gly Ala Gln Asp Ala Leu Asn Pro Glu Asp Glu Val Asp Glu
    610                 615                 620

Phe Leu Gly Arg Ala Ile Asp Ala Arg Ser Ile Asp Gln Leu Arg Lys
625                 630                 635                 640

Asp His Val Arg Arg Phe Leu Leu Thr Phe Gln Arg Glu Asp Leu Glu
                645                 650                 655

Lys Lys Tyr Ser Arg Lys Val Asp Pro Arg Phe Gly Ala Tyr Val Ala
            660                 665                 670

Cys Ala Leu Leu Val Phe Cys Phe Ile Cys Phe Ile Gln Leu Leu Val
        675                 680                 685

Phe Pro Tyr Ser Thr Leu Ile Leu Gly Ile Tyr Ala Ala Ile Phe Leu
    690                 695                 700

Leu Leu Leu Val Thr Val Leu Ile Cys Ala Val Cys Ser Cys Gly Ser
705                 710                 715                 720

Phe Phe Pro Lys Ala Leu Gln Arg Leu Ser Arg Asn Ile Val Arg Ser
                725                 730                 735

Arg Val His Ser Thr Ala Val Gly Ile Phe Ser Val Leu Leu Val Phe
            740                 745                 750
```

[0028]

```
Ile Ser Ala Ile Ala Asn Met Phe Thr Cys Asn His Thr Pro Ile Arg
        755                 760                 765

Thr Cys Ala Ala Arg Met Leu Asn Leu Thr Pro Ala Asp Val Thr Ala
    770                 775                 780

Cys His Leu Gln Gln Leu Asn Tyr Ser Leu Gly Leu Asp Ala Pro Leu
785                 790                 795                 800

Cys Glu Gly Thr Ala Pro Thr Cys Ser Phe Pro Glu Tyr Phe Val Gly
                805                 810                 815

Asn Val Leu Leu Ser Leu Leu Ala Ser Ser Val Phe Leu His Ile Ser
            820                 825                 830

Ser Ile Gly Lys Leu Ala Met Thr Phe Ile Leu Gly Phe Thr Tyr Leu
        835                 840                 845

Val Leu Leu Leu Leu Gly Pro Pro Ala Ala Ile Phe Asp Asn Tyr Asp
    850                 855                 860

Leu Leu Leu Gly Val His Gly Leu Ala Ser Ser Asn Glu Thr Phe Asp
865                 870                 875                 880

Gly Leu Asp Cys Pro Ala Val Gly Arg Val Ala Leu Lys Tyr Met Thr
                885                 890                 895

