



(51) International Patent Classification:

A61K 48/00 (2006.01) C12N 15/00 (2006.01)
A61K 38/00 (2006.01) C07H 21/04 (2006.01)

(21) International Application Number:

PCT/US2017/035897

(22) International Filing Date:

05 June 2017 (05.06.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/345,273 03 June 2016 (03.06.2016) US

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

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,

(54) Title: METHODS AND COMPOSITIONS FOR TREATING HEART FAILURE

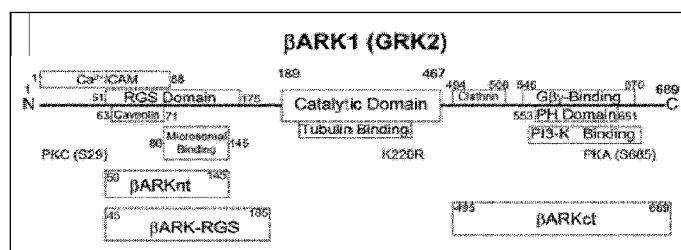


Figure 1A

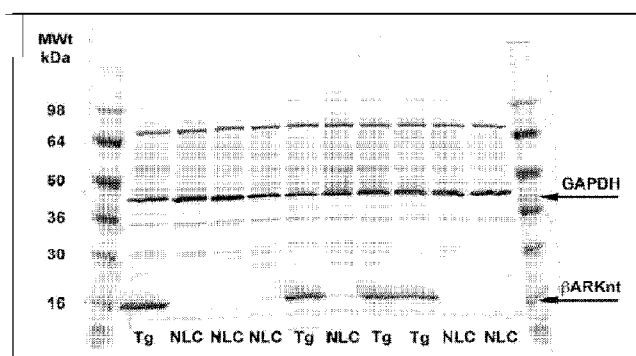


Figure 1B

(57) Abstract: This invention is generally related to novel compositions for treating or preventing heart failure. Specifically, the disclosure provides a composition comprising an amino-terminal domain of Beta. adrenergic receptor kinase-1 (Beta.ARKnt) peptide, or a nucleic acid encoding Beta.ARKnt. Further disclosed are methods of using the compositions for treating or preventing heart failure in a subject or for altering Beta.-adrenergic receptor (Beta.AR) density in a subject.



TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

Published:

- *with international search report (Art. 21(3))*
- *before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments (Rule 48.2(h))*
- *with sequence listing part of description (Rule 5.2(a))*

TITLE OF THE INVENTION

METHODS AND COMPOSITIONS FOR TREATING HEART FAILURE

CROSS-REFERENCE TO RELATED APPLICATIONS

5 The present application claims priority to U.S Provisional Patent
Application No. 62/345,273, filed June 3, 2016, which is hereby incorporated herein by
reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR
DEVELOPMENT

This invention was made with government support under Grant Numbers R37 HL061690 and P01 HL075443 awarded by National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Signaling via G protein-coupled receptors (GPCRs) is critical for normal heart function, and during disease GPCRs are uncoupled from their downstream effectors and receptor number is reduced. Signaling through these receptors is tightly controlled by GPCR kinases (GRKs) and one in particular, GRK2 (originally known as β ARK1), has been shown to be intimately involved in heart failure (HF) progression. GRK2 upregulation in failing hearts represents one of the first molecular changes that occur after cardiac injury, and lowering GRK2 restores GPCR levels and preserves cardiac function. However, ongoing research has demonstrated great diversity in the functional roles of GRK2, including phosphorylation of non-GPCR substrates and numerous phosphorylation-independent regulatory binding partners. Many binding sites within GRK2 have been identified for such protein-protein interactions, and several are important for adaptive and maladaptive myocyte growth. However, to date it was not known whether domain-specific interactions with signaling and regulatory molecules could lead to novel targets for HF therapy.

Thus, there is a need in the art for novel approaches for HF therapy, including through the use of GRK2/ β ARK1 based therapies. The present invention addresses this unmet need.

5

SUMMARY OF THE INVENTION

In one aspect, the present invention provides a β ARKnt peptide, a nucleic acid encoding a β ARKnt peptide, or a composition comprising a β ARKnt peptide or nucleic acid encoding a β ARKnt peptide. In one embodiment, the β ARKnt peptide comprises an amino acid sequence at least 90% homologous to SEQ ID NO:1. In one
10 embodiment, the β ARKnt peptide comprises the amino acid sequence of SEQ ID NO:1.

In one embodiment, the nucleic acid encoding a β ARKnt peptide is contained within a vector. In one embodiment, the vector is an AAV vector. In one embodiment, the AAV vector is an AAV6 or AAV9. In one embodiment, the AAV vector comprises an EF1alpha promoter. In one embodiment, the EF1alpha promoter
15 comprises mir122.

In another aspect, the invention provides a method for treating or preventing heart failure in a subject. In one embodiment, the method comprises β ARKnt peptide, a nucleic acid encoding a β ARKnt peptide, or a composition comprising a β ARKnt peptide or nucleic acid encoding a β ARKnt peptide. In one embodiment, the
20 β ARKnt peptide comprises an amino acid sequence at least 90% homologous to SEQ ID NO:1. In one embodiment, the β ARKnt peptide comprises the amino acid sequence of SEQ ID NO:1. In one embodiment, the subject is human.

In another aspect, the invention provides a method for altering β -adrenergic receptor (β AR) density in a subject. In one embodiment, the method
25 comprises β ARKnt peptide, a nucleic acid encoding a β ARKnt peptide, or a composition comprising a β ARKnt peptide or nucleic acid encoding a β ARKnt peptide. In one embodiment, the β ARKnt peptide comprises an amino acid sequence at least 90% homologous to SEQ ID NO:1. In one embodiment, the β ARKnt peptide comprises the amino acid sequence of SEQ ID NO:1.

30 In one embodiment, β AR density is increased. In one embodiment, the subject has low β AR density.

In one embodiment, the method further comprises administering a second therapeutic. In one embodiment, the second therapeutic is a β AR agonist.

BRIEF DESCRIPTION OF THE DRAWINGS

5 The following detailed description of preferred embodiments of the invention will be better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention, there are shown in the drawings embodiments which are presently preferred. It should be understood, however, that the invention is not limited to the precise arrangements and instrumentalities of the
10 embodiments shown in the drawings.

Figure 1, comprising Figure 1A and Figure 1B, depicts that domain-specific interactions with the amino-terminus of GRK2 may reveal novel roles in cardiac signaling and function. Figure 1A depicts a diagram of β ARK1 (GRK2) and Figure 1B depicts a gel confirming the presence of β ARK1 in transgenic animals.

