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(54) Title: INDUCING PROLIFERATION OF CARDIOMYOCYTES AND THERAPEUTIC USES RELATED THERETO

(57) Abstract: This disclosure relates to methods of treating or preventing heart malformations or cardiovascular diseases comprising administering an effective amount of thyroid hormone in combination with i) an agent that decreases DUSP5 and/or DUSP6 expression and/or ii) a beta-adrenergic blocking agent to a subject in need thereof. In certain embodiments, this disclosure relates to in vivo and in vitro methods of inducing proliferation of cardiomyocytes using agents or combinations of agents disclosed herein.



INDUCING PROLIFERATION OF CARDIOMYOCYTES AND THERAPEUTIC USES RELATED THERETO

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 62/718,583 filed August 14, 2018 and U.S. Provisional Application No. 62/813,467 filed March 4, 2019. The entirety of each of these applications is hereby incorporated by reference for all purposes.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under HL127726 awarded by the National Institutes of Health. The government has certain rights in the invention.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED AS A TEXT FILE VIA THE OFFICE ELECTRONIC FILING SYSTEM (EFS-WEB)

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is 18222PCT_ST25.txt. The text file is 22 KB, was created on August 14, 2019, and is being submitted electronically via EFSWeb.

BACKGROUND

Human hearts are typically unable to regenerate cardiac tissue following traumatic injury leading to heart failure. Thus, there is a need to identify methods of improving cardiac regeneration. Compared to low oxygen environment of the fetus, the oxygen-rich postnatal environment at birth induces physiological changes. Cardiomyocytes in the heart dramatically decrease spontaneous replication several weeks after birth. Nakada et al. report oxygen-dependent mitochondrial metabolism is a major driver of cell cycle arrest of cardiomyocytes. *Nature*, 2017, 541(7636):222-227. Thyroid hormone increases aerobic metabolism, induces mitochondrial biogenesis and activates oxidative phosphorylation (OXPHOS), a major source of ROS, in the early postnatal period. Li et al. *Stem Cell Res.* 2014, 13(3 Pt B):582-91.

The human heart contains a mixture of mononuclear and binuclear cardiomyocytes. Binuclear cardiomyocytes are believed to be more actively involved in cardiomyocyte replication and regeneration. Naqvi et al. report a thyroid hormone surge activates the IGF-1/IGF1-R/Akt pathway after birth and initiates a brief but intense proliferative burst of predominantly binuclear cardiomyocytes. *Cell*. 2014, 157(4): 795–807.

Molina et al. report zebrafish chemical screening reveals an inhibitor of DUSP6 that expands cardiac cell lineages. *Nat. Chem. Biol.* 2009, 5:680–687

Ueda et al. report dual-specificity phosphatase 5 (DUSP5) as a direct transcriptional target of tumor suppressor p53. *Oncogene*. 2003, 22(36):5586-91.

US Published Patent Application 2018/0000778 (Ramchandran et al.) reports small molecule antagonists of DUSP5. See also US 2007/0276135 (Khvorova et al.).

References cited herein are not an admission of prior art.

SUMMARY

This disclosure relates to methods of treating or preventing heart malformations or cardiovascular diseases comprising administering an effective amount of thyroid hormone in combination with i) an agent that decreases DUSP5 and/or DUSP6 expression and/or ii) a beta-adrenergic blocking agent to a subject in need thereof. In certain embodiments, this disclosure relates to in vivo and in vitro methods of inducing proliferation of cardiomyocytes using agents or combinations of agents disclosed herein.

In certain embodiments, this disclosure relates to methods of treating or preventing heart malformation or cardiovascular disease comprising administering an effective amount of thyroid hormone in combination with a beta-adrenergic blocking agent to a subject in need thereof. In certain embodiments, this disclosure relates to methods of inducing proliferation of cardiomyocytes comprising administering an effective amount of a DUSP5 and/or DUSP6 inhibitor to a subject.

In certain embodiments, the beta-adrenergic blocking agent is selected from acebutolol, atenolol, betaxolol, bisoprolol, carteolol, carvedilol, esmolol, labetalol, metoprolol, nadolol, nebivolol, penbutolol, pindolol, propranolol, sotalol, timolol, or salts thereof. In certain embodiments, the beta-adrenergic blocking agent is administered in combination with a mitogen. In certain embodiments, the mitogen is neuregulin, IGF-1, YAP1, ERBB2.

In certain embodiments, the cardiovascular disease is selected from coronary artery disease, heart failure, cardiomyopathy, heart valve disease, and cardiac arrhythmias. In certain embodiments, the subject is diagnosed with a heart attack, angina, or stroke, diabetes, angina pectoris due to coronary atherosclerosis, hypertension, migraine headaches, glaucoma, hyperthyroidism, fibromyalgia, generalized anxiety disorder, parkinsonian tremor, and atrial fibrillation.

In certain embodiments, this disclosure relates to methods of inducing proliferation of cardiomyocytes comprising administering an effective amount of thyroid hormone in combination with a beta-adrenergic blocking agent to a subject in need thereof. In certain embodiments, the beta-adrenergic blocking agent is siRNA. In certain embodiments, the combination is administered to a newborn within 1 to 7 days of birth. In certain embodiments, the combination is administered to a newborn within 8 to 14 days of birth. In certain embodiments, the combination is administered to a newborn within 15 to 21 days of birth. In certain embodiments, the combination is administered to a subject more than 21 days after birth.

In certain embodiments, the combination is administered by intravenous (IV) injection, direct injection into the cardiac tissue, direct injection into apical cardiac tissue, or pericardial cavity. In certain embodiments, the combination is administered to a subject at risk of, exhibiting symptoms of, or diagnosed with a cardiovascular disease, condition, or injury. In certain embodiments, the combination is administered to a subject at risk of, exhibiting symptoms of, or diagnosed with heart disease, heart attack, stroke, heart failure, arrhythmia, heart valve disease, congenital heart defect, patent ductus arteriosus, ventricular septal defect, truncus arteriosus, atrioventricular septal defect, tetralogy of Fallot, transposition of the great arteries, hypoplastic left heart syndrome, tricuspid atresia, or heart murmur, for use in the treatment or prevention thereof.

In certain embodiments, this disclosure relates to an in vitro method of inducing proliferation of cardiomyocytes comprising administering an effective amount of a thyroid hormone in combination with a beta-adrenergic blocking agent to a cardiac cell.

In certain embodiments, this disclosure relates to an in vitro method of inducing proliferation of cardiomyocytes comprising administering an effective amount of a thyroid hormone in combination with a DUSP5 or DUSP6 inhibitor to a cardiac cell.

In certain embodiments, this disclosure relates to methods of inducing proliferation of cardiomyocytes comprising administering an effective amount of a DUSP5 and/or DUSP6

inhibitor to a subject, wherein the DUSP5 or DUSP6 inhibitor is a small molecule or wherein the DUSP5 or DUSP6 inhibitor is a polysulfonated aromatic compound with a carbazole or naphthalene scaffold.

In certain embodiments, the DUSP5 or DUSP6 inhibitor is a compound selected from 2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one (BCI), carbazole-1,3,6-trisulfonic acid, carbazole-1,3,6,8-tetrasulfonic acid, 8-hydroxynaphthalene-1,6-disulfonic acid (RR527), 8-amino-4-hydroxynaphthalene-2-sulfonic acid (RR535), 8,8'-(diazene-1,2-diyl)bis(4-hydroxy naphthalene-2-sulfonic acid) (RR601), 1²,3²,5²,7²-tetrahydroxy-1,3,5,7(1,3)-tetrabenzene cyclooctaphane-1⁵,3⁵,5⁵,7⁵-tetrasulfonic acid (RR701), derivatives, prodrugs, alkylesters, or alternative salts thereof.

In certain embodiments, the DUSP5 or DUSP6 inhibitor is siRNA or an antisense therapy.

In certain embodiments, the DUSP5 or DUSP6 inhibitor is siRNA comprising a sense region and an antisense region, wherein said sense region and said antisense region together form a duplex region, said antisense region and said sense region are each 18-30 nucleotides in length and said antisense region comprises a sequence a sequence selected from the group consisting of SEQ ID Nos: 1-120 or variant thereof.

In certain embodiments, the DUSP5 or DUSP6 inhibitor is administered to a newborn within 1 to 7 days of birth.

In certain embodiments, the DUSP5 or DUSP6 inhibitor is administered to a newborn within 8 to 14 days of birth. In certain embodiments, the DUSP5 or DUSP6 inhibitor is administered to a newborn within 15 to 21 days of birth. In certain embodiments, the DUSP5 or DUSP6 inhibitor is administered to a subject more than 21 days after birth

In certain embodiments, the DUSP5 or DUSP6 inhibitor is administered by intravenous (IV) injection, direct injection into the cardiac tissue, direct injection into apical cardiac tissue, or pericardial cavity.

In certain embodiments, the DUSP5 or DUSP6 inhibitor is administered at risk of, exhibiting symptoms of, or diagnosed with a cardiovascular disease, condition, or injury. In certain embodiments, the DUSP5 or DUSP6 inhibitor is administered at risk of, exhibiting symptoms of, or diagnosed with heart disease, heart attack, stroke, heart failure, arrhythmia, heart valve disease, congenital heart defect, patent ductus arteriosus, ventricular septal defect, truncus arteriosus,

atrioventricular septal defect, tetralogy of Fallot, transposition of the great arteries, hypoplastic left heart syndrome, tricuspid atresia, or heart murmur, for use in the treatment or prevention thereof.

In certain embodiments, this disclosure relates to an in vitro method of inducing proliferation of cardiomyocytes comprising administering an effective amount of a DUSP5 and/or DUSP6 inhibitor to a cardiac cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows data indicating developmental loss of T₃-induced proliferative signaling in postnatal cardiomyocytes.

Figure 1B shows data indicating an effect of in vivo T₃ treatment on regional cyclin D1, A2, and B1 expression in the post-neonatal heart.

Figure 1C shows representative immunoblots indicating expression levels of proteins in whole cell lysates of cardiomyocytes of the LV apex (a) and base (b) at P2 (neonatal), P8 (post-neonatal/early preadolescent) and P16 (preadolescent), both with and without in vivo T₃ treatment.

Figure 2A shows data indicating cardiomyocytes (CM) of the early post-neonatal LV apex retain proliferative capacity. Cardiomyocytes were identified by cTnT labeling. T₃ effects on DNA synthesis (EdU) (A) and in LV apical (a) and basal (b) cardiomyocytes.

Figure 2B shows data on mitosis (pH3).

Figure 2C illustrates multicolor labeling of post-neonatal mouse LV cardiomyocytes. Limited 4-hydroxytamoxifen-induced recombination of paired loxP sites (black triangles) leads to expression of green fluorescent protein (GFP), yellow fluorescent protein (YFP), red fluorescent protein (RFP) or cyan fluorescent protein (CFP) in a few cardiomyocytes, randomly positioned within the entire LV. Insets show examples of monochromatic clusters.

Figure 2D show data on total ventricular cardiomyocyte cell numbers.

Figure 3A shows data indicating T₃-stimulated cyclin expression in early post-neonatal apical cardiomyocytes is mediated by IGF-1/IGF-1R signaling. In vivo cardiomyocyte-specific IGF-1R knockdown prevented T₃-induced increases in cyclins in LV apical cardiomyocytes. Floxed Igf1r mice (Igf1rflox/flox) were crossed with Myh6-MerCreMer mice in which the transgene consists of the mouse cardiac-specific Myh6 promoter directing expression of a 4-hydroxytamoxifen-inducible Cre recombinase (MerCreMer) in postnatal cardiomyocytes. To

achieve such a cell type-specific knockdown, 4-hydroxytamoxifen was administered at P6 to Myh6-MerCreMer::Igf1rflox/flox mice.

Figure 3B shows data from immunoblots indicating expression levels of indicated proteins in post-neonatal apical (a) and basal (b) cardiomyocytes after in vivo T₃ or vehicle treatment. Bar graph shows fold changes in expression 40 h after T₃ treatment, relative to vehicle treatment.

Figure 4A shows data indicating DUSP5 inhibits T₃-induced proliferative signaling in early post-neonatal LV cardiomyocytes (CMs). Protocol for T₃ administration, cardiomyocyte isolation and preparation of whole cell, cytosolic or nuclear lysates for immunoblotting is shown in the schematic. Representative immunoblots show expression levels of indicated proteins in cardiomyocytes of the LV apex and base at 0 (baseline) and 24 h after T₃ administration. Bar graphs show quantification of the phospho-MEK1/2, and nuclear phospho-ERK1/2 and DUSP5.

Figure 4B shows data where DUSP5 or scrambled (control) siRNAs were administered intraperitoneally (i.p.) to mice at P6 and P7, followed by T₃ (+) or vehicle (–) treatment at P8. siRNAs were dissolved in the in vivo-jetPEI and 10% glucose mixture. Forty hours later, CMs were isolated and whole cell lysates prepared. Lysates were then subjected to immunoblotting for the indicated proteins.

Figure 4C shows a model illustrating the proposed mechanism underlying the selective loss of nuclear phosphorylated ERK1/2 in LV basal cardiomyocytes of early post-neonatal mice.

Figure 5A shows data indicating acute DUSP5 depletion reverses cell cycle block in LV cardiomyocytes (CMs) of 6-week-old mice. Representative immunoblots showing developmental changes in myocardial DUSP5, tyrosine hydroxylase and β 1-adrenergic receptor (β 1-AR) levels in the LV apex (a) and base (b) of early postnatal hearts.

Figure 5B shows DUSP5 knockdown in cardiomyocytes of the LV apex and base, using acute in vivo siRNA treatment, increased ERK1/2 phosphorylation and cyclin expression in response to T₃. Scrambled siRNA was used as a siRNA control. Whole cell lysates were used in these studies.

Figure 5C shows data indicating total ventricular cardiomyocyte numbers were greater in T₃- (dots) versus vehicle (dots)-treated mice that had been pretreated with DUSP5 siRNA, but not in those pretreated with scrambled siRNA.

Figure 6A shows data indicating cardiomyocytes of the P8 LV apex and base have similar morphology. Comparison of mono- (1N), bi- (2N) and multi-nuclear (>2N) cardiomyocyte (CM)

populations in the P8 LV apex (a) and base (b). Examples of mono- (1N), bi- (2N) and multi-nuclear (>2N) CMs are shown in insets, where CMs are identified by cTnT labeling and nuclei by DAPI.

Figure 6B shows a comparison of cell areas between P8 LV apical and base CMs.

Figure 7A. β 1-adrenergic receptors (β 1-AR) regulate DUSP5 expression in LV cardiomyocytes. β 1-adrenergic receptor knockdown in cardiomyocytes of the early post-neonatal LV apex and base, using acute in vivo siRNA treatment, decreased DUSP5 levels and increased ERK1/2 phosphorylation and cyclin expression in response to T₃. Scrambled siRNA was used as a siRNA control.

Figure 7B shows data for 5-week-old mice.

Figure 7C shows data using a β 1-AR blocker (metoprolol) pretreatment also blocks DUSP5 expression in young adult cardiomyocytes of the LV apex and base and increased cyclin expression in response to T₃.

DETAILED DISCUSSION

Before the present disclosure is described in greater detail, it is to be understood that this disclosure is not limited to embodiments described, and as such may, of course, vary. It is also to be understood that the terminology used herein is for describing particular embodiments only, and is not intended to be limiting, since the scope of the present disclosure will be limited only by the appended claims.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present disclosure, the preferred methods and materials are now described.

All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference and are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present disclosure is not entitled to antedate such publication by prior disclosure. Further,

the dates of publication provided could be different from the actual publication dates that may need to be independently confirmed.

As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present disclosure. Any recited method can be carried out in the order of events recited or in any other order that is logically possible.

Embodiments of the present disclosure will employ, unless otherwise indicated, techniques of medicine, organic chemistry, biochemistry, molecular biology, pharmacology, and the like, which are within the skill of the art. Such techniques are explained fully in the literature.

It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. In this specification and in the claims that follow, reference will be made to a number of terms that shall be defined to have the following meanings unless a contrary intention is apparent.

As used in this disclosure and claim(s), the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") have the meaning ascribed to them in U.S. Patent law in that they are inclusive or open-ended and do not exclude additional, unrecited elements or method steps. The term "comprising" in reference to an oligonucleotide having a nucleic acid sequence refers to an oligonucleotide that may contain additional 5' (5' terminal end) or 3' (3' terminal end) nucleotides, i.e., the term is intended to include the oligonucleotide sequence within a larger nucleic acid. "Consisting essentially of" or "consists of" or the like, when applied to methods and compositions encompassed by the present disclosure refers to compositions like those disclosed herein that exclude certain prior art elements to provide an inventive feature of a claim, but which may contain additional composition components or method steps, etc., that do not materially affect the basic and novel characteristic(s) of the compositions or methods, compared to those of the corresponding compositions or methods disclosed herein. The term "consisting of" in reference to an oligonucleotide having a nucleotide sequence refers an oligonucleotide having the exact number of nucleotides in the sequence and not

more or having not more than a range of nucleotide expressly specified in the claim. For example, “5’ sequence consisting of” is limited only to the 5’ end, i.e., the 3’ end may contain additional nucleotides. Similarly, a “3’ sequence consisting of” is limited only to the 3’ end, and the 5’ end may contain additional nucleotides.