Pro Val Ile Leu Leu Val Phe Ala Leu Ala Leu Tyr Leu His Ala Gln
```

900 905 910

Gln Val Glu Ser Thr Ala Arg Leu Asp Phe Leu Trp Lys Leu Gln Ala
915 920 925

Thr Gly Glu Lys Glu Glu Met Glu Glu Leu Gln Ala Tyr Asn Arg Arg
930 935 940

Leu Leu His Asn Ile Leu Pro Lys Asp Val Ala Ala His Phe Leu Ala
945 950 955 960

Arg Glu Arg Arg Asn Asp Glu Leu Tyr Tyr Gln Ser Cys Glu Cys Val
965 970 975

Ala Val Met Phe Ala Ser Ile Ala Asn Phe Ser Glu Phe Tyr Val Glu
980 985 990

Leu Glu Ala Asn Asn Glu Gly Val Glu Cys Leu Arg Leu Leu Asn Glu
995 1000 1005

Ile Ile Ala Asp Phe Asp Glu Ile Ile Ser Glu Glu Arg Phe Arg
1010 1015 1020

Gln Leu Glu Lys Ile Lys Thr Ile Gly Ser Thr Tyr Met Ala Ala
1025 1030 1035

[0029]

Ser Gly Leu Asn Ala Ser Thr Tyr Asp Gln Val Gly Arg Ser His
1040 1045 1050

Ile Thr Ala Leu Ala Asp Tyr Ala Met Arg Leu Met Glu Gln Met
1055 1060 1065

Lys His Ile Asn Glu His Ser Phe Asn Asn Phe Gln Met Lys Ile
1070 1075 1080

Gly Leu Asn Met Gly Pro Val Val Ala Gly Val Ile Gly Ala Arg
1085 1090 1095

Lys Pro Gln Tyr Asp Ile Trp Gly Asn Thr Val Asn Val Ser Ser
1100 1105 1110

Arg Met Asp Ser Thr Gly Val Pro Asp Arg Ile Gln Val Thr Thr
1115 1120 1125

Asp Leu Tyr Gln Val Leu Ala Ala Lys Gly Tyr Gln Leu Glu Cys
1130 1135 1140

Arg Gly Val Val Lys Val Lys Gly Lys Gly Glu Met Thr Thr Tyr
1145 1150 1155

Phe Leu Asn Gly Gly Pro Ser Ser
1160 1165

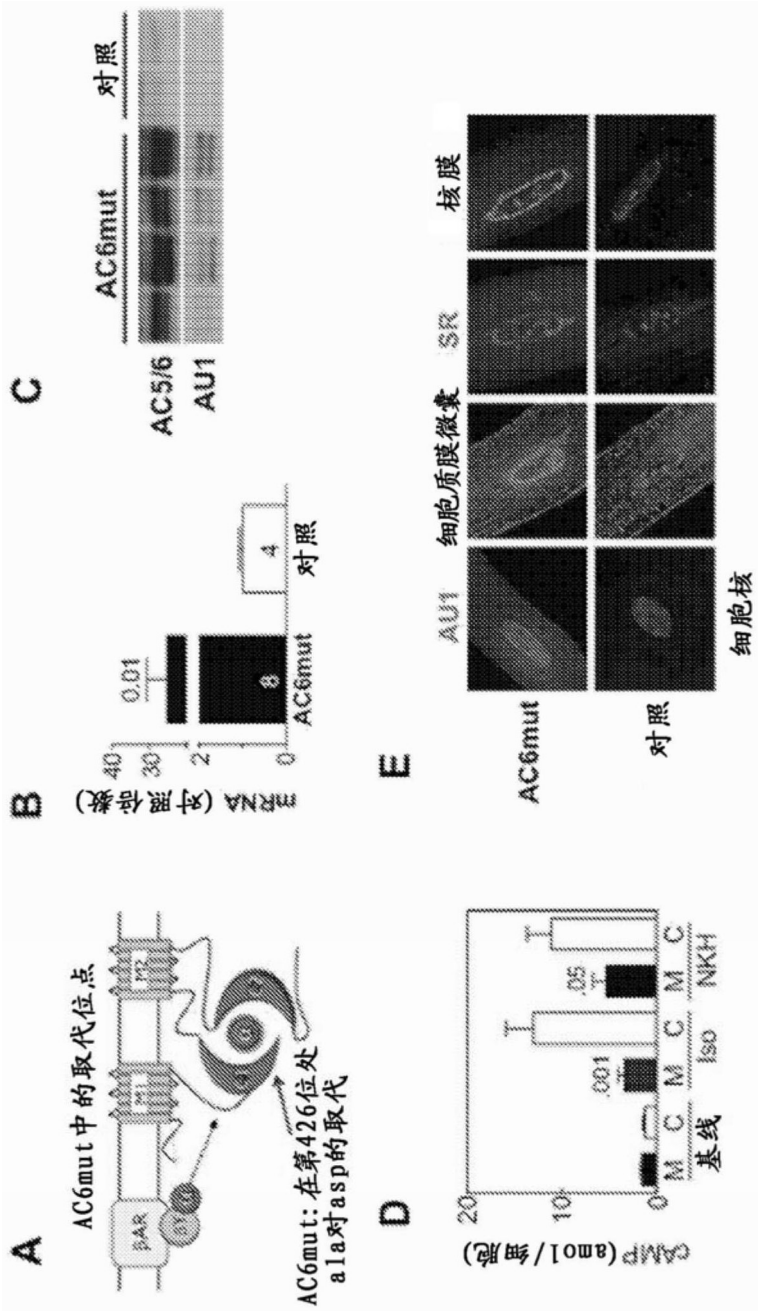