15 Figure 2 depicts a diagram and text outlining the experimental design utilized in example experiments.

Figure 3 depicts a mouse model of cardiac pressure overload using trans-aortic constriction (TAC). ***, $p < 0.0001$ by two-way ANOVA with Bonferroni post-hoc test relative to NLC Sham. $n = 7-26$ mice per group.

20 Figure 4, comprising Figure 4A through Figure 4D, depicts results from example experiments demonstrating that cardiac-specific β ARKnt expression exacerbates left ventricular hypertrophy after TAC. Figure 4A depicts ejection fraction plotted as a function of group (NLC Sham, Tg β ARKnt Sham, NLC TAC, Tg β ARKnt TAC), at 0 and 4 weeks. Figure 4B depicts left ventricular mass plotted as a function of time (data taken
25 at week 0, week 2, and week 4) for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 4C depicts Left Ventricular Posterior Wall diastole (LVPWd, in mm) for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 4D depicts Left Ventricular Posterior Wall systole (LVPWs, in mm) for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). * $P = 0.01$; *** $P <$
30 0.0001 by two-way ANOVA with repeated measures and Bonferroni relative to NLC

Sham. $^{***}P < 0.0001$ by two-way ANOVA with repeated measures and Bonferroni relative to NLC TAC. $n = 9$ to 17 mice per group.

Figure 5, comprising Figure 5A through 5D, depicts results from example experiments demonstrating that increased HW and LAW is similar in Tg β ARKnt and NLC mice 4 weeks after TAC. Figure 5A depicts HW/TL (mg/mm) plotted as a function of group (NLC Sham, Tg β ARKnt Sham, NLC TAC, Tg β ARKnt TAC). Figure 5B depicts % change in HW/TL plotted as a function of group (NLC Sham, Tg β ARKnt Sham, NLC TAC, Tg β ARKnt TAC). Figure 5C depicts left atrial weight plotted as a function of group (NLC Sham, Tg β ARKnt Sham, NLC TAC, Tg β ARKnt TAC). Figure 5D depicts tibia length (mm) plotted as a function of group (NLC Sham, Tg β ARKnt Sham, NLC TAC, Tg β ARKnt TAC). $^{**}P = 0.001$; $^{***}P < 0.0001$ by one-way ANOVA with Tukey relative to NLC Sham. $n = 9$ to 17 mice per group.

Figure 6, comprising Figure 6A through Figure 6C, depicts results from example experiments demonstrating that fetal gene induction is similar in β ARKnt and NLC mice 4 wks post-TAC. Figure 6A depicts ANF (Fold Change) plotted as a function of group (NLC Sham, Tg β ARKnt Sham, NLC TAC, Tg β ARKnt TAC). Figure 6B depicts BNP (Fold Change) plotted as a function of group (NLC Sham, Tg β ARKnt Sham, NLC TAC, Tg β ARKnt TAC). Figure 6C depicts β MHC (Fold Change) plotted as a function of group (NLC Sham, Tg β ARKnt Sham, NLC TAC, Tg β ARKnt TAC). $n = 12, 11, 12, 12$. $^{*}P = 0.01$; $^{***}P < 0.0001$ by nonparametric one-way ANOVA with Dunns post-hoc test.

Figure 7, comprising Figure 7A through Figure 7E, depicts results from example experiments demonstrating that hypertrophy is unaltered in TgGRK2 mice after TAC. Figure 7A depicts a diagram depicting signaling pathways relevant in heart failure. Figure 7B depicts LVPWs (mm) plotted as a function of time (data taken at week 0, week 2, and week 4), for the four groups (NLC Sham, TgGRK2 Sham, NLC TAC, TgGRK2 TAC). Figure 7C depicts LV mass (mg) as a function of time (data taken at week 0, week 2, and week 4), for the four groups (NLC Sham, TgGRK2 Sham, NLC TAC, TgGRK2 TAC). Figure 7D depicts HW/TL (mg/mm) plotted as a function of group (NLC Sham, TgGRK2 Sham, NLC TAC, TgGRK2 TAC). Figure 7E depicts left atrial weight (g) plotted as a function of group (NLC Sham, TgGRK2 Sham, NLC TAC, TgGRK2 TAC).

*P = 0.01; **P = 0.001; ***P < 0.0001 by two-way ANOVA with repeated measures and Bonferroni or one-way ANOVA with Tukey relative to NLC sham. n = 8 to 14 mice per group. See Schumacher SM, et al. Sci. Signal. 22 Mar 2016 Vol. 9, Issue 420, pp. ra30.

Figure 8, comprising Figure 8A through Figure 8E, depicts results from
 5 example experiments demonstrating that hypertrophy is unaltered in Tg β ARKct mice after TAC. Figure 8A depicts relevant signaling pathways. Figure 8B depicts LVPWs (mm) plotted as a function of time (data taken at week 0, week 2, and week 4), for the four groups (NLC Sham, β ARKct Sham, NLC TAC, β ARKct TAC). Figure 8C depicts LV mass (mg) plotted as a function of time (data taken at week 0, week 2, and week 4),
 10 for the four groups (NLC Sham, β ARKct Sham, NLC TAC, β ARKct TAC). Figure 8D depicts HW/TL (mg/mm) plotted as a function of group (NLC Sham, Tg β ARKct Sham, NLC TAC, Tg β ARKct TAC). Figure 8E depicts left atrial weight plotted as a function of group (NLC Sham, Tg β ARKct Sham, NLC TAC, Tg β ARKct TAC). *P = 0.01; **P = 0.001; ***P < 0.0001 by two-way ANOVA with repeated measures and Bonferroni or
 15 one-way ANOVA with Tukey relative to NLC sham. n = 8 to 14 mice per group. See Schumacher SM, et al. Sci. Signal. 22 Mar 2016 Vol. 9, Issue 420, pp. ra30.

Figure 9, comprising Figure 9A through Figure 9D, depicts results from
 example experiments demonstrating that chronic pressure-overload induced HF is
 blocked by cardiac β ARKnt expression. Figure 9A depicts ejection fraction (%) measured
 20 over time for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 9B depicts LV mass (mg) measured over time for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 9C depicts LVPWs (mm) measured over time for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 9D depicts LVPWd (mm) measured over time for the four groups (NLC Sham,
 25 β ARKnt Sham, NLC TAC, β ARKnt TAC). *, p = 0.01; **, p = 0.001; ***, p < 0.0001 by two-way ANOVA with repeated measures and Bonferroni post-hoc test relative to NLC Sham mice. τ , p = 0.01; $\tau\tau$, p = 0.001; $\tau\tau\tau$, p < 0.0001 by two-way ANOVA with repeated measures and Bonferroni post-hoc test relative to NLC TAC mice. n = 9-15 mice per group.