The following abbreviations and acronyms are used: β 1-AR (β 1-adrenergic receptor), CM (cardiomyocyte), DUSP5 (dual specificity phosphatase 5), EdU (5-ethynyl-2'-deoxyuridine), EF (ejection fraction), ERK1/2 (extracellular signal-regulated kinase-1/2), FS (fractional shortening), FWd (left ventricle free wall dimension at diastole), FWs (left ventricle free wall dimension at systole), IGF-1 (insulin-like growth factor-1), IGF-1R (insulin-like growth factor-1 receptor), IVSd (intraventricular septum dimension at diastole), IVSs (intraventricular septum dimension at systole), LV (left ventricle), LVEDD (left ventricle end-diastolic dimension), LVESD (left ventricle end-systolic dimension), MAPK (mitogen-activated protein kinase), MEK (mitogen-activated protein kinase kinase), P (postnatal day), pH3 (phospho-histone H3), RV (right ventricle).

As sued herein, the terms “T₃” and “thyroid hormone” refer to the compound, 3,3',5-triiodo-L-thyronine, IUPAC name (2S)-2-amino-3-[4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenyl]propanoic acid and salts thereof. T₃ derivatives are contemplated as a substitution of T₃ for uses in all methods and compositions disclosed herein. Example derivatives include tyrosine substituted with one or more iodine and deaminated or decarboxylated derivatives such as, thyroxine (T₄), 3,3',5'-triiodothyronine (rT₃), diiodothyronine (T₂), monoiodothyronine (T₁), 3,5,3'-triiodothyroacetic acid (Triac), 3,3',5,5'-tetraiodoacetic acid (Tetrac), 3,5,3'-triiodothyropropionic acid, 3-iodo-thyroacetic acid, 3,3',5-triiodothyronamine (Triam), 3,5-diiodo-thyronamine (T₂AM), 3-iodothyronamine (3-T₁AM), thyronamine (T₀AM), prodrugs, carboxylic acid esters, and salts thereof.

As used herein, the term “derivative” refers to a structurally similar compound that retains sufficient functional attributes of the identified analogue. The derivative may be structurally similar because it is lacking one or more atoms, substituted, a salt, in different hydration/oxidation states, or because one or more atoms within the molecule are switched, such as, but not limited to, replacing an oxygen atom with a sulfur atom or replacing an amino group with a hydroxyl group. The derivative may be a prodrug. Derivatives may be prepared by any variety of synthetic methods or appropriate adaptations presented in synthetic or organic chemistry text books, such as those

provide in March's Advanced Organic Chemistry: Reactions, Mechanisms, and Structure, Wiley, 6th Edition (2007) Michael B. Smith or Domino Reactions in Organic Synthesis, Wiley (2006) Lutz F. Tietze hereby incorporated by reference.

The term "substituted" refers to a molecule wherein at least one hydrogen atom is replaced with a substituent. When substituted, one or more of the groups are "substituents." The molecule may be multiply substituted. In the case of an oxo substituent ("=O"), two hydrogen atoms are replaced. Example substituents within this context may include halogen, hydroxy, alkyl, alkoxy, nitro, cyano, oxo, carbocyclyl, carbocycloalkyl, heterocarbocyclyl, heterocarbocycloalkyl, aryl, arylalkyl, heteroaryl, heteroarylalkyl, $-NR_aR_b$, $-NR_aC(=O)R_b$, $-NR_aC(=O)NR_aNR_b$, $-NR_aC(=O)OR_b$, $-NR_aSO_2R_b$, $-C(=O)R_a$, $-C(=O)OR_a$, $-C(=O)NR_aR_b$, $-OC(=O)NR_aR_b$, $-OR_a$, $-SR_a$, $-SOR_a$, $-S(=O)_2R_a$, $-OS(=O)_2R_a$ and $-S(=O)_2OR_a$. R_a and R_b in this context may be the same or different and independently hydrogen, halogen hydroxyl, alkyl, alkoxy, alkyl, amino, alkylamino, dialkylamino, carbocyclyl, carbocycloalkyl, heterocarbocyclyl, heterocarbocycloalkyl, aryl, arylalkyl, heteroaryl, and heteroarylalkyl.

The term "prodrug" refers to an agent that is converted into a biologically active form in vivo. Prodrugs are often useful because, in some situations, they may be easier to administer than the parent compound. They may, for instance, be bioavailable by oral administration whereas the parent compound is not. The prodrug may also have improved solubility in pharmaceutical compositions over the parent drug. A prodrug may be converted into the parent drug by various mechanisms, including enzymatic processes and metabolic hydrolysis. Typical prodrugs are pharmaceutically acceptable esters. Prodrugs include compounds wherein a hydroxy, amino or mercapto group is bonded to any group that, when the prodrug of the active compound is administered to a subject, cleaves to form a free hydroxy, free amino or free mercapto group, respectively. Examples of prodrugs include, but are not limited to, acetate, formate and benzoate derivatives of an alcohol or acetamide, formamide and benzamide derivatives of an amine functional group in the active compound and the like.

For example, if a disclosed compound or a pharmaceutically acceptable form of the compound contains a carboxylic acid functional group, a prodrug can comprise a pharmaceutically acceptable ester formed by the replacement of the hydrogen atom of the acid group with a group such as (C_1-C_8) alkyl, (C_2-C_{12}) alkanoyloxymethyl, 1-(alkanoyloxy)ethyl having from 4 to 9 carbon atoms, 1-methyl-1-(alkanoyloxy)-ethyl having from 5 to 10 carbon atoms,

alkoxycarbonyloxymethyl having from 3 to 6 carbon atoms, 1-(alkoxycarbonyloxy)ethyl having from 4 to 7 carbon atoms, 1-methyl-1-(alkoxycarbonyloxy)ethyl having from 5 to 8 carbon atoms, N-(alkoxycarbonyl)aminomethyl having from 3 to 9 carbon atoms, 1-(N-(alkoxycarbonyl)amino)ethyl having from 4 to 10 carbon atoms, 3-phthalidyl, 4-crotonolactonyl, gamma-butyrolacton-4-yl, di-N,N-(C₁-C₂)alkylamino(C₂-C₃)alkyl (such as beta-dimethylaminoethyl), carbamoyl-(C₁-C₂)alkyl, N,N-di(C₁-C₂)alkylcarbamoyl-(C₁-C₂)alkyl and piperidino-, pyrrolidino- or morpholino(C₂-C₃)alkyl.

If a disclosed compound or a pharmaceutically acceptable form of the compound contains an alcohol functional group, a prodrug can be formed by the replacement of the hydrogen atom of the alcohol group with a group such as (C₁-C₆)alkanoyloxymethyl, 1-((C₁-C₆)alkanoyloxy) ethyl, 1-methyl-1-((C₁-C₆)alkanoyloxy)ethyl (C₁-C₆)alkoxycarbonyloxymethyl, -N-(C₁-C₆)alkoxycarbonylaminomethyl, succinoyl, (C₁-C₆)alkanoyl, alpha-amino(C₁-C₄)alkanoyl, arylacyl and alpha-aminoacyl, or alpha-aminoacyl-alpha-aminoacyl, where each alpha-aminoacyl group is independently selected from naturally occurring L-amino acids P(O)(OH)₂, -P(O)(O(C₁-C₆)alkyl)₂, and glycosyl (the radical resulting from the removal of a hydroxyl group of the hemiacetal form of a carbohydrate).

If a disclosed compound or a pharmaceutically acceptable form of the compound incorporates an amine functional group, a prodrug can be formed by the replacement of a hydrogen atom in the amine group with a group such as R-carbonyl, RO-carbonyl, NRR'-carbonyl where R and R' are each independently (C₁-C₁₀)alkyl, (C₃-C₇)cycloalkyl, benzyl, a natural alpha-aminoacyl, -C(OH)C(O)OY¹ wherein Y¹ is H, (C₁-C₆)alkyl or benzyl, -C(OY²)Y³ wherein Y² is (C₁-C₄) alkyl and Y³ is (C₁-C₆)alkyl, carboxy(C₁-C₆)alkyl, amino(C₁-C₄)alkyl or mono-N or di-N,N-(C₁-C₆)alkylaminoalkyl, -C(Y⁴)Y⁵ wherein Y⁴ is H or methyl and Y⁵ is mono-N- or di-N,N-(C₁-C₆)alkylamino, morpholino, piperidin-1-yl or pyrrolidin-1-yl.

As used herein, "pharmaceutically acceptable esters" include, but are not limited to, alkyl, alkenyl, alkynyl, aryl, arylalkyl, and cycloalkyl esters of acidic groups, including, but not limited to, carboxylic acids, phosphoric acids, phosphinic acids, sulfonic acids, sulfinic acids, and boronic acids.

As used herein, "pharmaceutically acceptable enol ethers" include, but are not limited to, derivatives of formula -C=C(OR) where R can be selected from alkyl, alkenyl, alkynyl, aryl, aralkyl, and cycloalkyl. Pharmaceutically acceptable enol esters include, but are not limited to,

derivatives of formula $-C=C(OC(O)R)$ where R can be selected from hydrogen, alkyl, alkenyl, alkynyl, aryl, aralkyl, and cycloalkyl.

The term “patient” and “subject” are interchangeable and may be taken to mean any living organism which may be treated with compounds of the present disclosure. As such, the terms “patient” and “subject” may include, but is not limited to, any non-human mammal, primate or human. In some embodiments, the “patient” or “subject” is a mammal, such as mice, rats, other rodents, rabbits, dogs, cats, swine, cattle, sheep, horses, primates, or humans. In some embodiments, the patient or subject is an adult, child or infant. In some embodiments, the patient or subject is a human.

As used herein, the terms "treat" and "treating" are not limited to the case where the subject (e.g. patient) is cured and the disease is eradicated. Rather, embodiments of the present disclosure also contemplate treatment that merely reduces symptoms, and/or delays disease progression.

As used herein, “pharmaceutical formulation” and “pharmaceutical composition” can be used interchangeably. The composition may be administered to patients in an amount effective, especially to enhance pharmacological response in an animal or human organism. As used herein, the term “effective amount” refers to an amount sufficient to realize a desired biological effect. The appropriate dosage may vary depending upon known factors such as the pharmacodynamic characteristics of the particular active agent, age, health, and weight of the host organism; the condition(s) to be treated, nature and extent of symptoms, kind of concurrent treatment, frequency of treatment, the need for prevention or therapy and/or the effect desired. The dosage will also be calculated dependent upon the particular route of administration selected. Further refinement of the calculations necessary to determine the appropriate dosage for treatment is routinely made by a practitioner, in the light of the relevant circumstances. The titer may be determined by conventional techniques.

Methods of Use

The issue of how and when cardiomyocytes lose their ability to divide has captured the attention of specialists interested in cardiovascular biology. These endeavors have impacted diverse fields such as regenerative repair of the heart, and congenital heart conditions in which the postnatal cardiomyocyte endowment is diminished by disease. There is considerable debate as to when postnatal cardiomyocytes lose proliferative capacity. The consensus view is that in mice it

occurs by the end of the neonatal period (by postnatal day 6), and involves all ventricular cardiomyocytes (Tzahor et al., Science 356, 1035, 2017).

Experimental evidence indicates that in cardiac muscle cells it occurs gradually, extending from the base of the left ventricle to its apex, over postnatal days-7–15, and loss of proliferative capacity is caused by the post-neonatal expression of DUSP5, a nuclear p-ERK1/2-specific phosphatase that prevents sustained ERK1/2 activation in the nucleus (this activation is a prerequisite for proliferative signaling by all growth factors). DUSP5 expression not only initiates loss of proliferative capacity but it also maintains it. This latter finding suggests a strategy to reversibly activate proliferative competence in adult cardiac muscle cells.

Experimental evidence also indicates that cardiomyocyte DUSP5 expression is caused by sympathetic β_1 -adrenergic receptor activation. This discovery offers immediate solutions for regenerative therapies. Because many β_1 -selective antagonists are used clinically, it may be possible to engage in cardiac regenerative therapies.

In certain embodiments, this disclosure relates to in vivo and in vitro methods of inducing proliferation of cardiomyocytes by administering or mixing an agent or combination of agents disclosed herein and uses in managing diseases and conditions relates thereto.

In certain embodiments, this disclosure relates to methods of treating or preventing heart malformation or cardiovascular disease comprising administering an effective amount of an individual agent or combination of agents such as thyroid hormone, a beta-adrenergic blocking agent, and/or DUSP5 or DUSP6 inhibitor.

In certain embodiments, this disclosure relates to methods of treating or preventing heart malformation or cardiovascular disease comprising administering an effective amount of thyroid hormone in combination with i) an agent that decreases DUSP5 and/or DUSP6 expression and/or ii) a beta-adrenergic blocking agent to a subject in need thereof.

In certain embodiments, this disclosure relates to methods disclosed herein which are a combination of beta-blocker and/or siRNA (to suppress DUSP5) and mitogens.

In certain embodiments, this disclosure relates to methods disclosed herein which are an agent for beta-adrenergic blockage in combination with thyroid hormone or mitogens for cardiac regeneration.

In certain embodiments, this disclosure relates to methods of treating or preventing heart malformation or cardiovascular disease comprising administering an effective amount a beta-adrenergic blocking agent to a subject in need thereof.

In certain embodiments, the method comprises administering an effective amount of thyroid hormone in combination with a beta-adrenergic blocking agent.

In certain embodiments, the beta-adrenergic blocking agent is selected from acebutolol, atenolol, betaxolol, bisoprolol, carteolol, carvedilol, esmolol, labetalol, metoprolol, nadolol, nebivolol, penbutolol, pindolol, propranolol, sotalol, timolol, or salts thereof.

In certain embodiments, the thyroid hormone and an agent that decrease DUSP5 expression and/or a beta-adrenergic blocking agent are administered in combination with a mitogen. In certain embodiments, the mitogen is neuregulin, IGF-1, YAP1, ERBB2.

In certain embodiments, the individual agent or combination of agents are administered in combination with a mitogen. In certain embodiments, the mitogen is neuregulin, IGF-1, YAP1, ERBB2.

In certain embodiments, the cardiovascular disease is selected from coronary artery disease, heart failure, cardiomyopathy, heart valve disease, and cardiac arrhythmias.

In certain embodiments, the subject is diagnosed with a heart attack, angina, or stroke, diabetes, angina pectoris due to coronary atherosclerosis, hypertension, migraine headaches, glaucoma, hyperthyroidism, fibromyalgia, generalized anxiety disorder, parkinsonian tremor, and atrial fibrillation.

In certain embodiments, this disclosure relates to methods of inducing proliferation of cardiomyocytes comprising administering an effective amount of an individual agent or combination of agents such as thyroid hormone, beta-adrenergic blocking agent, and/or DUSP5 or DUSP6 inhibitor.

In certain embodiments, this disclosure relates to methods of inducing proliferation of cardiomyocytes comprising administering an effective amount of thyroid hormone in combination with i) an agent that decreases DUSP5 and/or DUSP6 expression and/or ii) a beta-adrenergic blocking agent to a subject in need thereof.

In certain embodiments, the individual agent or combination of agents are administered in combination with a mitogen. In certain embodiments, the mitogen is neuregulin, IGF-1, YAP1, ERBB2.

In certain embodiments, this disclosure relates to methods of inducing proliferation of cardiomyocytes comprising administering an effective amount of thyroid hormone in combination with a beta-adrenergic blocking agent to a subject in need thereof.

In certain embodiments, this disclosure relates to methods of inducing proliferation of cardiomyocytes comprising administering an effective amount of a beta-adrenergic blocking agent to a subject in need thereof.

In certain embodiments, the beta-adrenergic blocking agent, DUSP5, or DUSP6 inhibitor is a small molecule.

In certain embodiments, the DUSP5 or DUSP6 inhibitor is a polysulfonated aromatic compound with a carbazole or naphthalene scaffold.

In certain embodiments, the DUSP5 or DUSP6 inhibitor is a compound selected from 2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one (BCI), carbazole-1,3,6-trisulfonic acid, carbazole-1,3,6,8-tetrasulfonic acid, 8-hydroxynaphthalene-1,6-disulfonic acid (RR527), 8-amino-4-hydroxynaphthalene-2-sulfonic acid (RR535), 8,8'-(diazene-1,2-diyl)bis(4-hydroxynaphthalene-2-sulfonic acid)(RR601), 1²,3²,5²,7²-tetrahydroxy-1,3,5,7(1,3)-tetrabenzenacyclooctaphane-1⁵,3⁵,5⁵,7⁵-tetrasulfonic acid (RR701), derivatives, prodrugs, alkylesters, or alternative salts thereof.