A
AC6mut中的取代位点
BAR
P1
P2
AC6mut:在第426位处
ala对asp的取代
B
ERKs (对照倍数)
40
30
21
0
0.01
8
4
AC6mut
对照
C
AC6mut
对照
AC5/6
AU1
D
cAMP (amol/细胞)
20
10
0
M C M C M C
基线
Iso
NKH
E
AU1
细胞质膜微囊
SR
核膜
AC6mut
对照
细胞核

图1

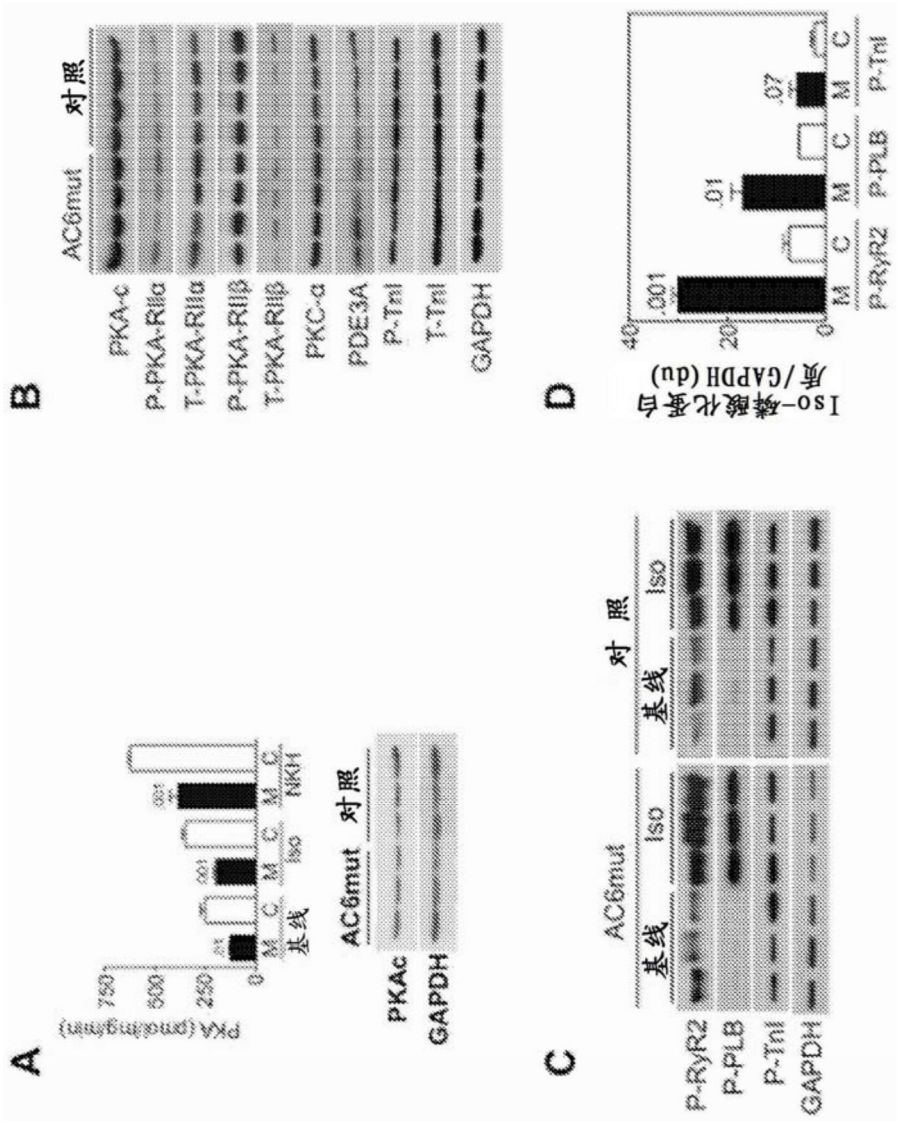

A
PKA (pmol/mg/min)
750
500
250
0
基线
Iso
NKH
AC6mut
对照
PKAc
GAPDH
B
AC6mut
对照
PKA-c
P-PKA-RIIα
T-PKA-RIIα
P-PKA-RIIβ
T-PKA-RIIβ
PKC-α
PDE3A
P-TnI
T-TnI
GAPDH
C
AC6mut
对照
基线
Iso
P-RyR2
P-PLB
P-TnI
GAPDH
D
Iso-磷酸化蛋白质/GAPDH (du)
P-RyR2
P-PLB
P-TnI

图2

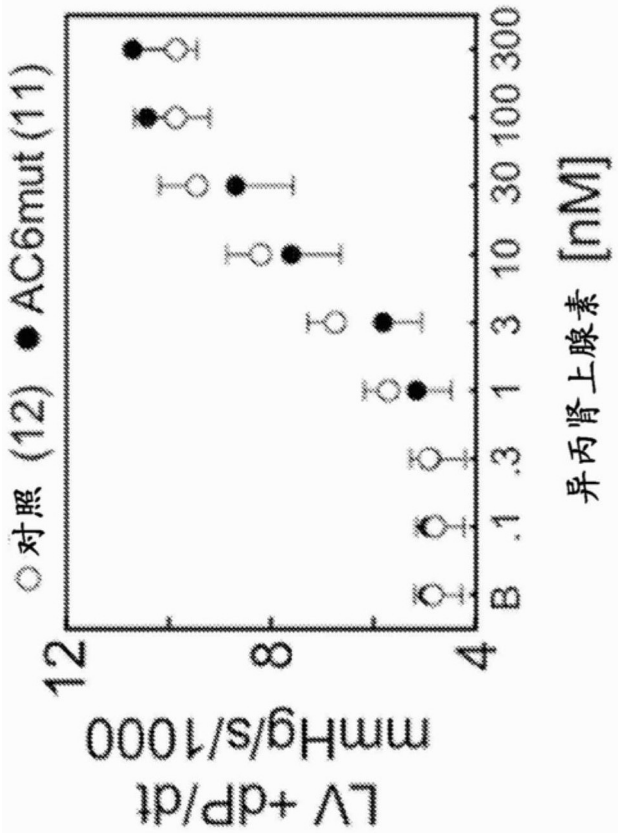

LV +dP/dt
mmHg/s/1000
12
8
4
B .1 .3 1 3 10 30 100 300
异丙肾上腺素 [nM]
○对照 (12) ●AC6mut (11)

图3

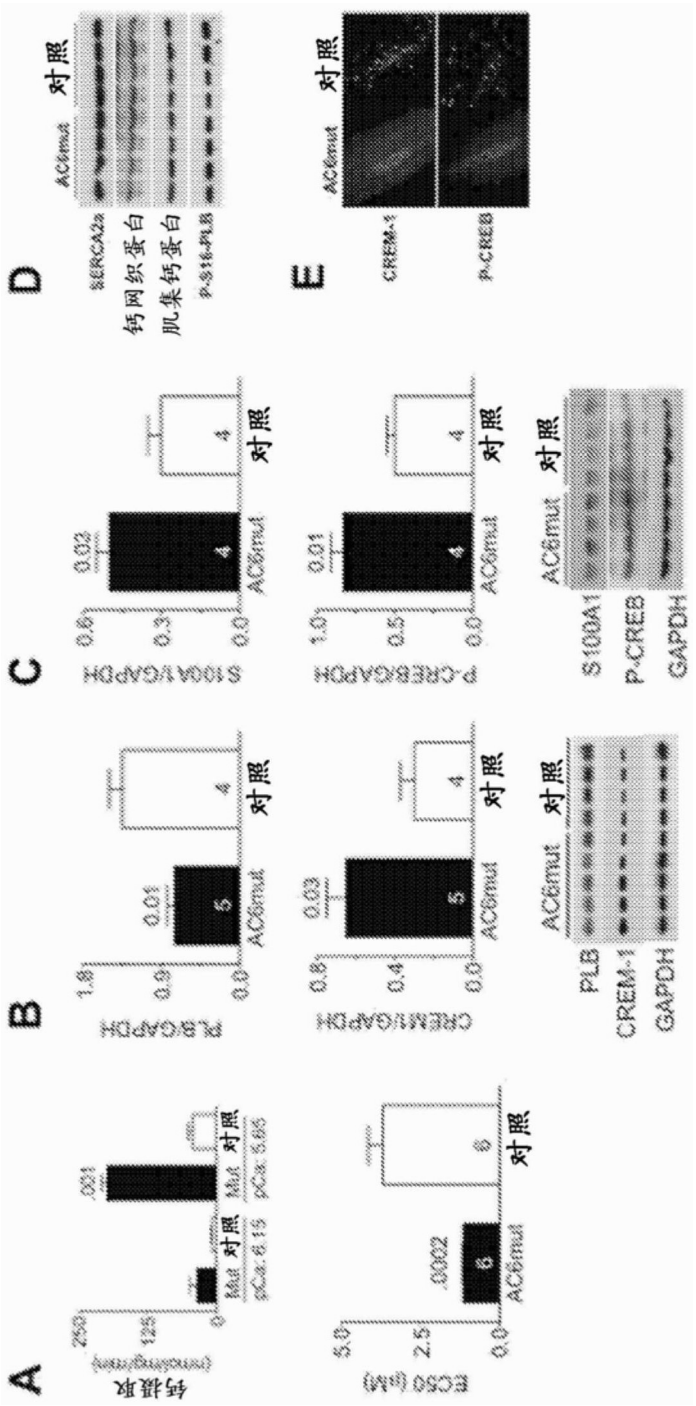

A
钙摄取
(nmol/mg/min)
250