30 Figure 10, comprising Figure 10A through Figure 10D, depicts results from example experiments demonstrating that chronic pressure-overload induced HF is

blocked by cardiac β ARKnt expression. Figure 10A depicts systolic pressure gradient (mm Hg) for the four groups (NLC Sham, Tg β ARKnt Sham, NLC TAC, Tg β ARKnt TAC). Figure 10B depicts HW/TL (mg/mm) for the four groups (NLC Sham, Tg β ARKnt Sham, NLC TAC, Tg β ARKnt TAC). Figure 10C depicts left atrial weight for the four groups (NLC Sham, Tg β ARKnt Sham, NLC TAC, Tg β ARKnt TAC). Figure 10D depicts tibia length (mm) for the four groups (NLC Sham, Tg β ARKnt Sham, NLC TAC, Tg β ARKnt TAC). *, $p = 0.01$; **, $p = 0.001$; ***, $p < 0.0001$ by Kruskal-Wallis nonparametric one-way ANOVA and Dunns post-hoc test relative to NLC Sham mice. $n = 9-15$ mice per group.

Figure 11, comprising Figure 11A through Figure 11C, depicts results from example experiments demonstrating that fetal gene expression is normalized in β ARKnt mice compared to NLC 14 weeks post-TAC. Figure 11A depicts ANF (Fold Change) plotted as a function of group (NLC Sham, Tg β ARKnt Sham, NLC TAC, Tg β ARKnt TAC). Figure 11B depicts BNP (Fold Change) plotted as a function of group (NLC Sham, Tg β ARKnt Sham, NLC TAC, Tg β ARKnt TAC). Figure 11C depicts β MHC (Fold Change) plotted as a function of group (NLC Sham, Tg β ARKnt Sham, NLC TAC, Tg β ARKnt TAC). $n = 9, 11, 13, 12$. * $P = 0.01$; ** $P = 0.001$; *** $P < 0.0001$ by nonparametric one-way ANOVA with Dunns post-hoc test.

Figure 12, comprising Figure 12A through Figure 12D, depicts results from example experiments demonstrating that chronic pressure-overload induced heart failure is inhibited by cardiac β ARKct expression. Figure 12A depicts ejection fraction (%) plotted as a function of time (weeks) for each group (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 12B depicts LV mass (mg) plotted as a function of time (weeks) for each group (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 12C depicts LVIDs (mm) plotted as a function of time (weeks) for each group (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 12D depicts LVPWs (mm) plotted as a function of time (weeks) for each group (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). ** $P = 0.001$; *** $P < 0.0001$ by two-way ANOVA with repeated measures and Bonferroni relative to NLC Sham. $^{\circ}P = 0.01$ by two-way ANOVA with repeated measures and Bonferroni relative to NLC TAC. $n = 9$ to 13 mice per group.

Figure 13, comprising Figure 13A through Figure 13C, depicts results from example experiments demonstrating that chronic pressure-overload induced heart failure is inhibited by cardiac β ARKct expression. Figure 13A depicts HW/TL (mg/mm) plotted as a function of group (NLC Sham, Tg β ARKct Sham, NLC TAC, Tg β ARKct TAC). Figure 13B depicts left atrial weight plotted as a function of group (NLC Sham, Tg β ARKct Sham, NLC TAC, Tg β ARKct TAC). Figure 13C depicts tibia length (mm) plotted as a function of group (NLC Sham, Tg β ARKct Sham, NLC TAC, Tg β ARKct TAC). **P = 0.001; ***P < 0.0001 by one-way ANOVA with Tukey relative to NLC Sham. n = 9 to 13 mice per group.

Figure 14, comprising Figure 14A through Figure 14C, depicts results from example experiments, demonstrating that chronic pressure-overload induced heart failure is inhibited by cardiac β ARKct expression. Figure 14A depicts ANF (Fold Change) plotted as a function of group (NLC Sham, Tg β ARKct Sham, NLC TAC, Tg β ARKct TAC). Figure 14B depicts BNP (Fold Change) plotted as a function of group (NLC Sham, Tg β ARKct Sham, NLC TAC, Tg β ARKct TAC). Figure 14C depicts β MHC (Fold Change) plotted as a function of group (NLC Sham, Tg β ARKct Sham, NLC TAC, Tg β ARKct TAC). *P = 0.01; ***P < 0.0001 by one-way ANOVA with Tukey relative to NLC Sham. n = 9 to 13 mice per group.

Figure 15, comprising Figure 15A through Figure 15D, depicts results from example experiments, demonstrating that cardiac GRK2 overexpression hastens progression to heart failure during chronic pressure-overload. Figure 15A depicts ejection fraction (%) plotted as a function of time (weeks), for each group (NLC Sham, TgGRK2 Sham, NLC TAC, TgGRK2 TAC). Figure 15B depicts LV mass (mg) plotted as a function of time (weeks), for each group (NLC Sham, TgGRK2 Sham, NLC TAC, TgGRK2 TAC). Figure 15C depicts LVIDs (mm) plotted as a function of time (weeks), for each group (NLC Sham, TgGRK2 Sham, NLC TAC, TgGRK2 TAC). Figure 15D depicts LVPWs (mm) plotted as a function of time (weeks), for each group (NLC Sham, TgGRK2 Sham, NLC TAC, TgGRK2 TAC). *P = 0.01 **P = 0.001; ***P < 0.0001 by two-way ANOVA with repeated measures and Bonferroni relative to NLC Sham. n = 9 to 12 mice per group.

Figure 16, comprising Figure 16A through Figure 16C, depicts results from example experiments demonstrating that cardiac GRK2 overexpression hastens progression to heart failure during chronic pressure-overload. Figure 16A depicts HW/TL (mg/mm) plotted as a function of group (NLC Sham, TgGRK2 Sham, NLC TAC, TgGRK2 TAC). Figure 16B depicts left atrial weight plotted as a function of group (NLC Sham, TgGRK2 Sham, NLC TAC, TgGRK2 TAC). Figure 16C depicts tibia length (mm) plotted as a function of group (NLC Sham, TgGRK2 Sham, NLC TAC, TgGRK2 TAC). ***P < 0.0001 by one-way ANOVA with Tukey relative to NLC Sham. n = 9 to 12 mice per group.