In certain embodiments, the beta-adrenergic blocking agent, DUSP5 or DUSP6 inhibitor is siRNA or an antisense therapy.

In certain embodiments, the DUSP5 or DUSP6 inhibitor is siRNA comprising a sense region and an antisense region, wherein said sense region and said antisense region together form a duplex region, said antisense region and said sense region are each 18-30 nucleotides in length and said antisense region comprises a sequence a sequence selected from the group consisting of SEQ ID Nos: 1-120 or variant thereof.

In certain embodiments, the individual agent or combination of agents such as thyroid hormone, beta-adrenergic blocking agent, and/or DUSP5 or DUSP6 inhibitor is administered to a newborn within 1 to 7 days of birth.

In certain embodiments, the individual agent or combination of agents such as thyroid hormone, beta-adrenergic blocking agent, and/or DUSP5 or DUSP6 inhibitor is administered to a newborn within 8 to 14 days of birth.

In certain embodiments, the individual agent or combination of agents such as thyroid hormone, beta-adrenergic blocking agent, and/or DUSP5 or DUSP6 inhibitor is administered to a newborn within 15 to 21 days of birth.

In certain embodiments, the individual agent or combination of agents such as thyroid hormone, beta-adrenergic blocking agent, and/or DUSP5 or DUSP6 inhibitor is administered to a subject more than 21 days after birth.

In certain embodiments, the individual agent or combination of agents such as thyroid hormone, beta-adrenergic blocking agent, and/or DUSP5 or DUSP6 inhibitor is administered by intravenous (IV) injection, direct injection into the cardiac tissue, direct injection into apical cardiac tissue, or pericardial cavity.

In certain embodiments, the individual agent or combination of agents such as thyroid hormone, beta-adrenergic blocking agent, and/or DUSP5 or DUSP6 inhibitor is administered to a subject at risk of, exhibiting symptoms of, or diagnosed with a cardiovascular disease, condition, or injury.

In certain embodiments, the individual agent or combination of agents such as thyroid hormone, beta-adrenergic blocking agent, and/or DUSP5 or DUSP6 inhibitor is administered to a subject at risk of, exhibiting symptoms of, or diagnosed with heart disease, heart attack, stroke, heart failure, arrhythmia, heart valve disease, congenital heart defect, patent ductus arteriosus, ventricular septal defect, truncus arteriosus, atrioventricular septal defect, tetralogy of Fallot, transposition of the great arteries, hypoplastic left heart syndrome, tricuspid atresia, or heart murmur, for use in the treatment or prevention thereof.

In certain embodiments, this disclosure relates to in vitro methods of inducing proliferation of cardiomyocytes comprising administering an effective amount of an individual agent or combination of agents such as thyroid hormone, beta-adrenergic blocking agent, and/or DUSP5 or DUSP6 inhibitor to a cardiac cell. In certain embodiments, this disclosure relates to in vitro methods of inducing proliferation of cardiomyocytes comprising administering to a cardiac cell an effective amount of a thyroid hormone in combination with i) an agent that decreases DUSP5 and/or DUSP6 expression and/or ii) a beta-adrenergic blocking agent.

In certain embodiments, this disclosure relates to pharmaceutical composition comprising combinations of agents disclosed herein and a pharmaceutically acceptable excipient. In certain

embodiments, the composition is in the form of a tablet, pill, capsule, granule or gel. In certain embodiments the pharmaceutical composition is in the form of a pH buffered saline solution.

Antisense and RNA induced silencing (RNAi) therapies

Antisense therapy is a form of treatment for genetic disorders or infections. When the genetic sequence of a particular gene is known to be causative of a particular disease, it is possible to synthesize a strand of nucleic acid (DNA, RNA or a chemical analogue) that will bind to the messenger RNA (mRNA) produced by that gene and inactivate it, effectively turning that gene "off". This is because mRNA has to be single stranded for it to be translated. Alternatively, the strand might be targeted to bind a splicing site on pre-mRNA and modify the exon content of an mRNA.

This synthesized nucleic acid is termed an "anti-sense" oligonucleotide (AON) because its base sequence is complementary to the messenger RNA (mRNA), which is called the "sense" sequence. Because nucleases that cleave the phosphodiester linkage in DNA are expressed in almost every cell, unmodified DNA molecules are generally degraded before they reach their targets. Therefore, antisense therapeutics are generally modified. Modifications include morpholino oligomers, peptide nucleic acids, and locked nucleic acids. A morpholino oligomer or a phosphorodiamidate morpholino oligomer (PMO) has DNA bases attached to a backbone of methylenemorpholine rings linked through phosphorodiamidate groups. Peptide nucleic acids (PNAs) are composed of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds. The various purine and pyrimidine bases are linked to the backbone by a methylene bridge (-CH₂-) and a carbonyl group (-C=O-). Locked nucleic acids (LNA) have a ribose moiety modified with an extra bridge connecting the 2' oxygen and 4' carbon. The bridge "locks" the ribose in the 3'-endo conformation,

Double stranded RNA ("dsRNA") can be used to inhibit protein expression. Double stranded RNA induced gene silencing can occur on at least three different levels: (i) transcription inactivation, which refers to RNA guided DNA or histone methylation; (ii) siRNA induced mRNA degradation; and (iii) mRNA induced transcriptional attenuation. mRNA degradation is generally considered to be the major mechanism of RNA induced silencing (RNA interference, or RNAi) in mammalian cells. Initial attempts to use RNAi in mammalian cells focused on the use of long strands of dsRNA. However, these attempts to induce RNAi met with limited success, due in part

to the induction of the interferon response, which results in a general, as opposed to a target-specific, inhibition of protein synthesis. Thus, long dsRNA is not a viable option for RNAi in mammalian systems.

When short (18-30 bp) RNA duplexes are introduced into mammalian cells in culture, sequence-specific inhibition of target mRNA can be realized without inducing an interferon response. Certain of these short dsRNAs, referred to as small inhibitory RNAs (“siRNAs”), can act catalytically at sub-molar concentrations to cleave greater than 95% of the target mRNA in the cell. A description of the mechanisms for siRNA activity, as well as some of its applications are described in Provost et al. (2002) Ribonuclease Activity and RNA Binding of Recombinant Human Dicer, *EMBO J* 21(21): 5864 -5874; Tabara et al. (2002) The dsRNA Binding Protein RDE-4 Interacts with RDE-1, DCR-1 and a DexH-box Helicase to Direct RNAi in *C. elegans*, *Cell* 109(7):861 -71; Ketting et al. (2002) Dicer Functions in RNA Interference and in Synthesis of Small RNA Involved in Developmental Timing in *C. elegans*; Martinez et al., Single-Stranded Antisense siRNAs Guide Target RNA Cleavage in RNAi, *Cell* 110(5):563; Hutvagner & Zamore (2002) A microRNA in a multiple-turnover RNAi enzyme complex, *Science* 297:2056.

From a mechanistic perspective, introduction of long double stranded RNA into plants and invertebrate cells is broken down into siRNA by a Type II endonuclease known as Dicer. Sharp, RNA interference—2001, *Genes Dev.* 2001, 15:485. Dicer, a ribonuclease-III-like enzyme, processes the dsRNA into 19-23 base pair short interfering RNAs with characteristic two base 3' overhangs. Bernstein, Caudy, Hammond, & Hannon (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference, *Nature* 409:363. The siRNAs are then incorporated into an RNA-induced silencing complex (RISC) where one or more helicases unwind the siRNA duplex, enabling the complementary antisense strand to guide target recognition. Nykanen, Haley, & Zamore (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway, *Cell* 107:309. Upon binding to the appropriate target mRNA, one or more endonucleases within the RISC cleaves the target to induce silencing. Elbashir, Lendeckel, & Tuschl (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs, *Genes Dev.* 15:188.

The interference effect can be long lasting and may be detectable after many cell divisions. Moreover, RNAi exhibits sequence specificity. Kieselow, M. et al. (2002) Isoform-specific knockdown and expression of adaptor protein ShcA using small interfering RNA, *J. Biochem.* 363:1-5. Thus, the RNAi machinery can specifically knock down one type of transcript, while not

affecting closely related mRNA. These properties make siRNA a potentially valuable tool for inhibiting gene expression and studying gene function and drug target validation. Moreover, siRNAs are potentially useful as therapeutic agents against: (1) diseases that are caused by over-expression or misexpression of genes; and (2) diseases brought about by expression of genes that contain mutations.

Successful siRNA-dependent gene silencing depends on a number of factors. One of the most contentious issues in RNAi is the question of the necessity of siRNA design, i.e., considering the sequence of the siRNA used. Early work in *C. elegans* and plants circumvented the issue of design by introducing long dsRNA (see, for instance, Fire, A. et al. (1998) *Nature* 391:806-811). In this primitive organism, long dsRNA molecules are cleaved into siRNA by Dicer, thus generating a diverse population of duplexes that can potentially cover the entire transcript. One or more have the potential to be highly functional, thereby silencing the gene of interest and alleviating the need for siRNA design.

The term “siRNA” refers to small inhibitory RNA duplexes that induce the RNA interference (RNAi) pathway. These molecules can vary in length (generally 18-30 base pairs) and contain varying degrees of complementarity to their target mRNA in the antisense strand. Some, but not all, siRNA have unpaired overhanging bases on the 5' or 3' end of the sense strand and/or the antisense strand. The term “siRNA” includes duplexes of two separate strands, as well as single strands that can form hairpin structures comprising a duplex region.

As used herein, unless otherwise specified, “non-functional siRNA” are defined as those siRNA that induce less than 50% (<50%) target silencing, “semi-functional siRNA” induce 50-79% target silencing, “functional siRNA” are molecules that induce 80-95% gene silencing, and “highly-functional siRNA” are molecules that induce greater than 95% gene silencing. These definitions are not intended to be rigid and can vary depending upon the design and needs of the application. For instance, it is possible that a researcher attempting to map a gene to a chromosome using a functional assay, may identify an siRNA that reduces gene activity by only 30%. While this level of gene silencing may be “non-functional” for, e.g., therapeutic needs, it is sufficient for gene mapping purposes and is, under these uses and conditions, “functional.” For these reasons, functional siRNA can be defined as those molecules having greater than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% silencing capabilities at 100 nM transfection conditions. Similarly, depending upon the needs of the study and/or application, non-functional and semi-functional

siRNA can be defined as having different parameters. For instance, semi-functional siRNA can be defined as being those molecules that induce 20%, 30%, 40%, 50%, 60%, or 70% silencing at 100 nM transfection conditions. Similarly, non-functional siRNA can be defined as being those molecules that silence gene expression by less than 70%, 60%, 50%, 40%, 30%, or less.

siRNAs that Target Phosphatase 5 (DUSP5)

AAACCCAUUUCACAAGAGA (SEQ ID NO: 1);
AAACUGGGAUGGAGGAAUC (SEQ ID NO: 2);
AAGAGAAGAUUGAGAGUGA (SEQ ID NO: 3);
AAGCACAAUUUCCACCUUA (SEQ ID NO: 4);
AAGGAAGGCCAAGCCAUUA (SEQ ID NO: 5);
AAGGGUACUUGCUAGGUUAU (SEQ ID NO: 6);
ACCCUGAAAUGUUGUGUAG (SEQ ID NO: 7);
ACUGUGGACUUCUGGGAUU (SEQ ID NO: 8);
AGACUUUCUACUCGGAAUA (SEQ ID NO: 9);
AGAGUUCGCCUUUUCAUUU (SEQ ID NO: 10);
AGCAGGCUCUUCACUGAUA (SEQ ID NO: 11);
AGUCAUACUUGAACUUGUC (SEQ ID NO: 12);
AGUGUUGCGUGGAUGUAAA (SEQ ID NO: 13);
CAAAUGGAUCCCUGUGGAA (SEQ ID NO: 14);
CAACGGCCACAUCCUGCUA (SEQ ID NO: 15);
CAACGUGGGAGAAAGAAGU (SEQ ID NO: 16);
CAAGAGCAACUGUGAUUUU (SEQ ID NO: 17);
CAAUAAAUACCUGCAGCAA (SEQ ID NO: 18);
CAAUACUGAAGACCUCAUU (SEQ ID NO: 19);
CACAAUUUCCACCUUAUUU (SEQ ID NO: 20);
CACCAGGCUUGCAAAUGAA (SEQ ID NO: 21);
CACCUACACUACAAAUGGA (SEQ ID NO: 22);
CAGCAACGUGGGAGAAAGA (SEQ ID NO: 23);
CAGCAGAAGCCCUGUGGCA (SEQ ID NO: 24);
CAGGGUGGCCCAGUUGAAA (SEQ ID NO: 25);

CAUGGUCUCGCCCCAACUUU (SEQ ID NO: 26);
CAUUAGCUCCACUUUCA (SEQ ID NO: 27);
CAUCCACCUCUUCUCAGA (SEQ ID NO: 28);
CCAAGAGCAACUGUGAUUU (SEQ ID NO: 29);
CCAAGCAGUCCGCCUGAA (SEQ ID NO: 30);
CCACACGGCUGACAUUAGC (SEQ ID NO: 31);
CCACCUACACUACAAAUGG (SEQ ID NO: 32);
CCACUUUCAAGAAGCAAUA (SEQ ID NO: 33);
CCAUUUCACAAGAGAAGAU (SEQ ID NO: 34);
CCCAAGAGCAACUGUGAUU (SEQ ID NO: 35);
CCUGAAAUGUUGUGUAGAC (SEQ ID NO: 36);
CGGAAUAUCCUGAGUGUUG (SEQ ID NO: 37);
CUAAGACCCGUGUGAAUGU (SEQ ID NO: 38);
CUGCAUGGCUUACCUUAUG (SEQ ID NO: 39);
GAAGAAAAGCAGUAUGUUA (SEQ ID NO: 40);
GAAGACCUCAUUCUGUCAU (SEQ ID NO: 41);
GAAGAUUGAGAGUGAGAGA (SEQ ID NO: 42);
GAAGCACAAUUUCCACCUU (SEQ ID NO: 43);
GAAGGAAGGCCAAGCCAUU (SEQ ID NO: 44);
GAAGGGUACUUGCUAGGUA (SEQ ID NO: 45);
GACAUUAGCUCCACUUUC (SEQ ID NO: 46);
GACUUUGGCAUGAUUCUUA (SEQ ID NO: 47);
GAGAAAAGGCAGUUAUGAA (SEQ ID NO: 48);
GAGAAGAUUGAGAGUGAGA (SEQ ID NO: 49);
GAGACUUUCUACUCGGAAU (SEQ ID NO: 50);
GAGGCAAGGUCCUGGUCCA (SEQ ID NO: 51);
GAGGUAGUUGGUUGAAGUA (SEQ ID NO: 52);
GAGUGUUGCGUGGAUGUAA (SEQ ID NO: 53);
GAUAGGCCAUUUGCAGACA (SEQ ID NO: 54);
GAUAGAGACUUUCUACUC (SEQ ID NO: 55);
GAUGUUGGCUUUUCUGGAU (SEQ ID NO: 56);

GAUUCUUAGUCAUACUUGA (SEQ ID NO: 57);
GCAACGUGGUACUACUUUU (SEQ ID NO: 58);
GCAAGAUGCUCGCAAGGA (SEQ ID NO: 59);
GCACAAUUUCCACCUUAUU (SEQ ID NO: 60);
GCAGAAGCCCUGUGGCAAC (SEQ ID NO: 61);
GCAGCAACGUGGGAGAAAG (SEQ ID NO: 62);
GCAGCAGGCUCUUCACUGA (SEQ ID NO: 63);
GCAGGCUCUUCACUGAUAG (SEQ ID NO: 64);
GCAGUUAUGAAGCCAAUUC (SEQ ID NO: 65);
GCAUGACCCACCUACACUA (SEQ ID NO: 66);
GCCAUGGGUUCUUCACUGA (SEQ ID NO: 67);
GCGGCUCGCUAACGUCAA (SEQ ID NO: 68);
GCGGGUCUACUCCUCAA (SEQ ID NO: 69);
GCUACAGGCCAGCUUAUGA (SEQ ID NO: 70);
GCUGAUCACCGUCUAGUUG (SEQ ID NO: 71);
GGAAGUGCCUACCAUGCAU (SEQ ID NO: 72);
GGAGAAAAGGCAGUUAUGA (SEQ ID NO: 73);
GGAGCAUGGUCUCGCCAA (SEQ ID NO: 74);
GGAGGCAGCAGGCUCUUA (SEQ ID NO: 75);
GGCCUUCGAUUACAUAAG (SEQ ID NO: 76);
GGGGAAAAGGCAAUAAUUU (SEQ ID NO: 77);
GGUAGGUUCUCGGGACUGA (SEQ ID NO: 78);
GUAGAUUCCAGGAGGAGAA (SEQ ID NO: 79);
GUAGCAAGAUGUUGGCUUU (SEQ ID NO: 80);
GUAGGGACAUGAUCAGCAU (SEQ ID NO: 81);
GUAGUUGGUUGAAGUAGCA (SEQ ID NO: 82);
GUGCUUAUGUCUCUUGUGA (SEQ ID NO: 83);
GUUCUUCACUGACCUUGGA (SEQ ID NO: 84);
UAAAACCCAUUUCACAAGA (SEQ ID NO: 85);
UAAGACCCGUGUGAAUGUG (SEQ ID NO: 86);
UAAGACUCAUGGACAUUUC (SEQ ID NO: 87);