125
0
Mut 对照
pCa 6.15
Mut 对照
pCa 5.85
.001
B
PLB/GAPDH
1.8
0.9
0.0
0.01
5
4
AC6mut 对照
CREM1/GAPDH
0.8
0.4
0.0
0.03
5
4
AC6mut 对照
C
S100A1/GAPDH
0.6
0.3
0.0
0.02
4
4
AC6mut 对照
P-CREB/GAPDH
1.0
0.5
0.0
0.01
4
4
AC6mut 对照
D
AC6mut 对照
SERCA2a
钙网织蛋白
肌集钙蛋白
P-S16-PLB
E
AC6mut 对照
CREM-1
P-CREB
AC6mut 对照
PLB
CREM-1
GAPDH
AC6mut 对照
S100A1
P-CREB
GAPDH
ECS0 (μM)
5.0
2.5
0.0
.0002
6
6
AC6mut 对照

图4

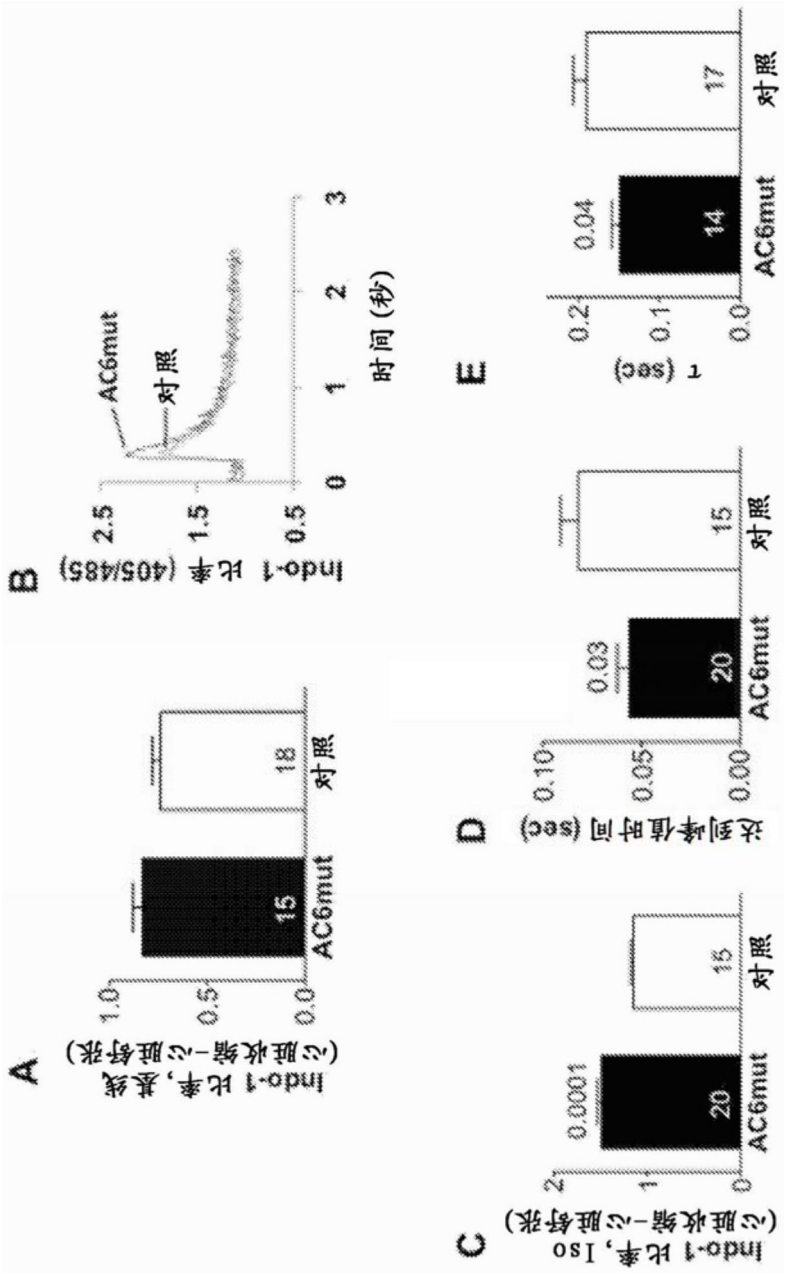

A
Indo-1 比率，基线
(心脏收缩-心脏舒张)
1.0
0.5
0.0
15
18
AC6mut
对照
B
Indo-1 比率 (405/485)
2.5
1.5
0.5
0
1
2
3
时间 (秒)
AC6mut
对照
C
Indo-1 比率，Iso
(心脏收缩-心脏舒张)
2
1
0
0.0001
20
15
AC6mut
对照
D
达到峰值时间 (sec)
0.10
0.05
0.00
0.03
20
15
AC6mut
对照
E
τ (sec)
0.2
0.1
0.0
0.04
14
17
AC6mut
对照

图5