Figure 17, comprising Figure 17A through Figure 17C, depicts results from example experiments demonstrating that cardiac GRK2 overexpression hastens progression to heart failure during chronic pressure-overload. Figure 17A depicts HW/TL (mg/mm) plotted as a function of group (NLC Sham, TgGRK2 Sham, NLC TAC, TgGRK2 TAC). Figure 17B depicts left atrial weight (g) plotted as a function of group (NLC Sham, TgGRK2 Sham, NLC TAC, TgGRK2 TAC). Figure 17C depicts tibia length (mm) plotted as a function of group (NLC Sham, TgGRK2 Sham, NLC TAC, TgGRK2 TAC). ***P < 0.0001 by one-way ANOVA with Tukey relative to NLC Sham. n = 9 to 12 mice per group.

Figure 18, comprising Figure 18A through Figure 18E, depicts results from example experiments demonstrating that the β ARKnt peptide does not interact with endogenous G α q or GRK2 in the heart. Figure 18A depicts immunoprecipitation using anti-FLAG antibody, followed by western blot probed with anti-G α q antibody. Figure 18B depicts SDS-PAGE analysis, showing pre-IP, post-FLAG-IP, and post-Ms-IgG-IP for TG Sham and TG TAC groups. Figure 18C depicts β ARKnt-flag/GAPDH for the three groups pre-IP, FLAG-IP, and Ms-IgG-IP (n=8). Figure 18D depicts immunoprecipitated (IP'd) β ARKnt-FLAG for sham and TAC groups (n=8). Figure 18E depicts G α q/GAPDH quantitation for the three groups pre-IP, FLAG-IP, and Ms-IgG-IP (n=8). ***, p < 0.0001 by nonparametric one-way ANOVA with Dunns relative to the Pre-immunoprecipitation control. n = 8 mice per group.

Figure 19, comprising Figure 19A and Figure 19B, depicts results from example experiments, demonstrating that no significant difference between NLC and

Tg β ARKnt IP₃ levels 4 weeks after Sham or TAC. Figure 19A depicts a signaling pathway (GqGPCRs). Figure 19B depicts IP₃ levels (pg/mL) for each of the four groups (NLC Sham, Tg β ARKnt Sham, NLC TAC, Tg β ARKnt TAC). ***, $p < 0.0001$ by one-way ANOVA with Tukey or Bonferroni post-hoc test. $n = 12, 6, 14, 10$ respectively.

5 Figure 20, comprising Figure 20A and Figure 20B, depicts results from example experiments demonstrating that β Adrenergic Receptor (β AR) is similar in β ARKnt and NLC mice 4 weeks post-TAC. Figure 20A depicts β_1 AR (Fold Change) plotted as a function of group (NLC Sham, Tg β ARKnt Sham, NLC TAC, Tg β ARKnt TAC). Figure 20B depicts β_2 AR (Fold Change) plotted as a function of group (NLC Sham, Tg β ARKnt Sham, NLC TAC, Tg β ARKnt TAC). $n = 12, 11, 12, 12$. * $P = 0.01$ by nonparametric one-way ANOVA with Dunns post-hoc test.

15 Figure 21, comprising Figure 21A and Figure 21B, depicts results from example experiments demonstrating that there is no significant difference in β_1 AR or β_2 AR mRNA expression in β ARKnt or NLC mice 14 weeks post-TAC. Figure 21A depicts β_1 AR (Fold Change) plotted as a function of group (NLC Sham, Tg β ARKnt Sham, NLC TAC, Tg β ARKnt TAC). Figure 21B depicts β_2 AR (Fold Change) plotted as a function of group (NLC Sham, Tg β ARKnt Sham, NLC TAC, Tg β ARKnt TAC). $n = 9, 11, 13, 12$.

20 Figure 22 depicts results from example experiments, demonstrating β AR density is significantly reduced in NLC mice 4 weeks post-TAC. Figure 22 depicts $B_{\max}$ (fmol/mg protein) plotted as a function of group (NLC Sham, Tg β ARKnt Sham, NLC TAC, Tg β ARKnt TAC). $n = 7, 8, 11, 12$. * $P = 0.01$ by nonparametric one-way ANOVA with Dunns post-hoc test.

25 Figure 23, comprising Figure 23A through Figure 23C, depicts results from example experiments demonstrating RT-PCR of angiogenesis markers in NLC and Tg β ARKnt mice 4 weeks post-TAC. Figure 23A depicts FGF5 (Fold Change) plotted as a function of group (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 23B depicts VE-Cadherin (Fold Change) plotted as a function of group (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 23C depicts eNOS (Fold Change) plotted as a function of group (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). $n = 8, 7, 11, 10$ each.

Figure 24, comprising Figure 24A through Figure 24D, depicts results from example experiments demonstrating RT-PCR of angiogenesis markers in NLC and Tg β ARKnt mice 4 weeks post-TAC. Figure 24A depicts VEGF A (Fold Change) plotted as a function of group (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 24B depicts CD31 (Fold Change) plotted as a function of group (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 24C depicts HIF1 α (Fold Change) plotted as a function of group (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 24D depicts VEGF R1 (Fold Change) plotted as a function of group (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). n = 8, 7, 11, 10 each. *P = 0.01; **P = 0.001; by nonparametric one-way ANOVA with Dunns post-hoc test.

Figure 25, comprising Figure 25A through 25C, depicts results from example experiments demonstrating RT-PCR of fibrosis markers in NLC and Tg β ARKnt mice 4 weeks post-TAC. Figure 25A depicts Col I (Fold Change) plotted as a function of group (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 25B depicts Col III (Fold Change) plotted as a function of group (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 25C depicts α SMA (Fold Change) plotted as a function of group (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). n = 11 per group. **P = 0.001; ***P < 0.0001 by nonparametric one-way ANOVA with Dunns post-hoc test.

Figure 26, comprising Figure 26A through Figure 26D, depicts results from example experiments demonstrating RT-PCR of fibrosis markers in NLC and Tg β ARKnt mice 4 weeks post-TAC. Figure 26A depicts MMP2 (Fold Change) plotted as a function of group (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 26B depicts MMP9 (Fold Change) plotted as a function of group (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 26C depicts TGF β (Fold Change) plotted as a function of group (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 26D depicts Osteopontin (Fold Change) plotted as a function of group (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). n = 11 per group. **P = 0.001; ***P < 0.0001 by nonparametric one-way ANOVA with Dunns post-hoc test.

Figure 27, comprising Figure 27A through Figure 27C, depicts results from example experiments demonstrating phospho-Akt is increased in β ARKnt mice compared to NLC. Figure 27A depicts an immunoblot for phospho-Akt for the four

groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 27B depicts an immunoblot for total Akt for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 27C depicts the ratio of phospho-Akt to total Akt for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC).