UACUUGAACUUGUCUCAUU (SEQ ID NO: 88);
UAGACUUCAUUGACUGUGU (SEQ ID NO: 89);
UAGCUCCCACUUUCAAGAA (SEQ ID NO: 90);
UCAAGCAUAAGCCAAUAAA (SEQ ID NO: 91);
UCACAAGAGAAGAUUGAGA (SEQ ID NO: 92);
UCACCAGGCUUGCAAUAUGA (SEQ ID NO: 93);
UCACUGACCUUGGACUUUG (SEQ ID NO: 94);
UCAUACCUGUGCAAUACUG (SEQ ID NO: 95);
UCUAAGACCCGUGUGAAUG (SEQ ID NO: 96);
UCUCAGAGCUCAGCAGAAG (SEQ ID NO: 97);
UGACAUUAGCUCCCACUUU (SEQ ID NO: 98);
UGACCAGGGUGGCCAGUU (SEQ ID NO: 99);
UGACCCACCUACACUACAA (SEQ ID NO: 100);
UGAGGUAGUUGGUUGAAGU (SEQ ID NO: 101);
UGGCUUACCUUAUGAAGAC (SEQ ID NO: 102);
UGGGCCAGCUCCUGCAGUA (SEQ ID NO: 103);
UUAAGACUCAUGGACAUUU (SEQ ID NO: 104);
UUAUGAAGACCAAGCAGUU (SEQ ID NO: 105);
UUCAUUGUCUGUCUCUGUU (SEQ ID NO: 106);
UUUCAAGCAUAAGCCAAUA (SEQ ID NO: 107);
UUUCAUACCUGUGCAAUAC (SEQ ID NO: 108);
UUUGAACCCUGAAAUGUUG (SEQ ID NO: 109); and,
UUUGAAGGAAGCACAAUUU (SEQ ID NO: 110).

SC-60554 : DUSP5 siRNA (human) is a pool of 3 different siRNA duplexes

A:

Sense: GCAUGGCUUACCUUAUGAATT (SEQ ID NO: 111)

Antisense: UUCAUAAGGUAAGCCAUGCTT (SEQ ID NO: 112)

B:

Sense: CGUCUAGUUGGGAAAGUAATT (SEQ ID NO: 113)

Antisense: UUACUUUCCCAACUAGACGTT (SEQ ID NO: 114)

C:

Sense: GUAGCAAGAUGUUGGCUUUTT (SEQ ID NO: 115)

Antisense: AAAGCCAACAUCUUGCUACTT (SEQ ID NO: 116)

sc-60555 : DUSP5 siRNA (mouse) is a pool of 2 different siRNA duplexes:

A:

Sense: GCAUGGCUUACCUCAUGAATT (SEQ ID NO: 117)

Antisense: UUCAUGAGGUAAGCCAUGCTT (SEQ ID NO: 118)

B:

Sense: GACAGCUCCUUCAGUAUGATT (SEQ ID NO: 119)

Antisense: UCAUACUGAAGGAGCUGUCTT (SEQ ID NO: 120)

Any of the methods disclosed herein can be used to silence one or more genes by introducing an siRNA selected, or designed, in accordance with any of the methods disclosed herein. The siRNA(s) can be introduced into the cell by any method known in the art, including passive uptake or through the use of one or more vectors.

Preferably, the siRNA is applied to a cell through transfection, employing standard transfection protocols. These methods are well known to persons skilled in the art and include the use of lipid-based carriers, electroporation, cationic carriers, and microinjection. Further, one could synthesize equivalent DNA sequences (either as two separate, complementary strands, or as hairpin molecules) instead of siRNA sequences and introducing them into cells through vectors. Once in the cells, the cloned DNA could be transcribed, thereby forcing the cells to generate the siRNA. Examples of vectors suitable for use with the present application include but are not limited to the standard transient expression vectors, adenoviruses, retroviruses, lentivirus-based vectors, as well as other traditional expression vectors. Any vector that has an adequate siRNA expression and procession module may be used. Furthermore, certain chemical modifications to siRNAs, including but not limited to conjugations to other molecules, may be used to facilitate delivery. For certain applications, it may be preferable to deliver molecules without transfection by simply formulating in a physiological acceptable solution.

Formulations

Pharmaceutical compositions disclosed herein may be combinations of agents for uses disclosed herein in the form of a single pharmaceutically acceptable composition. In certain embodiments, the pharmaceutical composition is in the form of a tablet, capsule, pill, or solution for injection. In certain embodiments, the pharmaceutical composition is in sterilized and pH buffered aqueous solution, e.g., phosphate buffered solution, optionally comprising a saccharide or polysaccharide.

When the compounds of the disclosure contain an acidic group as well as a basic group, the compounds of the disclosure may also form internal salts, and such compounds are within the scope of the disclosure. When a compound contains a hydrogen-donating heteroatom (e.g. NH), salts are contemplated to covers isomers formed by transfer of said hydrogen atom to a basic group or atom within the molecule.

Pharmaceutically acceptable salts of the compounds include the acid addition and base salts thereof. Suitable acid addition salts are formed from acids that form non-toxic salts. Examples include the acetate, adipate, aspartate, benzoate, besylate, bicarbonate/carbonate, bisulphate/sulphate, borate, camsylate, citrate, cyclamate, edisylate, esylate, formate, fumarate, gluceptate, gluconate, glucuronate, hexafluorophosphate, hibenzate, hydrochloride/chloride, hydrobromide/bromide, hydroiodide/iodide, isethionate, lactate, malate, maleate, malonate, mesylate, methylsulphate, naphthylate, 2-napsylate, nicotinate, nitrate, orotate, oxalate, palmitate, pamoate, phosphate/hydrogen phosphate/dihydrogen phosphate, pyroglutamate, saccharate, stearate, succinate, tannate, tartrate, tosylate, trifluoroacetate and xinofoate salts. Suitable base salts are formed from bases which form non-toxic salts. Examples include the aluminum, arginine, benzathine, calcium, choline, diethylamine, diolamine, glycine, lysine, magnesium, meglumine, olamine, potassium, sodium, tromethamine and zinc salts. Hemisalts of acids and bases may also be formed, for example, hemisulphate and hemicalcium salts. For a review on suitable salts, see Handbook of Pharmaceutical Salts: Properties, Selection, and Use by Stahl and Wermuth (Wiley-VCH, 2002), incorporated herein by reference.

The compounds described herein may be administered in the form of prodrugs. A prodrug can include a covalently bonded carrier that releases the active parent drug when administered to a mammalian subject. Prodrugs can be prepared by modifying functional groups present in the

compounds in such a way that the modifications are cleaved, either in routine manipulation or in vivo, to the parent compounds. Prodrugs include, for example, compounds wherein a hydroxyl group is bonded to any group that, when administered to a mammalian subject, cleaves to form a free hydroxyl group. Examples of prodrugs include, but are not limited to, acetate, formate and benzoate derivatives of alcohol functional groups in the compounds. Methods of structuring a compound as prodrugs can be found in the book of Testa and Mayer, *Hydrolysis in Drug and Prodrug Metabolism*, Wiley (2006). Typical prodrugs form the active metabolite by transformation of the prodrug by hydrolytic enzymes, the hydrolysis of amide, lactams, peptides, carboxylic acid esters, epoxides or the cleavage of esters of inorganic acids.

Pharmaceutical compositions for use in the present disclosure typically comprise an effective amount of a compound and a suitable pharmaceutical acceptable carrier. The preparations may be prepared in a manner known per se, which usually involves mixing the at least one compound according to the disclosure with the one or more pharmaceutically acceptable carriers, and, if desired, in combination with other pharmaceutical active compounds, when necessary under aseptic conditions. Reference is again made to U.S. Pat. No. 6,372,778, U.S. Pat. No. 6,369,086, U.S. Pat. No. 6,369,087 and U.S. Pat. No. 6,372,733 and the further references mentioned above, as well as to the standard handbooks, such as the latest edition of Remington's *Pharmaceutical Sciences*.

Generally, for pharmaceutical use, the compounds may be formulated as a pharmaceutical preparation comprising at least one compound and at least one pharmaceutically acceptable carrier, diluent or excipient and/or adjuvant, and optionally one or more further pharmaceutically active compounds.

The pharmaceutical preparations of the disclosure are preferably in a unit dosage form, and may be suitably packaged, for example in a box, blister, vial, bottle, sachet, ampoule or in any other suitable single-dose or multi-dose holder or container (which may be properly labeled); optionally with one or more leaflets containing product information and/or instructions for use. Generally, such unit dosages will contain between 1 and 1000 mg, and usually between 5 and 500 mg, of the at least one compound of the disclosure, e.g. about 10, 25, 50, 100, 200, 300 or 400 mg per unit dosage.

The compounds can be administered by a variety of routes including the oral, ocular, rectal, transdermal, subcutaneous, intravenous, intramuscular or intranasal routes, depending mainly on

the specific preparation used. The compound will generally be administered in an “effective amount”, by which is meant any amount of a compound that, upon suitable administration, is sufficient to achieve the desired therapeutic or prophylactic effect in the subject to which it is administered. Usually, depending on the condition to be prevented or treated and the route of administration, such an effective amount will usually be between 0.01 to 1000 mg per kilogram body weight of the patient per day, more often between 0.1 and 500 mg, such as between 1 and 250 mg, for example about 5, 10, 20, 50, 100, 150, 200 or 250 mg, per kilogram body weight of the patient per day, which may be administered as a single daily dose, divided over one or more daily doses. The amount(s) to be administered, the route of administration and the further treatment regimen may be determined by the treating clinician, depending on factors such as the age, gender and general condition of the patient and the nature and severity of the disease/symptoms to be treated. Reference is again made to U.S. Pat. No. 6,372,778, U.S. Pat. No. 6,369,086, U.S. Pat. No. 6,369,087 and U.S. Pat. No. 6,372,733 and the further references mentioned above, as well as to the standard handbooks, such as the latest edition of Remington's Pharmaceutical Sciences.

Depending upon the manner of introduction, the compounds described herein may be formulated in a variety of ways. Formulations containing one or more inhibitors can be prepared in various pharmaceutical forms, such as granules, tablets, capsules, suppositories, powders, controlled release formulations, suspensions, emulsions, creams, gels, ointments, salves, lotions, or aerosols and the like. Preferably, these formulations are employed in solid dosage forms suitable for simple, and preferably oral, administration of precise dosages. Solid dosage forms for oral administration include, but are not limited to, tablets, soft or hard gelatin or non-gelatin capsules, and caplets. However, liquid dosage forms, such as solutions, syrups, suspension, shakes, etc. can also be utilized. In another embodiment, the formulation is administered topically. Suitable topical formulations include, but are not limited to, lotions, ointments, creams, and gels. In a preferred embodiment, the topical formulation is a gel. In another embodiment, the formulation is administered intranasally.

Formulations containing one or more of the compounds described herein may be prepared using a pharmaceutically acceptable carrier composed of materials that are considered safe and effective and may be administered to an individual without causing undesirable biological side effects or unwanted interactions. The carrier is all components present in the pharmaceutical formulation other than the active ingredient or ingredients. As generally used herein “carrier”

includes, but is not limited to, diluents, binders, lubricants, disintegrators, fillers, pH modifying agents, preservatives, antioxidants, solubility enhancers, and coating compositions.

Carrier also includes all components of the coating composition which may include plasticizers, pigments, colorants, stabilizing agents, surfactants, and glidants. Delayed release, extended release, and/or pulsatile release dosage formulations may be prepared as described in standard references such as “Pharmaceutical dosage form tablets”, eds. Liberman et. al. (New York, Marcel Dekker, Inc., 1989), “Remington – The science and practice of pharmacy”, 20th ed., Lippincott Williams & Wilkins, Baltimore, MD, 2000, and “Pharmaceutical dosage forms and drug delivery systems”, 6th Edition, Ansel et al., (Media, PA: Williams and Wilkins, 1995). These references provide information on carriers, materials, equipment and process for preparing tablets and capsules and delayed release dosage forms of tablets, capsules, and granules.

Examples of suitable coating materials include, but are not limited to, cellulose polymers such as cellulose acetate phthalate, hydroxypropyl cellulose, hydroxypropyl methylcellulose, hydroxypropyl methylcellulose phthalate and hydroxypropyl methylcellulose acetate succinate; polyvinyl acetate phthalate, acrylic acid polymers and copolymers, and methacrylic resins, zein, shellac, and polysaccharides.

Optional pharmaceutically acceptable excipients present in the drug-containing tablets, beads, granules or particles include, but are not limited to, diluents, binders, lubricants, disintegrants, colorants, stabilizers, and surfactants. Diluents, also referred to as “fillers,” are typically necessary to increase the bulk of a solid dosage form so that a practical size is provided for compression of tablets or formation of beads and granules. Suitable diluents include, but are not limited to, dicalcium phosphate dihydrate, calcium sulfate, lactose, sucrose, mannitol, sorbitol, cellulose, microcrystalline cellulose, kaolin, sodium chloride, dry starch, hydrolyzed starches, pregelatinized starch, silicone dioxide, titanium oxide, magnesium aluminum silicate and powdered sugar.

Binders are used to impart cohesive qualities to a solid dosage formulation, and thus ensure that a tablet or bead or granule remains intact after the formation of the dosage forms. Suitable binder materials include, but are not limited to, starch, pregelatinized starch, gelatin, sugars (including sucrose, glucose, dextrose, lactose and sorbitol), polyethylene glycol, waxes, natural and synthetic gums such as acacia, tragacanth, sodium alginate, cellulose, including hydroxypropyl methylcellulose, hydroxypropyl cellulose, ethylcellulose, and magnesium aluminum silicate, and

synthetic polymers such as acrylic acid and methacrylic acid copolymers, methacrylic acid copolymers, methyl methacrylate copolymers, aminoalkyl methacrylate copolymers, polyacrylic acid/polymethacrylic acid and polyvinylpyrrolidone.

Lubricants are used to facilitate tablet manufacture. Examples of suitable lubricants include, but are not limited to, magnesium stearate, calcium stearate, stearic acid, glycerol behenate, polyethylene glycol, talc, and mineral oil.

Disintegrants are used to facilitate dosage form disintegration or “breakup” after administration, and generally include, but are not limited to, starch, sodium starch glycolate, sodium carboxymethyl starch, sodium carboxymethylcellulose, hydroxypropyl cellulose, pregelatinized starch, clays, cellulose, arginine, gums or cross-linked polymers, such as cross-linked PVP.

Stabilizers are used to inhibit or retard drug decomposition reactions which include, by way of example, oxidative reactions.

Surfactants may be anionic, cationic, amphoteric or nonionic surface-active agents. Suitable anionic surfactants include, but are not limited to, those containing carboxylate, sulfonate and sulfate ions. Examples of anionic surfactants include sodium, potassium, ammonium of long chain alkyl sulfonates and alkyl aryl sulfonates such as sodium dodecylbenzene sulfonate; dialkyl sodium sulfosuccinates, such as sodium dodecylbenzene sulfonate; dialkyl sodium sulfosuccinates, such as sodium bis-(2-ethylthioxy)-sulfosuccinate; and alkyl sulfates such as sodium lauryl sulfate. Cationic surfactants include, but are not limited to, quaternary ammonium compounds such as benzalkonium chloride, benzethonium chloride, cetrimonium bromide, stearyl dimethylbenzyl ammonium chloride, polyoxyethylene and coconut amine. Examples of nonionic surfactants include ethylene glycol monostearate, propylene glycol myristate, glyceryl monostearate, glyceryl stearate, polyglyceryl-4-oleate, sorbitan acylate, sucrose acylate, PEG-150 laurate, PEG-400 monolaurate, polyoxyethylene monolaurate, polysorbates, polyoxyethylene octylphenylether, PEG-1000 cetyl ether, polyoxyethylene tridecyl ether, polypropylene glycol butyl ether, stearyl monoisopropanolamide, and polyoxyethylene hydrogenated tallow amide. Examples of amphoteric surfactants include sodium N-dodecyl-beta-alanine, sodium N-lauryl-beta-iminodipropionate, myristoamphoacetate, lauryl betaine, and lauryl sulfobetaine.