5 Figure 28, comprising Figure 28A through Figure 28D, depicts results from example experiments demonstrating there is no difference in phospho and total I κ B in β ARKnt versus NLC mice 4 weeks post-TAC. Figure 28A depicts the ratio of phospho-I κ B to total I κ B in the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 28B depicts the ratio of phospho-I κ B to GAPDH in the four
10 groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 28C depicts the ratio of I κ B to GAPDH in the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 28D depicts the ratio of phospho-I κ B/I κ B to GAPDH for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC).

 Figure 29, comprising Figure 29A through Figure 29D, depicts results
15 from example experiments demonstrating an increase in total p65 (NF κ B) and decrease in phospho/total p65 in β ARKnt versus NLC mice 4 weeks post-TAC. Figure 29A depicts the ratio of phospho-p65 to total p65 for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 29B depicts the ratio of phospho-p65 to GAPDH for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 29C
20 depicts the ratio of p65 to GAPDH for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 29D depicts the ratio of phospho-p65/total p65 to GAPDH for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC).

 Figure 30, comprising Figure 30A through Figure 30D, depicts results from example experiments, demonstrating an increase in phospho/total Akt in β ARKnt
25 versus NLC mice 4 weeks post-TAC. Figure 30A depicts the ratio of phospho-Akt to total Akt for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 30B depicts the ratio of phospho-Akt to GAPDH for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 30C depicts the ratio of Akt to GAPDH for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC).
30 Figure 30D depicts the ratio of phospho-Akt/total Akt to GAPDH for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC).

Figure 31, comprising Figure 31A through Figure 31D, depicts results from example experiments demonstrating an increase in phospho/total GSK3 β in β ARKnt Sham mice 4 weeks post-TAC. Figure 31A depicts the ratio of phospho-GSK3 β to total GSK3 β for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 31B depicts the ratio of phospho-GSK3 β to GAPDH for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 31C depicts the ratio of GSK3 β to GAPDH for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Figure 31D depicts the ratio of phospho-GSK3 β /total GSK3 β to GAPDH for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC).

Figure 32 depicts results from example experiments, demonstrating a trend towards an increase in GRK2/GAPDH in β ARKnt versus NLC mice 4 weeks post-TAC. Figure 32 depicts the ratio of GRK2 to GAPDH for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC). Bottom panel: *P = 0.01 by nonparametric one-way ANOVA with Dunns post-hoc test relative to NLC Sham. n = 6 mice per group for signaling.

Figure 33, comprising Figure 33A and Figure 33B, depicts results from example experiments demonstrating no change in AP2 levels in Tg β ARKnt or NLC Sham and TAC mice at 4 weeks. Figure 33A depicts results from a gel electrophoresis experiment, showing AP2 levels for the four groups (NLC Sham, TG Sham, NLC TAC, TG TAC). Figure 33B depicts the ratio of AP2 to GAPDH for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC).

Figure 34, comprising Figure 34A and Figure 34B, depicts results from example experiments demonstrating an increase in clathrin in Tg β ARKnt Sham and TAC and NLC TAC mice at 4 weeks. Figure 34A depicts results from a gel electrophoresis experiment, showing clathrin levels for the four groups (NLC Sham, TG Sham, NLC TAC, TG TAC). Figure 34B depicts the ratio of clathrin to GAPDH for the four groups (NLC Sham, β ARKnt Sham, NLC TAC, β ARKnt TAC).

Figure 35, comprising Figure 35A and Figure 35B, depicts results from example experiments demonstrating a decrease in Caveolin 3 in Tg β ARKnt Sham and TAC and NLC TAC mice at 4 weeks. Figure 35A depicts results from a gel electrophoresis experiment, showing caveolin 3 levels for the four groups (NLC Sham,

TG Sham, NLC TAC, TG TAC). Figure 35B depicts the ratio of caveolin 3 to GAPDH for the four groups (NLC Sham, TG Sham, NLC TAC, TG TAC).

Figure 36, comprising Figure 36A through Figure 36D, depicts results from example experiments demonstrating cardiac-specific β ARKnt expression does not alter hemodynamic function. Figure 36A depicts systemic pressure for NLC and Tg β ARKnt groups. Figure 36B depicts mean heart rate (bpm) as a function of isoproterenol (ng) dose for NLC and Tg β ARKnt groups. Figure 36C depicts (+) dP/dt (mm Hg per second) as a function of isoproterenol (ng) dose for NLC and Tg β ARKnt groups. Figure 36D depicts (-) dP/dt (mm Hg per second) as a function of isoproterenol (ng) dose for NLC and Tg β ARKnt groups. n = 10 NLC, 9 Tg β ARKnt.

Figure 37, comprising Figure 37A through Figure 37E, depicts results from example experiments demonstrating cardiomyocyte cross-sectional area is enhanced in Tg β ARKnt Sham mice, but not after TAC. Cross-sectional area was measured in NLC Sham (Figure 37A), NLC TAC (Figure 37B), β ARKnt Sham (Figure 37C), and β ARKnt TAC (Figure 37D), and quantified in a graph (Figure 37E). ***, p < 0.0001 by one-way ANOVA with Bonferroni post-hoc test relative to corresponding NLC Sham. n = 5-10 hearts per group, 40 images per heart.

Figure 38 depicts results from example experiments demonstrating left ventricular structural remodeling is limited in Tg β ARKnt mice 4 weeks after TAC.

Figure 39 depicts results from example experiments demonstrating left ventricular structural remodeling is limited in Tg β ARKnt mice 4 weeks after TAC.

Figure 40 depicts results from example experiments demonstrating Cardiac signaling is altered in Tg β ARKnt mice compared to NLC 4 weeks after TAC. *P = 0.01, **P = 0.001; ***P < 0.0001 by one-way ANOVA with Bonferroni post-hoc test relative to NLC Sham. n = 6-10 mice per group.

DETAILED DESCRIPTION

The present invention is based on the discovery of a novel peptide, β ARKnt for the treatment or prevention of heart failure. β ARKnt can be delivered as a peptide or as a nucleic acid. In some embodiments, a nucleic acid encoding β ARKnt, a variant thereof or a fragment thereof is contained within an AAV vector. An AAV6 or

AAV9 vector may be used for cardiac selective expression of the peptide following systemic injection.

Thus, the present invention provides compositions comprising a β ARKnt peptide or a nucleic acid encoding a β ARKnt peptide. In some embodiments, the nucleic acid encoding a β ARKnt peptide is contained within an AAV6 or AAV9 vector.

The present invention also provides methods for treating or preventing a cardiovascular disease or disorder, such as heart failure, in a subject in need thereof by administering a peptide, or nucleic acid of the invention or composition comprising a peptide, or nucleic acid of the invention to the subject. In some embodiments, the methods of the invention increase β AR density, thereby treating or preventing cardiovascular diseases and disorders.

The present invention also provides methods for altering β AR density, in a subject in need thereof by administering a peptide, or nucleic acid of the invention or composition comprising a peptide, or nucleic acid of the invention to the subject. In some embodiments, the methods of the invention increase β AR density.

Definitions

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 the invention pertains. Although any methods and materials similar or equivalent to those described herein can be used in the practice of and/or for the testing of the present invention, the preferred materials and methods are described herein. In describing and claiming the present invention, the following terminology will be used according to how it is defined, where a definition is provided.

It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

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 the invention pertains. Although any methods and materials similar or equivalent to those described herein can be used in the practice of and/or for the testing of the present invention, the preferred materials and methods are described herein. In describing

and claiming the present invention, the following terminology will be used according to how it is defined, where a definition is provided.

It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

5 The articles “a” and “an” are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

 “About” as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of $\pm 20\%$ or
10 in some instances $\pm 10\%$, or in some instances $\pm 5\%$, or in some instances $\pm 1\%$, or in some instances $\pm 0.1\%$ from the specified value, as such variations are appropriate to perform the disclosed methods.

 A “disease” is a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal’s
15 health continues to deteriorate.

 In contrast, a “disorder” in an animal is a state of health in which the animal is able to maintain homeostasis, but in which the animal’s state of health is less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the animal’s state of health.

20 A disease or disorder is “alleviated” if the severity of a sign or symptom of the disease or disorder, the frequency with which such a sign or symptom is experienced by a patient, or both, is reduced.

 “Encoding” refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as
25 templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the
30 nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a

gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

“Expression vector” refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in an in vitro expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (e.g., naked or contained in liposomes) and viruses (e.g., lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

“Homologous” refers to the sequence similarity or sequence identity between two polypeptides or between two nucleic acid molecules. When a position in both of the two compared sequences is occupied by the same base or amino acid monomer subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then the molecules are homologous at that position. The percent of homology between two sequences is a function of the number of matching or homologous positions shared by the two sequences divided by the number of positions compared X 100. For example, if 6 of 10 of the positions in two sequences are matched or homologous then the two sequences are 60% homologous. By way of example, the DNA sequences ATTGCC and TATGGC share 50% homology. Generally, a comparison is made when two sequences are aligned to give maximum homology.

“Isolated” means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in a living animal is not “isolated,” but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural state is “isolated.” An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell.

In the context of the present invention, the following abbreviations for the commonly occurring nucleic acid bases are used. “A” refers to adenosine, “C” refers to cytosine, “G” refers to guanosine, “T” refers to thymidine, and “U” refers to uridine.

Unless otherwise specified, a “nucleotide sequence encoding an amino

acid sequence” includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. The phrase nucleotide sequence that encodes a protein or an RNA may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain an intron(s).

5 The terms “patient,” “subject,” “individual,” and the like are used interchangeably herein, and refer to any animal, or cells thereof whether in vitro or in situ, amenable to the methods described herein. In some embodiments, the patient, subject or individual is a mammal such as a non-primate (e.g., cows, pigs, horses, cats, dogs, rats, etc.) and a primate (e.g., monkey and human), most preferably a human. In
10 certain non-limiting embodiments, the patient, subject or individual is a human.

 The term “polynucleotide” as used herein is defined as a chain of nucleotides. Furthermore, nucleic acids are polymers of nucleotides. Thus, nucleic acids and polynucleotides as used herein are interchangeable. One skilled in the art has the general knowledge that nucleic acids are polynucleotides, which can be hydrolyzed into
15 the monomeric “nucleotides.” The monomeric nucleotides can be hydrolyzed into nucleosides. As used herein polynucleotides include, but are not limited to, all nucleic acid sequences which are obtained by any means available in the art, including, without limitation, recombinant means, i.e., the cloning of nucleic acid sequences from a recombinant library or a cell genome, using ordinary cloning technology and PCR™, and
20 the like, and by synthetic means.

 Unless otherwise specified, a “nucleotide sequence encoding an amino acid sequence” includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. The phrase nucleotide sequence that encodes a protein or an RNA may also include introns to the extent that the nucleotide
25 sequence encoding the protein may in some version contain an intron(s).

 As used herein, the terms “peptide,” “polypeptide,” and “protein” are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can comprise a
30 protein’s or peptide’s sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term

refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. "Polypeptides" include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or a combination thereof.

The term "promoter" as used herein is defined as a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a polynucleotide sequence.

As used herein, the term "promoter/regulatory sequence" means a nucleic acid sequence which is required for expression of a gene product operably linked to the promoter/regulatory sequence. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are required for expression of the gene product. The promoter/regulatory sequence may, for example, be one which expresses the gene product in a tissue specific manner.

As used herein, a "peptidomimetic" is a compound containing non-peptidic structural elements that is capable of mimicking the biological action of a parent peptide. A peptidomimetic may or may not comprise peptide bonds.

A "therapeutic" treatment is a treatment administered to a subject who exhibits signs of pathology, for the purpose of diminishing or eliminating those signs.

As used herein, "treating a disease or disorder" means reducing the frequency with which a symptom of the disease or disorder is experienced by a patient. Disease and disorder are used interchangeably herein.

The phrase "therapeutically effective amount," as used herein, refers to an amount that is sufficient or effective to prevent or treat (delay or prevent the onset of, prevent the progression of, inhibit, decrease or reverse) a disease or condition, including alleviating symptoms of such diseases.

To “treat” a disease as the term is used herein, means to reduce the frequency or severity of at least one sign or symptom of a disease or disorder experienced by a subject.

A “vector” is a composition of matter which comprises an isolated nucleic acid and which can be used to deliver the isolated nucleic acid to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term “vector” includes an autonomously replicating plasmid or a virus. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds, liposomes, and the like. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, and the like.

Ranges: throughout this disclosure, various aspects of the invention can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range.

Description

The present invention is based, in part, on the discovery that a fragment of the G protein-coupled receptor (GPCR) kinase (GRK) GRK2 terminal Regulator of G protein Signaling (RGS) domain (β ARKnt) regulates β -adrenergic receptor (β AR) density and provides cardio-protection during pressure-overload induced heart failure (HF).

Thus, the present invention provides compositions comprising a β ARKnt peptide or a nucleic acid encoding a β ARKnt peptide. In some embodiments, the nucleic acid encoding a β ARKnt peptide is contained within an AAV6 or AAV9 vector.