If desired, the tablets, beads, granules, or particles may also contain minor amount of nontoxic auxiliary substances such as wetting or emulsifying agents, dyes, pH buffering agents, or preservatives.

The concentration of the inhibitor(s) to carrier and/or other substances may vary from about 0.5 to about 100 wt % (weight percent). For oral use, the pharmaceutical formulation will generally contain from about 5 to about 100% by weight of the active material. For other uses, the pharmaceutical formulation will generally have from about 0.5 to about 50 wt % of the active material.

EXAMPLES

DUSP5 triggers and maintains cell cycle block in postnatal murine cardiomyocytes

Experimental evidence indicates cardiomyocyte endowment of the murine heart increases during preadolescence—that is, during the second week of life. This increase is triggered by a rise in circulating thyroid hormone (T_3) that starts at around postnatal day-11. These findings questioned long-held views about timing of cardiomyocyte cell cycle withdrawal in murine hearts. It is generally believed that murine cardiomyocytes withdraw from the cell cycle after postnatal day 6. Studies suggested that this developmental process does not occur until after the second week of life. T_3 has long been regarded as a cardiomyocyte maturation factor causing cardiomyocytes to hypertrophy. The fact that experimental evidence indicates developmental increases in T_3 cause cardiomyocytes to proliferate contradicts long-held views about the way by which this hormone regulates early postnatal heart growth: by cardiomyocyte hypertrophy, hyperplasia, or both.

The mechanism by which murine cardiomyocytes withdraw from the cell cycle and its timing were examined. Cell cycle block in post-neonatal cardiomyocytes occurs during the second week of life, progressively from left ventricular base to its apex, that is concordant with sympathetic innervation of the heart. This results in β_1 -adrenergic receptor-mediated expression of dual specificity phosphatase 5 (DUSP5). DUSP5 is a nuclear phosphatase that inactivates phosphorylated-ERK1/2; because nuclear ERK1/2 signaling is the common mediator of proliferative signaling by growth factors, integrins and a host of other mitogens, its expression in postnatal cardiomyocytes makes them resistant to proliferation. Due to the unique pattern of DUSP5 expression during early postnatal heart development, left ventricular cardiomyocytes at

the base of the heart withdraw from the cell cycle immediately after postnatal day-6, whereas those residing at the ventricular apex retain proliferative capacity until the end of the second week of life. Conclusions about the timing of cardiomyocyte cell cycle withdrawal must, therefore, take into consideration this critical region-specific difference in cardiomyocyte proliferative capacity if the timing of cardiomyocyte cell cycle withdrawal is to be fully understood—a factor not previously appreciated.

Importantly, experimental evidence indicates acute β 1-adrenergic receptor blockade suppresses DUSP5 expression in cardiomyocytes, which restores cardiomyocyte proliferative capacity even in adult 6-month-old hearts. This therapy, when combined with T₃ administration, causes hyperplastic heart growth in adult mice with important consequences for left ventricular function; a finding of potential major clinical significance for building muscle in failing hearts.

To determine when and how proliferative capacity is lost in murine cardiomyocytes. (1) A T₃ challenge coupled with genetic lineage tracing was used to assess in vivo cardiomyocyte proliferative capacity in post-neonatal hearts. Post-neonatal cardiomyocytes residing in the left ventricular (LV) apex, but not base, proliferated in response to T₃; this mitogenic effect was lost by P16. (2) The mechanism involved nuclear ERK1/2 signaling consequent on T₃-mediated enhancement of IGF-1 and IGF-1R expression. (3) The loss of T₃-stimulated proliferation in LV basal cardiomyocytes was caused by developmental expression of dual specificity phosphatase 5 (DUSP5), a nuclear phosphatase that inactivates phosphorylated-ERK1/2. (4) In early postnatal heart development, spatial and temporal restriction of DUSP5 expression is regulated by β 1-adrenergic receptor activation commensurate with progressive sympathetic innervation of the heart from base to apex. (6) Combination therapy with T₃ plus a β 1-adrenergic receptor blocker stimulated in vivo cardiomyogenesis and enhanced LV contractile function in adult hearts.

These findings indicate cell cycle block in post-neonatal cardiomyocytes occurs progressively from LV base to its apex during the second week of life and is caused by β 1-adrenergic receptor-mediated expression of DUSP5. A combination of β 1-adrenergic receptor blockade plus T₃ or derivatives can be useful for restoring cardiomyogenesis in diseased hearts.

Compound administration

T₃ was administered, intraperitoneally, to each mouse at the dose indicated. Vehicle was administered by the same route and animals thus treated served as controls. Phosphate buffered

saline (PBS) was the vehicle for T₃, and soybean oil (Sigma, S7381) was the vehicle for 4-hydroxytamoxifen. DUSP5 or β 1-adrenergic receptor-specific siRNA or scrambled siRNA (control) was administered using in vivo-jetPEI®, (VWR, 89129-960). Litters were not used if one or more mouse in the litter was observed to be sick or stunted. P7 and older mice were first anesthetized with 5% isoflurane and then hearts were harvested for further processing. Hearts were also collected at 12, 24 and 40 h after T₃ or vehicle treatment and ventricular cardiomyocytes were prepared from the apex and base for immunoblotting, RT-qPCR or immunocytochemistry. Hearts were also collected at various ages after birth for immunoblotting, immunocytochemistry, immunohistochemistry and cardiomyocyte number estimation.

Echocardiograph Analysis of Mice after T₃/ β 1-Adrenergic Receptor Blocker Therapy

Approximately 6-month-old mice were used. These mice were treated with the β 1-adrenergic receptor blocker, metoprolol succinate (6 μ g/g daily, i.p.) or vehicle (as control) for 19 days. On day-14 of this therapy, mice were additionally given 5 daily injections of T₃ (2 ng/g, i.p.) or vehicle. At 2 and 4 weeks after the cessation of T₃/ β 1-adrenergic receptor blocker mono- or combination therapy, the body weights of these mice were measured as well as their cardiac dimensions and function using transthoracic echocardiography.

LV Apical Cardiomyocytes of Early Post-Neonatal Hearts Retain Proliferative Capacity

An acute in vivo T₃ challenge was used to identify post-neonatal LV cardiomyocytes that retain proliferative capacity. Increases in myocardial cell cycle-promoting cyclins was explored as a surrogate for identifying cardiomyocytes that are able to enter the cell cycle. After 40 h an in vivo T₃ challenge in P8 mice, expression of cyclins D1, A2 and B1—which promote G1/S phase transition, S phase, and G2/M phase transition, respectively—was increased only in the LV and, that within the LV, these cyclins showed 2.5–100-fold higher expression in the apex than in the base ($P < 0.001$) (Figure 1A and B).

The ontogeny of the T₃-stimulated cyclin response during early heart development was examined by comparing cardiomyocytes from the LV apex and the base. In neonatal hearts, T₃ increased cyclins D1/A2/B1 similarly in LV apical and basal cardiomyocytes (Figure 1C). In P8 hearts, T₃ increased these cyclins ~2–5-fold more in apical cardiomyocytes than in basal cardiomyocytes (Figure 1C). However, T₃ did not increase cyclins in either apical or basal P16

cardiomyocytes. These findings indicate that, for ~1 week beyond the neonatal period, a population of LV cardiomyocytes is able to enter the cell cycle.

Experiments were performed to determine whether T₃-stimulated cell cycle entry in LV apical cardiomyocytes ends in mitosis and cell replication. Immunocytochemical studies on cardiomyocytes isolated from the post-P8 LV apex revealed that in vivo T₃ administration increased the number of cardiomyocytes in S phase (5-ethynyl-2'-deoxyuridine+ (EdU)+) and in mitosis (phosphorylated histone H3+ (pH3)+) (Figure 2A and 2B). Although S phase and mitosis were observed in both mono- and binucleated LV apical cardiomyocytes, their occurrence as a percentage of cell type was greater in mononuclear cells (Figure 2B). While an increase in S phase was observed in LV basal cardiomyocytes, these cells did not enter mitosis (Figure 2A and 2B).

Mitosis can result in cell replication, or it can stall prior to karyokinesis or cytokinesis. To determine if LV apical cardiomyocytes undergo cell replication, multicolor clonal analysis was used in double-transgenic Myh6-MerCreMer::Rosa26fs-Confetti mice to record the proliferative strategies of individual cardiomyocytes of the apex relative to those of the base. Limited Cre activation (by administration of 10 mg/kg 4-hydroxytamoxifen to P6 mice) yielded labeling in ~5% of ventricular cardiomyocytes with one of 4 unique colors. T₃ given daily over 3 days (P7–P9) increased the frequency of monochromatic cardiomyocyte clusters by ~6.5-fold in the LV apex relative to the base of the same mouse heart. The majority of monochromatic cardiomyocyte clusters in the LV apex consisted of 2 cells, and were distributed throughout the tissue section, indicating that cardiomyocyte proliferation resulted from multiple individual cell replication events rather than clonal expansion of a few highly proliferative cells.

To determine the effect of T₃ on the ventricular cardiomyocyte population in early post-neonatal mice, T₃ daily (i. p.), was administered from P7 to P9. At P12, hearts were enzymatically disaggregated and cell suspensions created from the cardiac ventricles. Digestion efficiencies were ~98%. Cardiomyocytes in these cell suspensions, identified by their size and by rod shape, were counted using a hemocytometer. T₃ increased ventricular cardiomyocyte numbers by ~18% (Figure 2D), which indicates an average replication rate of 3.6%/day between P7 and P12.

DUSP5 Expression Causes Loss of Proliferative Competence in Post-Neonatal Cardiomyocytes

Developmental increases in T₃ stimulate insulin-like growth factor-1 (IGF-1) receptor (IGF-1R) signaling in early post-neonatal cardiomyocytes. Experiments indicate that IGF-1R expression is required for T₃-stimulated cyclin expression in P8 LV apical cardiomyocytes (Figure 3A). However, while T₃-stimulated expression of IGF-1/IGF-1R is comparable in basal and apical cardiomyocytes (Figure 3B), expression of cyclins D1, A2 and B1 is ~2–3-fold lower in basal cardiomyocytes (P<0.001) (Figure 3B). These data suggest that IGF-1R proliferative signaling is intrinsically inhibited in basal cardiomyocytes.

Proliferative signaling by growth factors involves Ras/Raf/mitogen-activated protein kinase (MAPK) kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling. MEK1/2 activate ERK1/2 by phosphorylating T202 and Y204, which is required for ERK1/2 translocation to the nucleus. Sustained ERK1/2 phosphorylation in the nucleus activates multiple transcription factors ultimately resulting in effector protein synthesis and cell proliferation. A single dose of T₃ was administered to P8 mice, and cardiomyocytes were isolated from the LV apex or base either immediately after T₃ administration (0 h) or after 24 h. T₃ increased MEK1/2 phosphorylation (S218/S222) and nuclear translocation of ERK1/2 similarly in apical and basal cardiomyocytes (Figure 4A). However, the fraction of nuclear ERK1/2 that remained phosphorylated at 24 h post-T₃ treatment was ~70% lower in LV basal cardiomyocytes (Figure 4A).

These data suggest that cytoplasmic ERK1/2 phosphorylation by T₃/IGF-1R is similar in apical and basal P8–P9 LV cardiomyocytes, but after nuclear translocation its dephosphorylation is more rapid in basal cardiomyocytes. The potential involvement of the dual-specificity (T/Y) MAPK phosphatase, DUSP5, was studied. DUSP5 is an inducible nuclear phosphatase that attenuates MAPK signaling by specifically binding and dephosphorylating p-ERK1/2. DUSP5's involvement was supported by the finding that levels of DUSP5 were ~6-fold higher in LV basal versus apical cardiomyocytes (Figure 4A). In vivo siRNA knock-down of DUSP5 mRNA depleted DUSP5 in LV basal cardiomyocytes, and in this setting T₃ stimulated ERK1/2 phosphorylation and expression of cyclins (Figure 4B). Thus, DUSP5 inhibits T₃-stimulated ERK1/2 phosphorylation in post-neonatal LV basal cardiomyocytes (Figure 4C).

To determine if the developmental pattern of DUSP5 expression is consistent with a role in suppression of the proliferative potential of murine hearts, changes in DUSP5 expression were

examined in the LV apical and basal myocardium from birth to P16. DUSP5 was not detectable in the LV apex or base of neonatal animals (Figure 5A). After P6, DUSP5 expression increased in the LV base and remained high. In the LV apex, DUSP5 expression was first observed at P14, and by P15, it was uniformly high throughout the LV. This temporal expression of DUSP5 in the LV is concordant with developmental differences in T₃-stimulated cardiomyocyte cyclin expression (Figure 1C).

Additional experiments were performed to determine whether DUSP5 expression in adult cardiomyocytes necessary to sustain cell cycle block. In 6-week-old adult mice, acute DUSP5 depletion by siRNA enhanced T₃-induced ERK1/2 phosphorylation and cyclin expression in LV cardiomyocytes, without influencing IGF-1/IGF-1R expression or MEK1/2 phosphorylation (Figure 5B). Importantly, acute DUSP5 depletion coupled with T₃ treatment increased the cardiomyocyte endowment of adult hearts (Figure 5C). Collectively, the results of these experiments indicate that DUSP5 blocks the cell cycle in cardiomyocytes as its expression increases progressively from the LV base during the early post-neonatal period to the apex during preadolescence. It then maintains this block as cardiomyocytes mature into their adult form; inhibition of DUSP5 in adult hearts permits cell cycle progression in response to T₃.

DUSP5 Expression in Cardiomyocytes is Independent of Cell Maturation

Experiments were performed to determine if DUSP5 expression is secondary to cardiomyocyte maturation. Gene expression analyses revealed that DUSP5 mRNA levels were ~6-fold higher in cardiomyocytes of the P8 LV base than in those of the apex. However, no differences were seen in the expression of genes that regulate cardiomyocyte differentiation/dedifferentiation, oxidative phosphorylation, fatty acid synthesis, glycolysis, the DNA damage response or the cell cycle. Also, the distribution of mono-, bi- and multinucleated cardiomyocytes in these LV regions were similar (Figure 6A) as were their sizes (Figure 6B). These results indicate that DUSP5 expression in early post-neonatal LV cardiomyocytes is not related to a maturation-induced shift in metabolism, activation of the DNA damage response pathway, or cellular morphology.

Beta1-Adrenergic Receptors Promote DUSP5 Expression in Cardiomyocytes

As cardiomyocytes mature, they gain sensitivity to norepinephrine by increasing the number of β 1-adrenergic receptors; this enhanced sensitivity is associated with progressive innervation of the LV by sympathetic nerves. The cardiac-sympathetic axis becomes functional after the first week of life. Experiments were performed to determine whether β 1-adrenergic receptors regulate DUSP5 in LV cardiomyocytes. Sequential analyses, between P1 and P16, show associated developmental changes in LV β 1-adrenergic receptors, adrenergic nerves (using tyrosine hydroxylase as a marker) and DUSP5 (Figure 5A). To establish causality, β 1-adrenergic receptors were knocked-down in vivo in early post-neonatal and young adult LV cardiomyocytes, using β 1-adrenergic receptor siRNA, which inhibited cardiomyocyte DUSP5 expression and increased T₃-induced ERK1/2 phosphorylation (Figure 7A and 7B). DUSP5 expression was also suppressed after β 1-adrenergic receptor blocker pretreatment with metoprolol; this pretreatment permitted the expression of cyclins D1/A2/B1 in response to T₃ (Figure 7C). These results indicate that β 1-adrenergic receptors regulate DUSP5 expression in LV cardiomyocytes. Moreover, they suggest that as sympathetic innervation extends during postnatal development from the LV base to its apex, it progressively triggers and then maintains cell cycle block in cardiomyocytes via β 1-adrenergic receptor-mediated DUSP5 expression.

T₃ Stimulates Cardiac Muscle Formation in β 1-Adrenergic Receptor Blocker Treated Mice

Experiments indicate reversal of cell cycle block in cardiomyocytes is not sufficient for the activation of proliferation. Rather, a pro-proliferative stimulus is needed to get these cells to replicate. Experiments were performed to determine whether reversal of cell cycle block using β 1-blockade coupled with acute T₃ treatment may be sufficient to expand the cardiomyocyte endowment of the adult murine heart. Adult mice (26-week-old) were given metoprolol (6 μ g/g, daily, i.p.) or vehicle for 19 days; over the last 5 days of this β 1-blockade (or vehicle control) the mice were additionally given T₃ (2 ng/g, daily, i.p.) or vehicle. Two weeks after cessation of these therapies LV dimensions and contractility were analyzed by echocardiography as well as ventricular cardiomyocyte numbers. The data are summarized in Table 1 below.