5 The present invention also provides methods for treating or preventing a cardiovascular disease or disorder, such as heart failure, in a subject in need thereof by administering a β ARKnt peptide or a nucleic acid encoding a β ARKnt peptide or composition comprising a β ARKnt peptide or a nucleic acid encoding a β ARKnt peptide to the subject. In some embodiments, the methods of the invention increase β AR density, thereby treating or preventing cardiovascular diseases and disorders.

10 The present invention also provides methods for altering β AR density, in a subject in need thereof by administering a β ARKnt peptide or a nucleic acid encoding a β ARKnt peptide or composition comprising a β ARKnt peptide or a nucleic acid encoding a β ARKnt peptide to the subject. In some embodiments, the methods of the invention increase β AR density.

15

Peptides

In one aspect, the present invention provides isolated peptides and compositions for treating heart failure. For example, in certain instances the compositions improve regulate β AR density. In some instances, the compositions increase β AR density and prevent progression to HF.

20 In one embodiment, the composition comprises isolated peptides derived from the GRK2-RGS domain. In one embodiment, the composition comprises a β ARKnt peptide, or a derivative or fragment thereof. In one embodiment, the isolated peptide comprises an amino acid sequence of

25 RGEVTFEKIFSQKLG YLLFRDFCLKHLEEAKPLVEFYEEIKKYEKLETEEERLVCS
REIFDTYIMKELLACSHPFKSAIEHVQGHLVKKQVPPDL (SEQ ID NO:1), a
derivative thereof or a fragment thereof.

The peptide of the present invention may be made using chemical methods. For example, peptides can be synthesized by solid phase techniques (Roberge J
30 Y et al (1995) Science 269: 202-204), cleaved from the resin, and purified by preparative high performance liquid chromatography. Automated synthesis may be achieved, for

example, using the ABI 431 A Peptide Synthesizer (Perkin Elmer) in accordance with the instructions provided by the manufacturer.

The invention should also be construed to include any form of a peptide having substantial homology to SEQ ID NO: 1. Preferably, a peptide which is

5 “substantially homologous” is about 50% homologous, more preferably about 70% homologous, even more preferably about 80% homologous, more preferably about 90% homologous, even more preferably, about 95% homologous, and even more preferably about 99% homologous to SEQ ID NO: 1.

The peptide may alternatively be made by recombinant means or by

10 cleavage from a longer polypeptide. The composition of a peptide may be confirmed by amino acid analysis or sequencing.

The variants of the peptides according to the present invention may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such

15 substituted amino acid residue may or may not be one encoded by the genetic code, (ii) one in which there are one or more modified amino acid residues, e.g., residues that are modified by the attachment of substituent groups, (iii) one in which the peptide is an alternative splice variant of the peptide of the present invention, (iv) fragments of the peptides and/or (v) one in which the peptide is fused with another peptide, such as a

20 leader or secretory sequence or a sequence which is employed for purification (for example, His-tag) or for detection (for example, Sv5 epitope tag). The fragments include peptides generated via proteolytic cleavage (including multi-site proteolysis) of an original sequence. Variants may be post-translationally, or chemically modified. Such variants are deemed to be within the scope of those skilled in the art from the teaching

25 herein.

As known in the art the “similarity” between two peptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to a sequence of a second polypeptide. Variants are defined to include peptide sequences different from the original sequence, preferably different from the

30 original sequence in less than 40% of residues per segment of interest, more preferably different from the original sequence in less than 25% of residues per segment of interest,

more preferably different by less than 10% of residues per segment of interest, most preferably different from the original protein sequence in just a few residues per segment of interest and at the same time sufficiently homologous to the original sequence to preserve the functionality of the original sequence and/or the ability to stimulate the
5 differentiation of a stem cell into the osteoblast lineage. The present invention includes amino acid sequences that are at least 60%, 65%, 70%, 72%, 74%, 76%, 78%, 80%, 90%, or 95% similar or identical to the original amino acid sequence. The degree of identity between two peptides is determined using computer algorithms and methods that are widely known for the persons skilled in the art. The identity between two amino acid
10 sequences is preferably determined by using the BLASTP algorithm [BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, Md. 20894, Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990)].

The peptides of the invention can be post-translationally modified. For example, post-translational modifications that fall within the scope of the present
15 invention include signal peptide cleavage, glycosylation, acetylation, isoprenylation, proteolysis, myristoylation, protein folding and proteolytic processing, etc. Some modifications or processing events require introduction of additional biological machinery. For example, processing events, such as signal peptide cleavage and core glycosylation, are examined by adding canine microsomal membranes or *Xenopus* egg
20 extracts (U.S. Pat. No. 6,103,489) to a standard translation reaction.

The peptides of the invention may include unnatural amino acids formed by post-translational modification or by introducing unnatural amino acids during translation. A variety of approaches are available for introducing unnatural amino acids during protein translation.

25 A peptide or protein of the invention may be conjugated with other molecules, such as proteins, to prepare fusion proteins. This may be accomplished, for example, by the synthesis of N-terminal or C-terminal fusion proteins provided that the resulting fusion protein retains the functionality of the β ARKnt peptide.

A peptide or protein of the invention may be phosphorylated using
30 conventional methods such as the method described in Reedijk et al. (The EMBO Journal 11(4):1365, 1992).

Cyclic derivatives of the peptides of the invention are also part of the present invention. Cyclization may allow the peptide to assume a more favorable conformation for association with other molecules. Cyclization may be achieved using techniques known in the art. For example, disulfide bonds may be formed between two appropriately spaced components having free sulfhydryl groups, or an amide bond may be formed between an amino group of one component and a carboxyl group of another component. Cyclization may also be achieved using an azobenzene-containing amino acid as described by Ulysse, L., et al., J. Am. Chem. Soc. 1995, 117, 8466-8467. The components that form the bonds may be side chains of amino acids, non-amino acid components or a combination of the two. In an embodiment of the invention, cyclic peptides may comprise a beta-turn in the right position. Beta-turns may be introduced into the peptides of the invention by adding the amino acids Pro-Gly at the right position.

It may be desirable to produce a cyclic peptide which is more flexible than the cyclic peptides containing peptide bond linkages as described above. A more flexible peptide may be prepared by introducing cysteines at the right and left position of the peptide and forming a disulphide bridge between the two cysteines. The two cysteines are arranged so as not to deform the beta-sheet and turn. The peptide is more flexible as a result of the length of the disulfide linkage and the smaller number of hydrogen bonds in the beta-sheet portion. The relative flexibility of a cyclic peptide can be determined by molecular dynamics simulations.