Table 1. Comparison of Echocardiography Dimensions and LV Systolic Function in Adult 6-Month-Old Mice given T³ and Metoprolol Monotherapies or T³ + Metoprolol Combination Therapy

	No therapy*	T ³ monotherapy*	Metoprolol monotherapy*	T ³ +Metoprolol combination therapy*
Echocardiography, <i>n</i>	19	20	20	20
FW diastole, mm	0.84±0.02	0.87±0.01 ^{NS}	0.92±0.02	1.01±0.02***
FW systole, mm	1.13±0.03	1.19±0.03 ^{NS}	1.20±0.03	1.33±0.03**
IVS diastole, mm	0.95±0.01	0.94±0.01 ^{NS}	0.96±0.02	1.09±0.02**
IVS systole, mm	1.31±0.03	1.29±0.02 ^{NS}	1.29±0.02	1.43±0.03***
LV end-diastolic dimension, mm	3.53±0.06	3.60±0.03 ^{NS}	3.53±0.07	3.66±0.06 ^{NS}
LV end-systolic dimension, mm	2.72±0.07	2.70±0.05 ^{NS}	2.71±0.06	2.25±0.07***
Fractional shortening, %	23.0±1.4	24.8±1.4 ^{NS}	23.4±0.6	38.5±1.5***
Ejection fraction, %	53.5±1.5	55.7±1.4 ^{NS}	55.4±1.8	78.0±2.2***
LV mass, mg	109±2	110±2 ^{NS}	113±2	141±3***
Body weight, g	33.3±0.4	32.9±0.4 ^{NS}	33.2±0.5	33.1±0.3 ^{NS}
Euthanasia, <i>n</i>	9	8	9	7
Cardiomyocyte numbers, (millions) [<i>n</i>]	2.37±0.029	2.33±0.039 ^{NS}	2.44±0.026	2.88±0.056***

*Six-month-old mice were given metoprolol (6 µg/g daily, i.p.) or vehicle (as control) pretreatment for 19 days. On day-14 of this therapy, they were additionally given 5 daily injections of T₃ (2 ng/g, i.p.) or vehicle. At 2 weeks after the cessation of mono- or combination therapy body weights of these mice were determined, and their cardiac dimensions and function determined by transthoracic echocardiography.

In vehicle pretreated mice, T³ treatment did not significantly affect body weight, ventricular cardiomyocyte numbers, LV mass or any measured LV dimension. By contrast, in metoprolol-treated mice, T₃ treatment increased ventricular cardiomyocyte numbers by 18% (that is, by ~450,000 cardiomyocytes) (P<0.001), LV mass by 25% (P<0.001), and LV free wall dimension at diastole by 11% (P<0.001), without significantly changing LV end-diastolic

dimension. These functional parameters are consistent with heart growth without chamber occlusion. Metoprolol/T₃ treatment did not increase mortality—no animals died as a result of this therapy—or influence body weight. The most striking effect of the combination therapy, relative to metoprolol monotherapy, was an about 40% increase in ejection fraction ($P < 0.001$) which, in a cohort of animals given combination therapy, was mostly sustained over the 4-week post-therapy follow up interval (ejection fraction: $83.6\% \pm 1.04\%$ and $82.4\% \pm 1.05\%$ at 2 and 4 weeks after cessation of combination therapy, respectively; number of pairs=8; $P=0.031$, paired 2-tailed Student's t-test).

CLAIMS

1. A method of treating or preventing heart malformation or cardiovascular disease comprising administering an effective amount of thyroid hormone in combination with a beta-adrenergic blocking agent to a subject in need thereof.
2. The method of Claim 1, wherein the beta-adrenergic blocking agent is selected from acebutolol, atenolol, betaxolol, bisoprolol, carteolol, carvedilol, esmolol, labetalol, metoprolol, nadolol, nebivolol, penbutolol, pindolol, propranolol, sotalol, timolol, or salts thereof.
3. The method of Claim 1, wherein the beta-adrenergic blocking agent is administered in combination with a mitogen.
4. The method of Claim 4, wherein the mitogen is neuregulin, IGF-1, YAP1, ERBB2.
5. The method of Claim 1, wherein the cardiovascular disease is selected from coronary artery disease, heart failure, cardiomyopathy, heart valve disease, and cardiac arrhythmias.
6. The method of Claim 1, wherein the subject is diagnosed with a heart attack, angina, or stroke, diabetes, angina pectoris due to coronary atherosclerosis, hypertension, migraine headaches, glaucoma, hyperthyroidism, fibromyalgia, generalized anxiety disorder, parkinsonian tremor, and atrial fibrillation.
7. A method of inducing proliferation of cardiomyocytes comprising administering an effective amount of thyroid hormone in combination with a beta-adrenergic blocking agent to a subject in need thereof.
8. The method of Claim 7, wherein the beta-adrenergic blocking agent is siRNA.
9. The method of Claim 7, wherein the combination is administered is administered to a newborn within 1 to 7 days of birth.

10. The method of Claim 7, wherein the combination is administered to a newborn within 8 to 14 days of birth.
11. The method of Claim 7, wherein the combination is administered to a newborn within 15 to 21 days of birth.
12. The method of Claim 7, wherein the combination is administered to a subject more than 21 days after birth.
13. The method of Claim 7, wherein the combination is administered by intravenous (IV) injection, direct injection into the cardiac tissue, direct injection into apical cardiac tissue, or pericardial cavity.
14. The method of Claim 7, wherein the combination is administered to a subject at risk of, exhibiting symptoms of, or diagnosed with a cardiovascular disease, condition, or injury.
15. The method of Claim 7, wherein the combination is administered to a subject at risk of, exhibiting symptoms of, or diagnosed with heart disease, heart attack, stroke, heart failure, arrhythmia, heart valve disease, congenital heart defect, patent ductus arteriosus, ventricular septal defect, truncus arteriosus, atrioventricular septal defect, tetralogy of Fallot, transposition of the great arteries, hypoplastic left heart syndrome, tricuspid atresia, or heart murmur, for use in the treatment or prevention thereof.
16. A method of inducing proliferation of cardiomyocytes comprising administering an effective amount of a DUSP5 and/or DUSP6 inhibitor to a subject in need thereof.
17. The method of Claim 16, wherein the DUSP5 or DUSP6 inhibitor is a polysulfonated aromatic compound with a carbazole or naphthalene scaffold.
18. The method of Claim 16, wherein the DUSP5 or DUSP6 inhibitor is siRNA or an antisense therapy.

19. The method of Claim 1, wherein the DUSP5 or DUSP6 inhibitor is administered at risk of, exhibiting symptoms of, or diagnosed with a cardiovascular disease, condition, or injury.

20. An in vitro method of inducing proliferation of cardiomyocytes comprising mixing a DUSP5 and/or DUSP6 inhibitor with a cardiac cell.

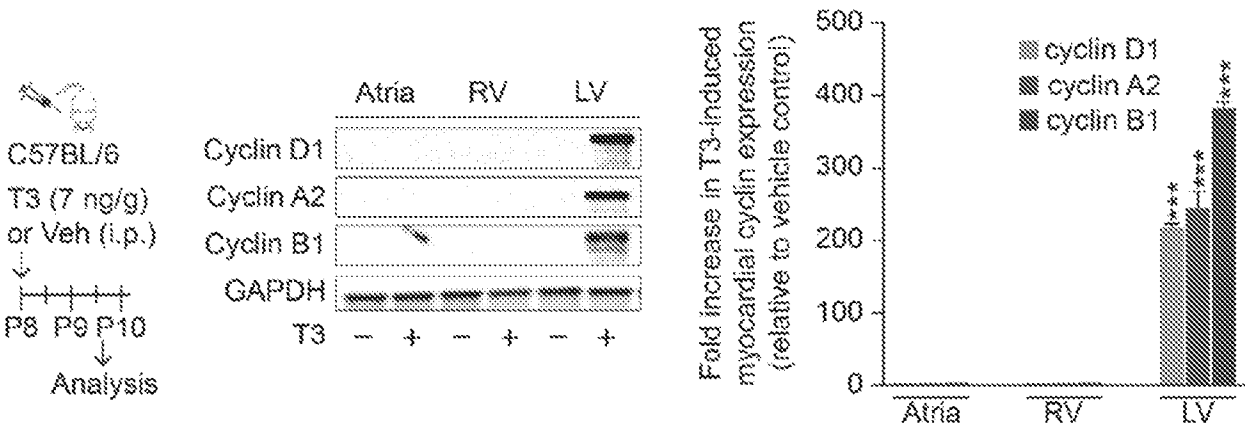


FIG. 1A

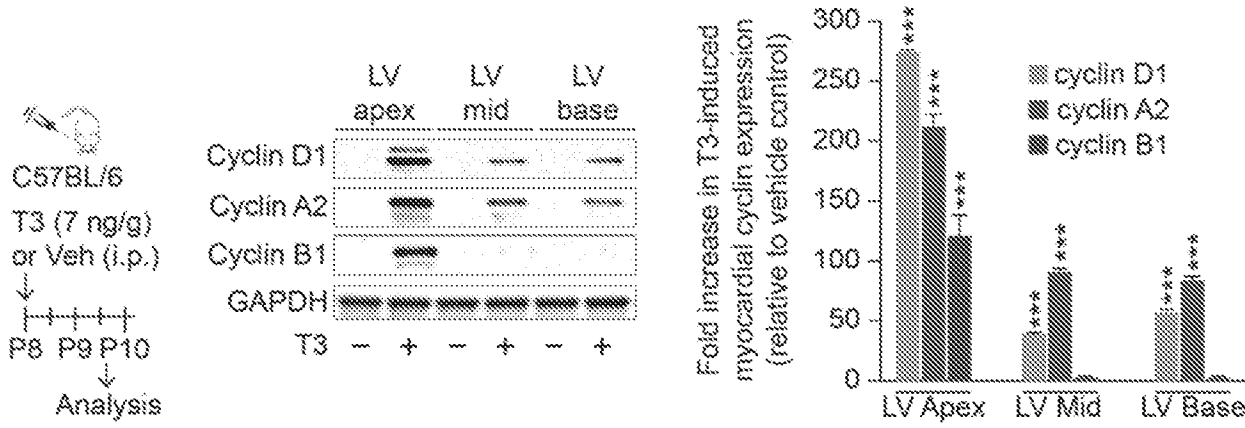


FIG. 1B

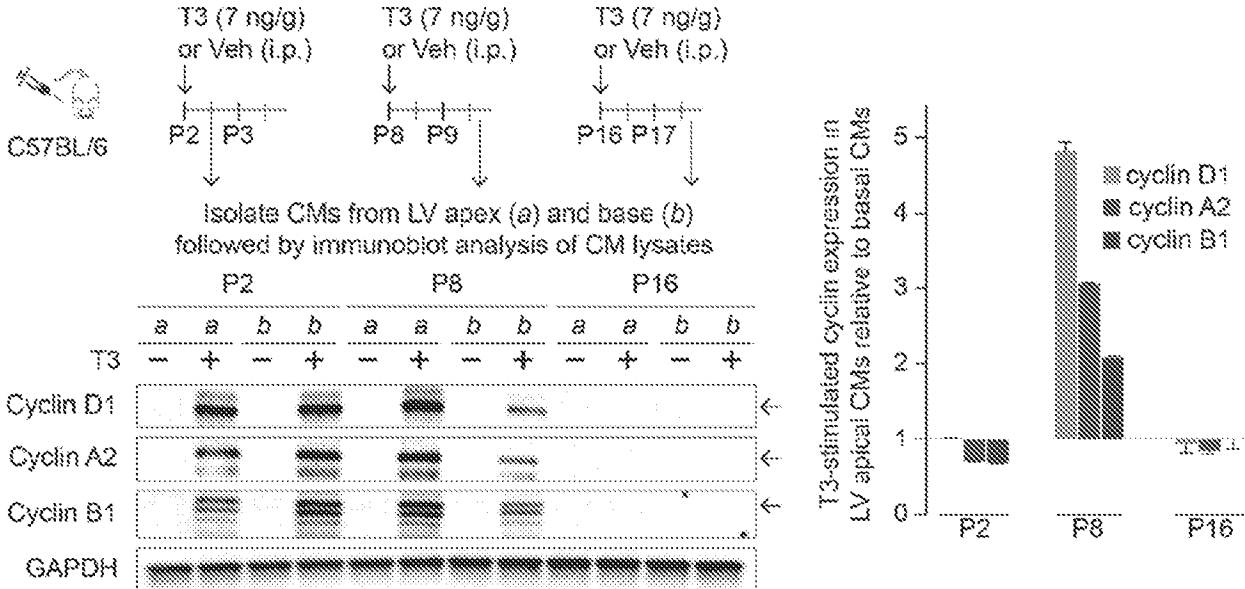


FIG. 1C

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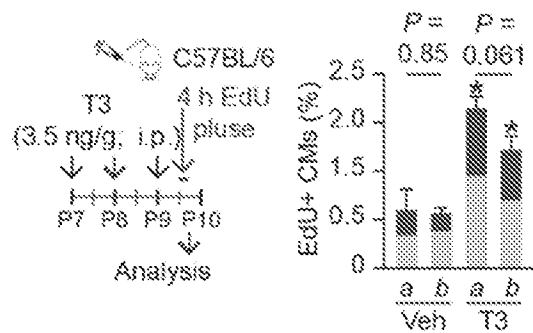


FIG. 2A

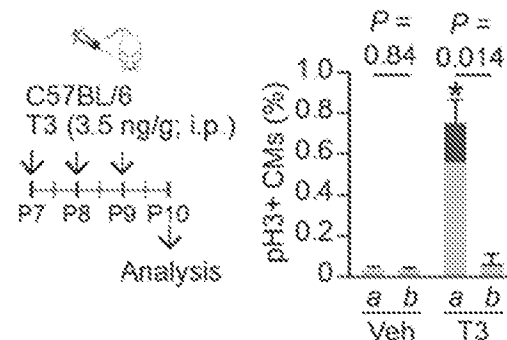


FIG. 2B

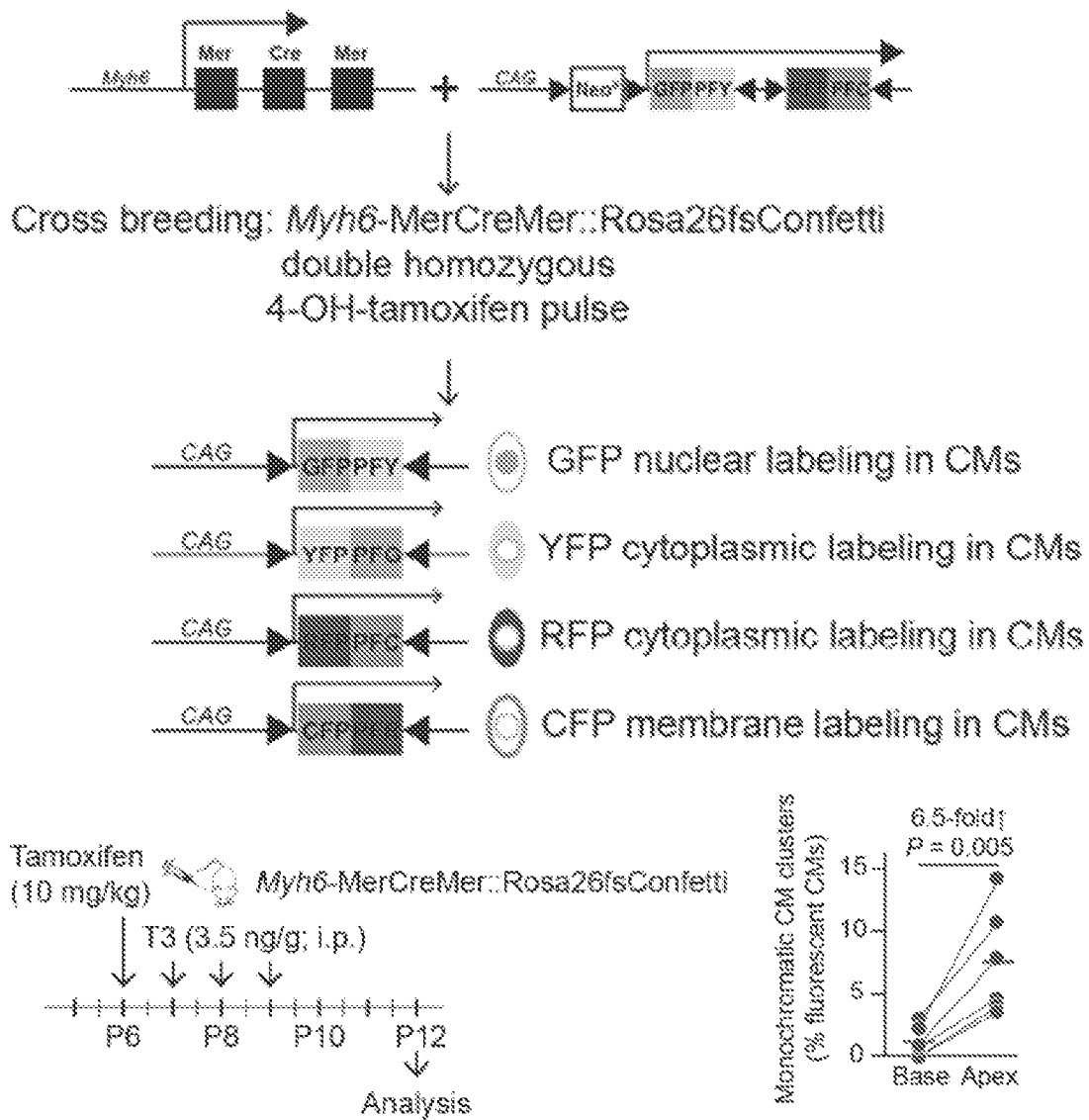


FIG. 2C

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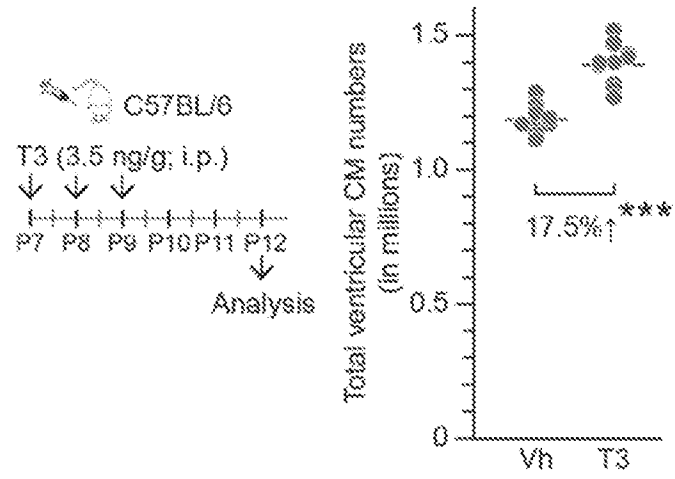


FIG. 2D

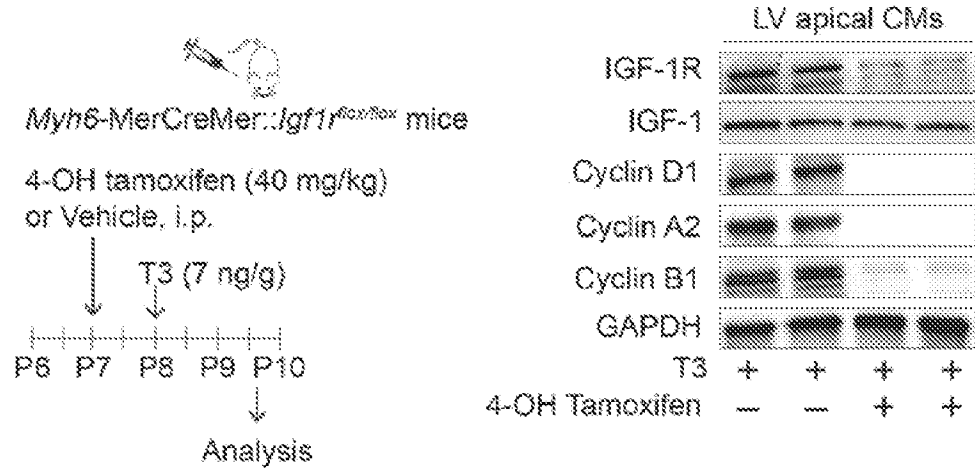


FIG. 3A

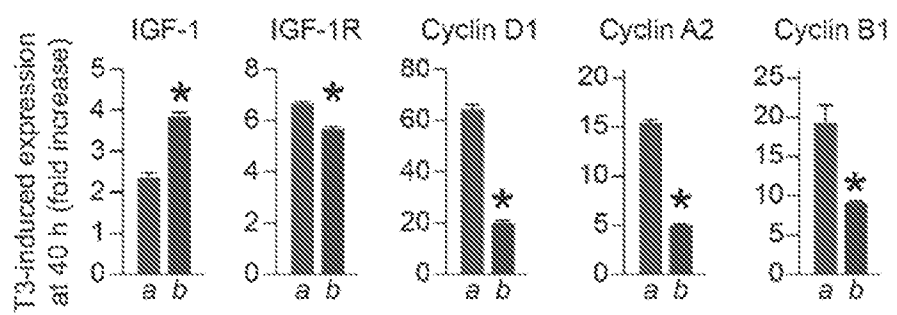


FIG. 3B

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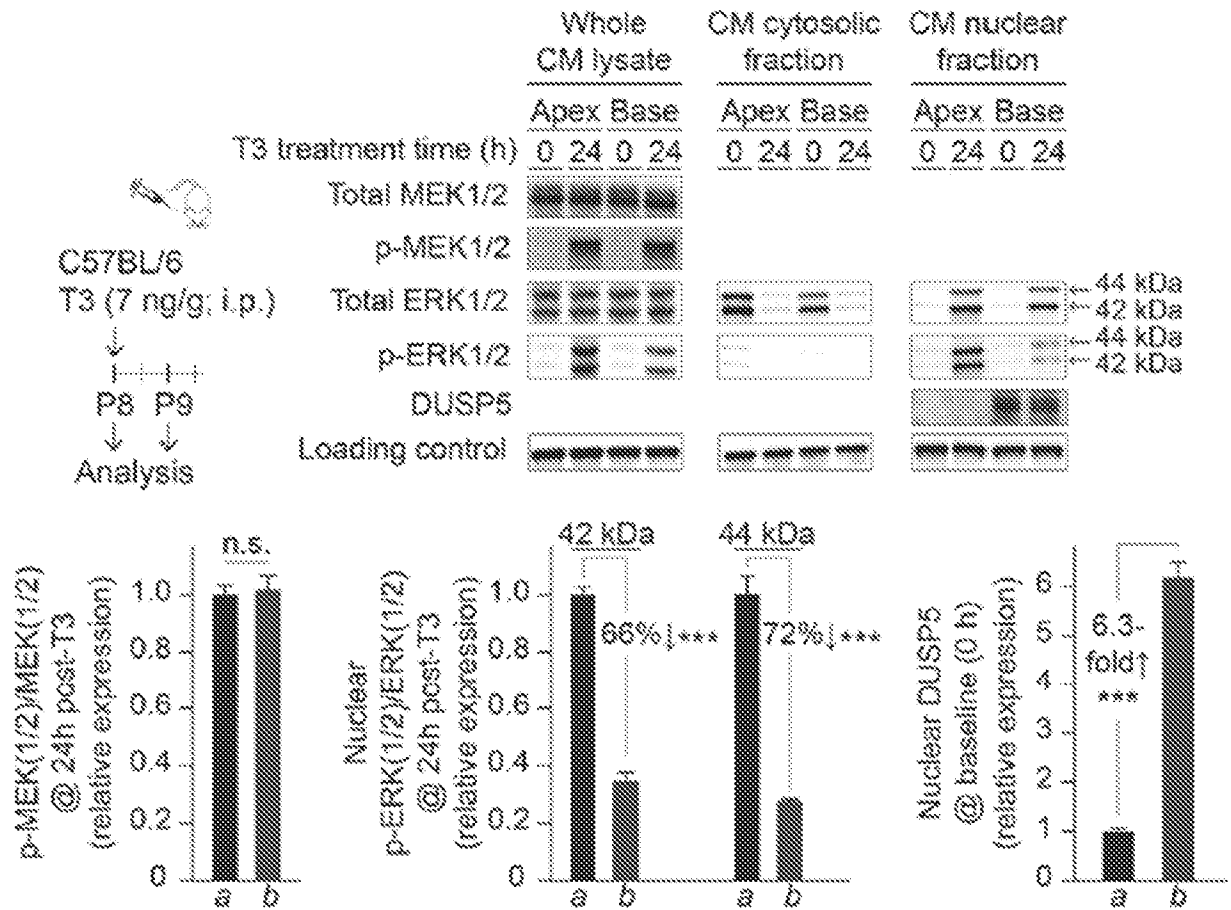


FIG. 4A

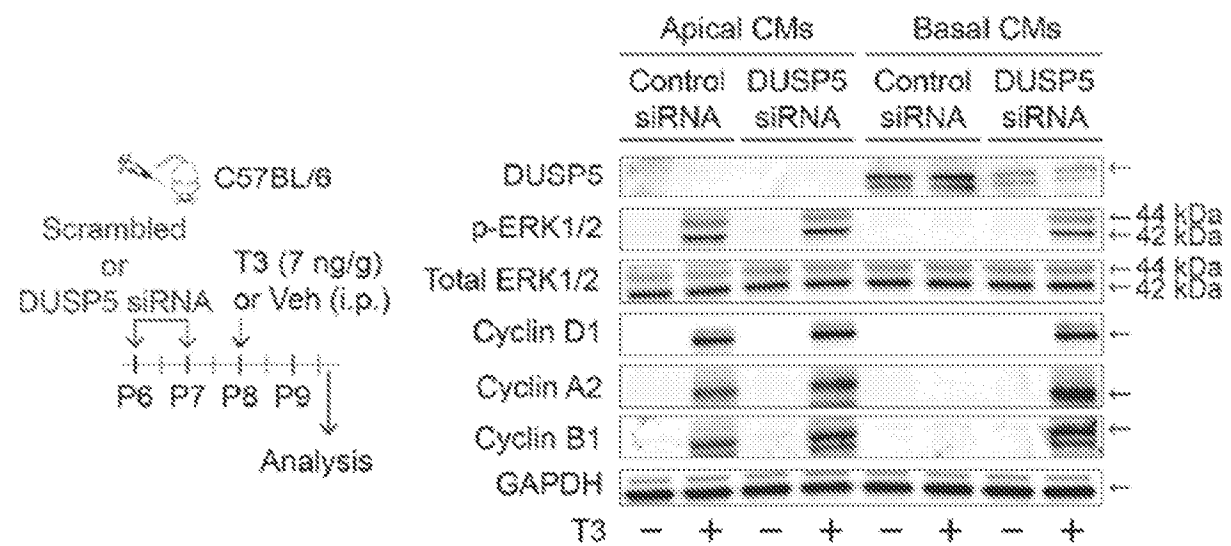


FIG. 4B

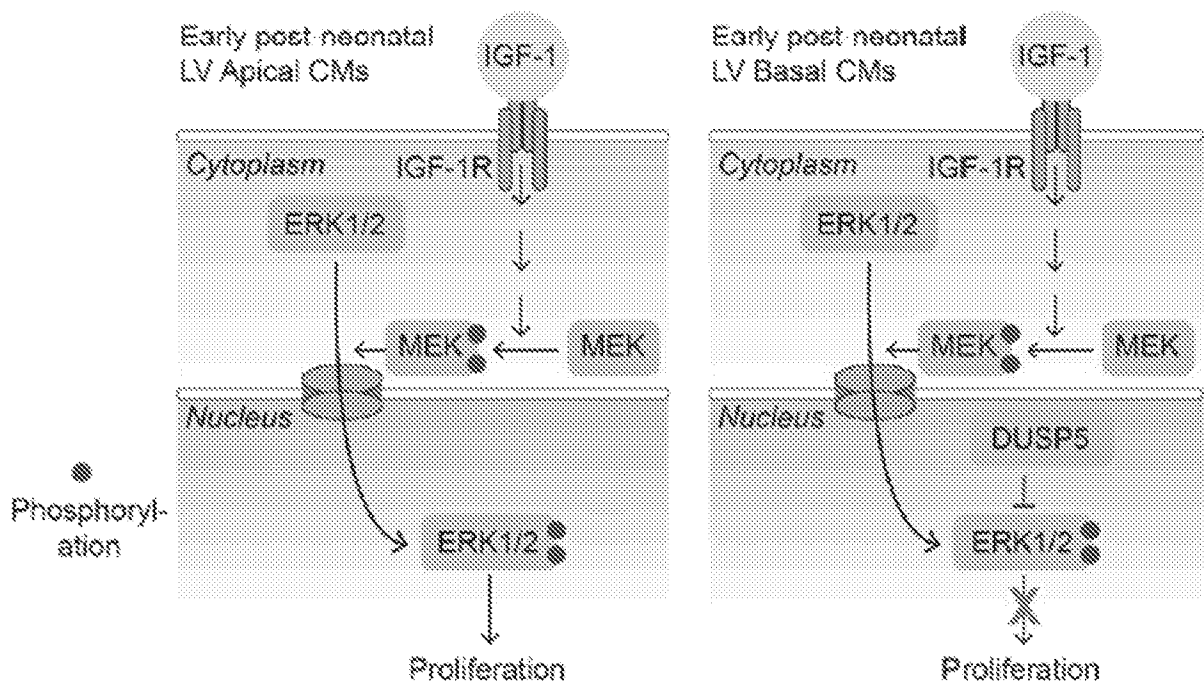


FIG. 4C

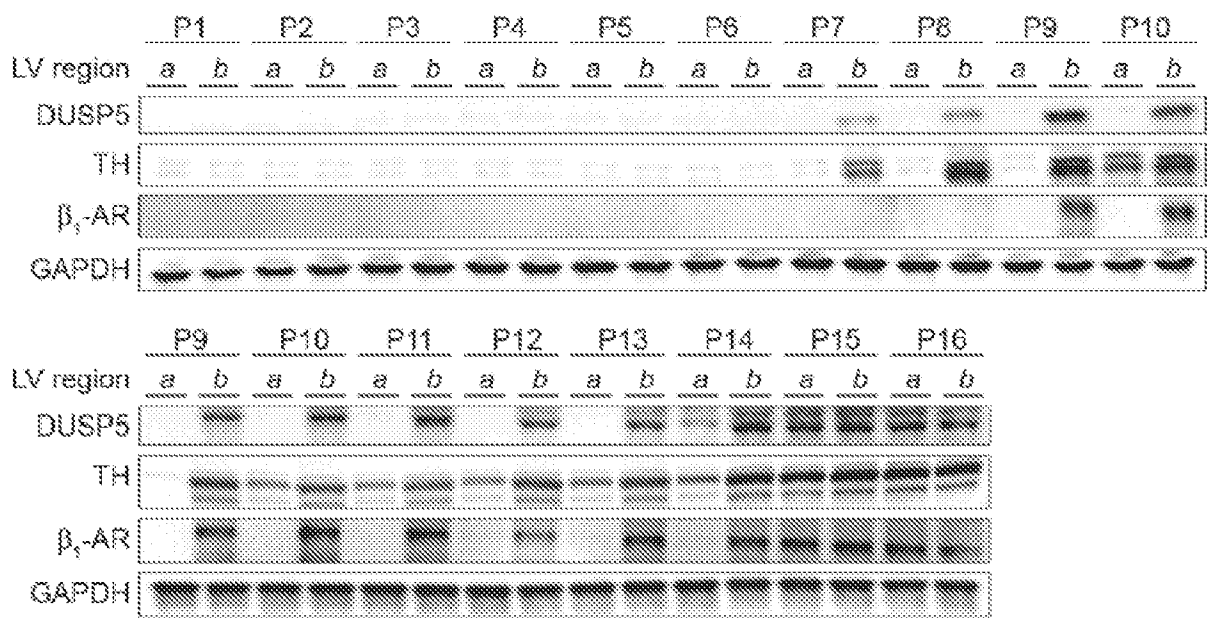


FIG. 5A

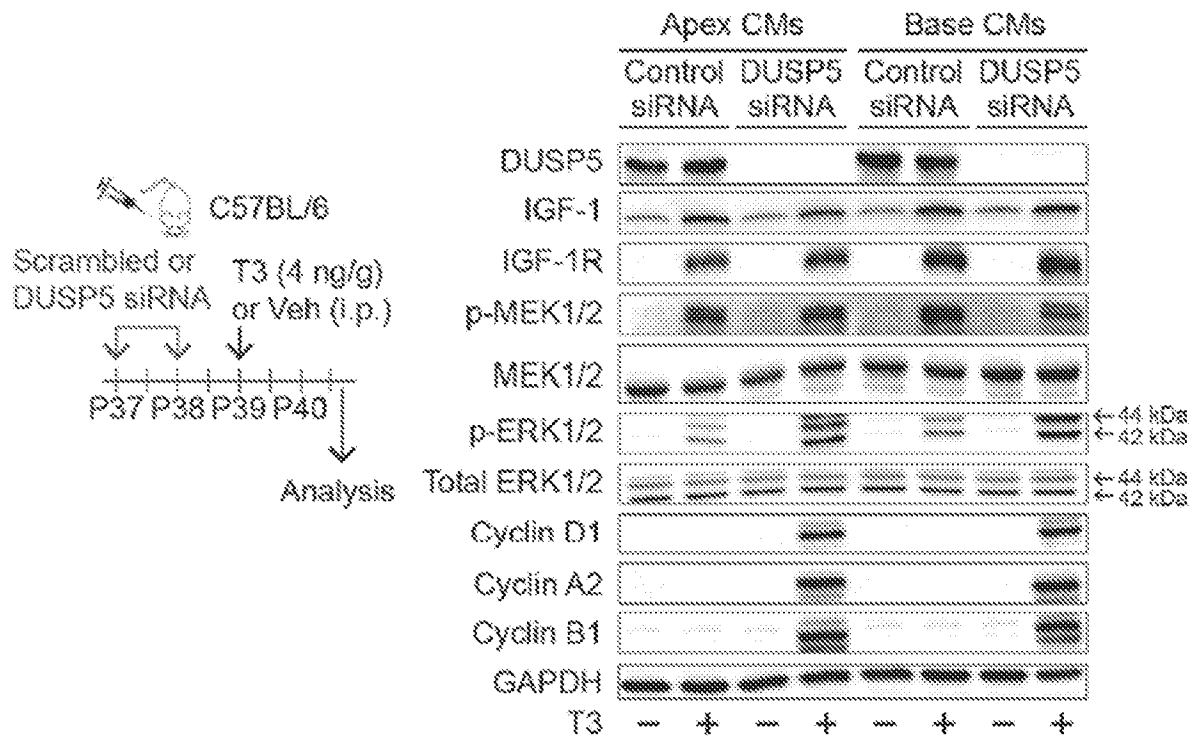


FIG. 5B

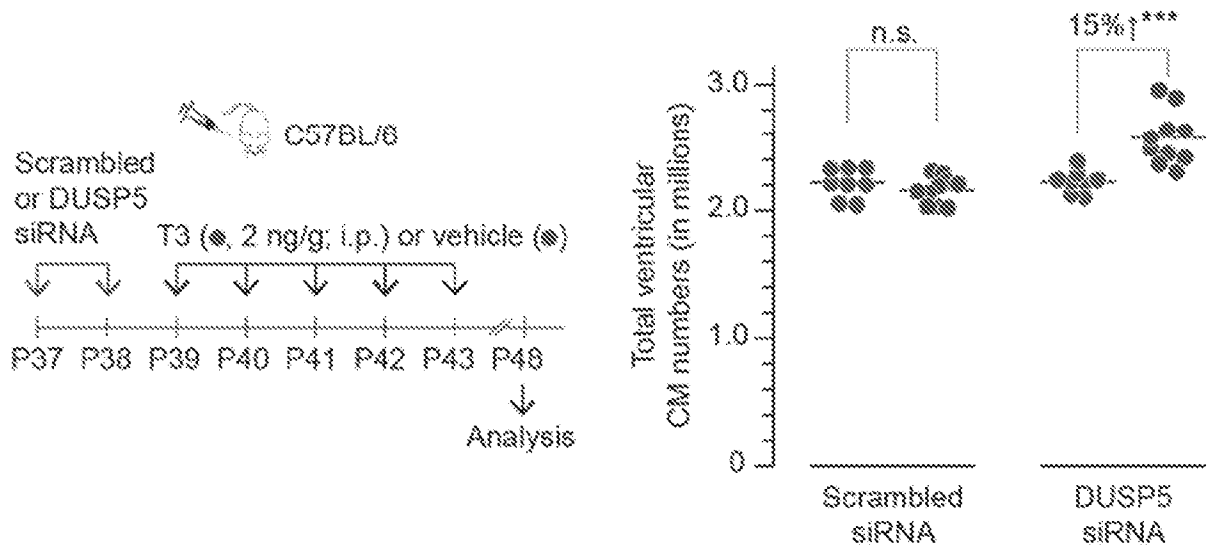


FIG. 5C

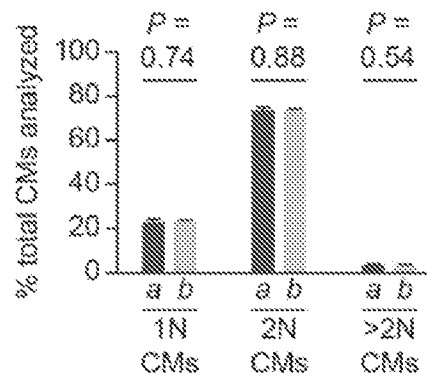


FIG. 6A

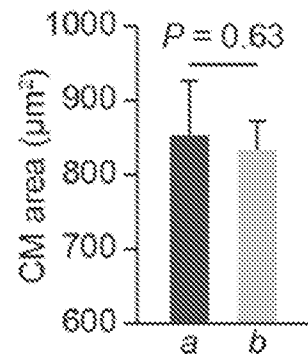


FIG. 6B

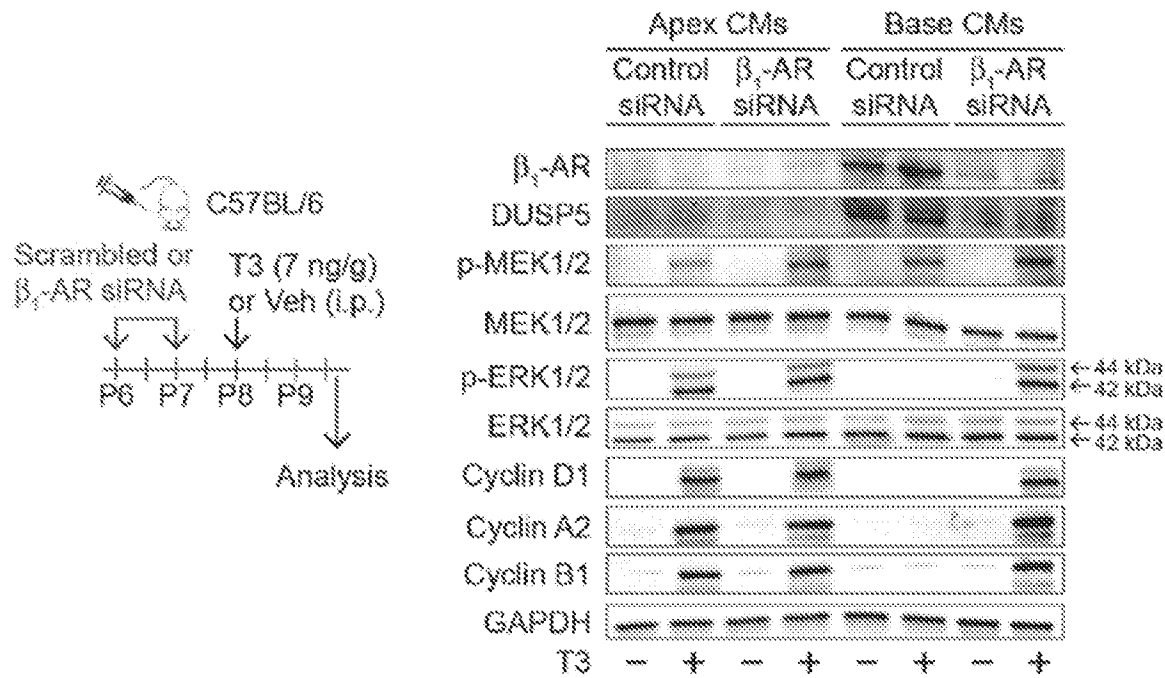


FIG. 7A

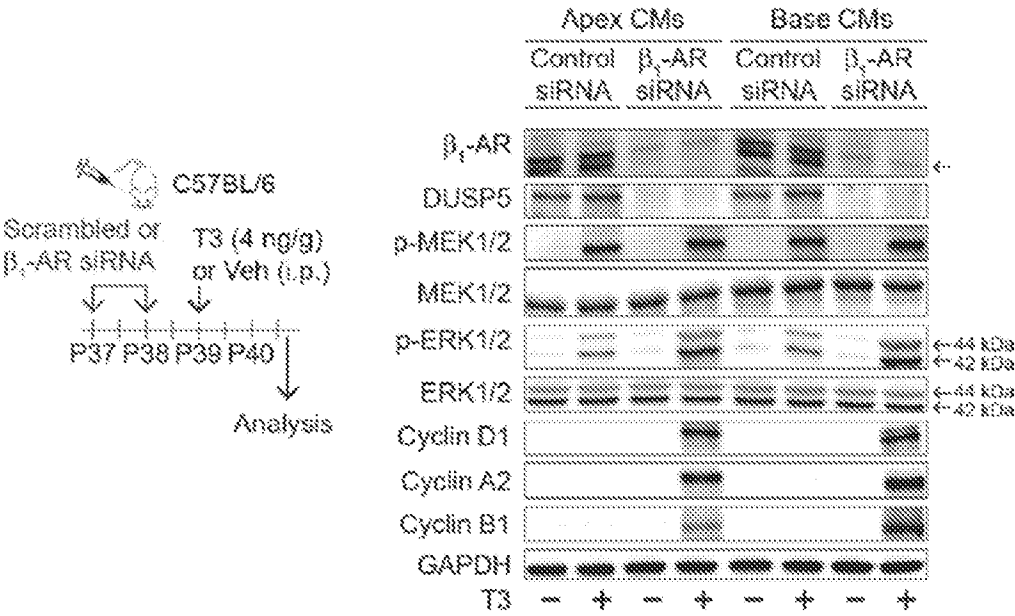


FIG. 7B

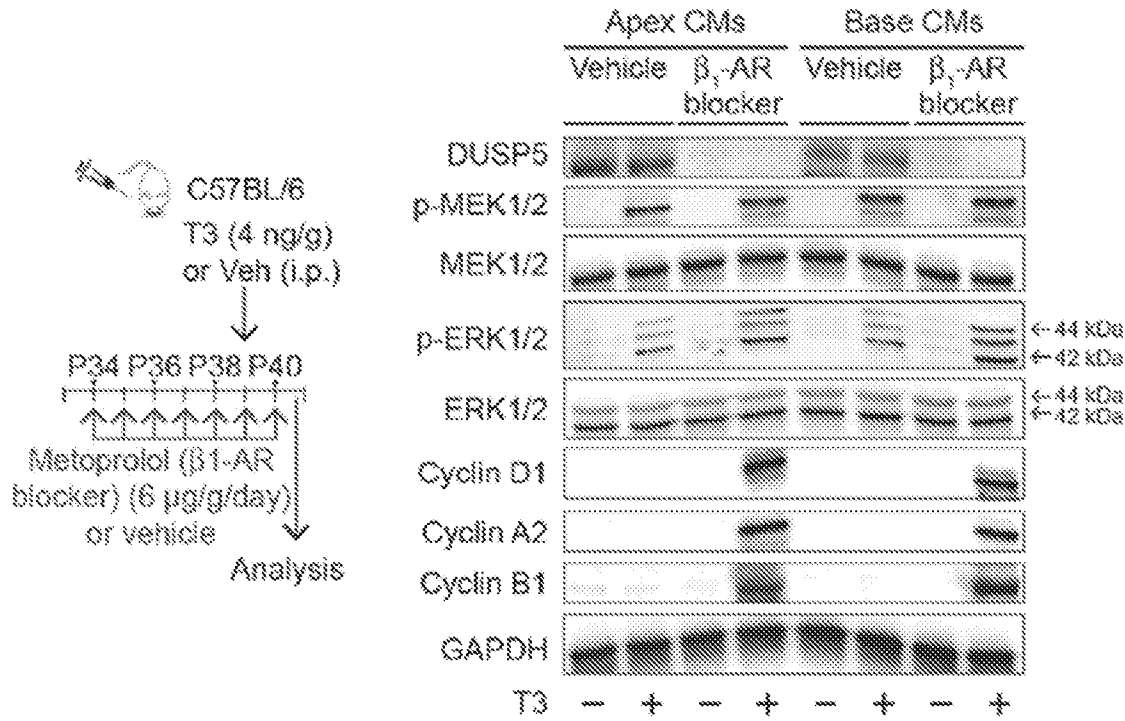


FIG. 7C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 19/46566

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 31/216, A61K 31/197, A61K 31/137, A61K 31/138, A61K 31/166, A61K 31/403 (2019.01)

CPC - C07C 59/64, C07C 217/76, C07C 235/34, C07C 2601/14, C07C 323/25, C07C 2601/02, C07C 2601/04, C07C 235/52, A61F9/00, A61K31/00, A61K31/137, A61K 31/138, A61K 31/166, A61K 31/403, A61K 31/404, A61K 31/4045, A61K 31/4704, A61K 31/5377, G01N 33/74, G01N 2333/726, G01N 2500/04, G01N 2800/40

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)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	Nagele, et al. Combination therapy with carvedilol and amiodarone in patients with severe heart failure. Eur J Heart Fail. 2000, 2(1):71-79; Abstract, pg 73, Table 1; pg 74, col 1; pg 76, col 1	1, 2, 5, 6 ----- 3, 4
A	Razvi, et al. Thyroid Hormones and Cardiovascular Function and Diseases. Journal of the American College of Cardiology April 2018, 71(16):1781-1796; pg 1792, col 2 and Fig 6 and its legend	1-6
Y	US 2017/368140 A1 (ZENSUN (SHANGHAI) SCIENCE & TECH CO LTD.[CN]) 28 December 2017 (28.12.2017) claims 7, 13-15	3, 4
Y	Cai, et al. Repair Injured Heart by Regulating Cardiac Regenerative Signals. Stem Cells Int. 2016; 2016:6193419; pg 2, Table 1; pg 3; pg 10, col 1	7-15
Y	Arnold, et al. Specific beta1-adrenergic receptor silencing with small interfering RNA lowers high blood pressure and improves cardiac function in myocardial ischemia. J Hypertens. 2007, 25(1):197-205; Abstract, pg 197, col 1; pg 198	7-15

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"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

16 October 2019

Date of mailing of the international search report

30 DEC 2019

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

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Lee Young

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 19/46566

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Animal Resources Centre. Rat and Mice Weights, July 2018 [online] [Retrieved on 17 October 2019]. Retrieved from the Internet < URL: https://web.archive.org/web/20180717224335/https://www.arc.wa.gov.au/?page_id=125 > pg 2 to 3	11, 12

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 19/46566

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.:
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:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I, claims 1-15, directed to a method of treating cardiovascular disease/ inducing proliferation of cardiomyocytes by administering thyroid hormone in combination with a beta adrenergic blocker to a subject in need thereof.

Group II, claims 16-20, directed to a method of inducing proliferation of cardiomyocytes by administering a DUSP5 and/or DUSP6 inhibitor to a subject in need thereof.

***** See Supplemental Sheet to continue *****

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.:
1-15

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.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/US 19/46566

In Continuation of Box III. Observations where unity of invention is lacking:

The inventions listed as Group I and Group II do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The inventions of Group I share the special technical feature of a method comprising administering thyroid hormone in combination with a beta adrenergic blocker that is not required by Group II.

The inventions of Group II share the special technical feature of a method comprising administering a DUSP5 and/or DUSP6 inhibitor that is not required by Group I.

The inventions of Group I and Group II share the technical feature of a method of inducing proliferation of cardiomyocytes in a subject. However, this shared technical feature does not represent an improvement over prior art as being anticipated by a paper titled "ERBB2 triggers mammalian heart regeneration by promoting cardiomyocyte dedifferentiation and proliferation" by D'Uva, et al. (Nat Cell Biol. 2015, 17(5):627-38) (hereinafter "D'Uva").

D'Uva discloses a method of inducing proliferation of cardiomyocytes in a subject by administering ERBB2 to a subject (pg 2, col 1, "Transient caErbB2 induction in juvenile or adult mice stimulates CM [cardiomyocyte] proliferation and allowed anatomical and functional regeneration of hearts after ischaemic injury. Thus, augmentation of ERBB2 signalling awakens a dormant regenerative window in juvenile and adult mice... potentiation of ERBB2 signalling in adult CMs might represent a promising therapeutic approach for CM replacement in heart failure"; Abstract, "Induction of a constitutively active ERBB2 (caERBB2) in neonatal, juvenile and adult CMs resulted in cardiomegaly, characterized by extensive CM hypertrophy, dedifferentiation and proliferation... Transient induction of caERBB2 following myocardial infarction triggered CM dedifferentiation and proliferation followed by redifferentiation and regeneration. Thus, ERBB2 is both necessary for CM proliferation and sufficient to reactivate postnatal CM proliferative and regenerative potentials"; SUPPLEMENTARY INFORMATION, pg 1, Methods, col 1, "Doxycycline-inducible CM-restricted overexpression of a constitutively active Erbb2 (caErbb2) was generated by crossing the TetRE x caErbb2 mouse line65 (a B6SJLF1/J transgenic line in which this mutated Erbb2 is placed under the control of a minimal CMV promoter and the tetracycline-responsive element) with aMHC- tTA (ref. 66), which expresses the tetracycline-responsive transcriptional activator (tTA) under the control of the human alpha myosin heavy chain promoter (aMHC) promoter (see Supplementary Fig. 2a)"). As the technical feature was known in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the inventions.

Therefore, inventions of Group I and Group II lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.