The invention also relates to peptides comprising a β ARKnt peptide fused to, or integrated into, a target protein, and/or a targeting domain capable of directing the chimeric protein to a desired cellular component or cell type or tissue. The chimeric proteins may also contain additional amino acid sequences or domains. The chimeric proteins are recombinant in the sense that the various components are from different sources, and as such are not found together in nature (i.e., are heterologous).

In one embodiment, the targeting domain can be a membrane spanning domain, a membrane binding domain, or a sequence directing the protein to associate with for example vesicles or with the nucleus. In one embodiment, the targeting domain can target a peptide to a particular cell type or tissue. For example, the targeting domain can be a cell surface ligand or an antibody against cell surface antigens of a target tissue

(e.g., bone, regenerating bone, degenerating bone, cartilage). A targeting domain may target the peptide of the invention to a cellular component.

A peptide of the invention may be synthesized by conventional techniques. For example, the peptides or chimeric proteins may be synthesized by chemical synthesis using solid phase peptide synthesis. These methods employ either solid or solution phase synthesis methods (see for example, J. M. Stewart, and J. D. Young, Solid Phase Peptide Synthesis, 2nd Ed., Pierce Chemical Co., Rockford Ill. (1984) and G. Barany and R. B. Merrifield, The Peptides: Analysis Synthesis, Biology editors E. Gross and J. Meienhofer Vol. 2 Academic Press, New York, 1980, pp. 3-254 for solid phase synthesis techniques; and M Bodansky, Principles of Peptide Synthesis, Springer-Verlag, Berlin 1984, and E. Gross and J. Meienhofer, Eds., The Peptides: Analysis, Synthesis, Biology, supra, Vol 1, for classical solution synthesis). By way of example, a peptide of the invention may be synthesized using 9-fluorenyl methoxycarbonyl (Fmoc) solid phase chemistry with direct incorporation of phosphothreonine as the N-fluorenylmethoxy-carbonyl-O-benzyl-L-phosphothreonine derivative.

N-terminal or C-terminal fusion proteins comprising a peptide or chimeric protein of the invention conjugated with other molecules may be prepared by fusing, through recombinant techniques, the N-terminal or C-terminal of the peptide or chimeric protein, and the sequence of a selected protein or selectable marker with a desired biological function. The resultant fusion proteins contain a peptide comprising a β ARKnt peptide fused to the selected protein or marker protein as described herein. Examples of proteins which may be used to prepare fusion proteins include immunoglobulins, glutathione-S-transferase (GST), hemagglutinin (HA), and truncated myc.

Peptides of the invention may be developed using a biological expression system. The use of these systems allows the production of large libraries of random peptide sequences and the screening of these libraries for peptide sequences that bind to particular proteins. Libraries may be produced by cloning synthetic DNA that encodes random peptide sequences into appropriate expression vectors (see Christian et al 1992, J. Mol. Biol. 227:711; Devlin et al, 1990 Science 249:404; Cwirla et al 1990, Proc. Natl.

Acad, Sci. USA, 87:6378). Libraries may also be constructed by concurrent synthesis of overlapping peptides (see U.S. Pat. No. 4,708,871).

The peptides and chimeric proteins of the invention may be converted into pharmaceutical salts by reacting with inorganic acids such as hydrochloric acid, sulfuric acid, hydrobromic acid, phosphoric acid, etc., or organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, succinic acid, malic acid, tartaric acid, citric acid, benzoic acid, salicylic acid, benzenesulfonic acid, and toluenesulfonic acids.

10 Mimetics

In one embodiment, the present invention provides a β ARKnt peptidomimetics. Peptidomimetics are compounds based on, or derived from, peptides and proteins. The peptidomimetics of the present invention typically can be obtained by structural modification of a β ARKnt peptide using unnatural amino acids, conformational restraints, isosteric replacement, and the like. The subject peptidomimetics constitute the continuum of structural space between peptides and non-peptide synthetic structures; peptidomimetics may be useful, therefore, in delineating pharmacophores and in helping to translate peptides into nonpeptide compounds with the activity of the parent peptide.

Moreover, as is apparent from the present disclosure, mimetopes of the subject β ARKnt peptide can be provided. Such peptidomimetics can have such attributes as being non-hydrolyzable (e.g., increased stability against proteases or other physiological conditions which degrade the corresponding peptide), increased specificity and/or potency, and increased cell permeability for intracellular localization of the peptidomimetic. For illustrative purposes, peptide analogs of the present invention can be generated using, for example, benzodiazepines (e.g., see Freidinger et al. in *Peptides: Chemistry and Biology*, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey et al. in *Peptides: Chemistry and Biology*, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988, p123), C-7 mimics (Huffman et al. in *Peptides: Chemistry and Biology*, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988, p. 105), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med Chem* 29:295; and Ewenson et al. in *Peptides: Structure and Function*

(Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, Ill., 1985), β -turn dipeptide cores (Nagai et al. (1985) Tetrahedron Lett 26:647; and Sato et al. (1986) J Chem Soc Perkin Trans 1:1231), β -aminoalcohols (Gordon et al. (1985) Biochem Biophys Res Commun 126:419; and Dann et al. (1986) Biochem Biophys Res Commun 134:71), diaminoketones (Natarajan et al. (1984) Biochem Biophys Res Commun 124:141), and methyleneamino-modified (Roark et al. in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988, p134). Also, see generally, Session III: Analytic and synthetic methods, in in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988)

In addition to a variety of side chain replacements which can be carried out to generate the peptidomimetics, the present invention specifically contemplates the use of conformationally restrained mimics of peptide secondary structure. Numerous surrogates have been developed for the amide bond of peptides. Frequently exploited surrogates for the amide bond include the following groups (i) trans-olefins, (ii) fluoroalkene, (iii) methyleneamino, (iv) phosphoramides, and (v) sulfonamides.

Moreover, other examples of mimetopes include, but are not limited to, protein-based compounds, carbohydrate-based compounds, lipid-based compounds, nucleic acid-based compounds, natural organic compounds, synthetically derived organic compounds, anti-idiotypic antibodies and/or catalytic antibodies, or fragments thereof. A mimetope can be obtained by, for example, screening libraries of natural and synthetic compounds for compounds capable of binding to the β ARKnt peptide. A mimetope can also be obtained, for example, from libraries of natural and synthetic compounds, in particular, chemical or combinatorial libraries (i.e., libraries of compounds that differ in sequence or size but that have the same building blocks). A mimetope can also be obtained by, for example, rational drug design. In a rational drug design procedure, the three-dimensional structure of a compound of the present invention can be analyzed by, for example, nuclear magnetic resonance (NMR) or x-ray crystallography. The three-dimensional structure can then be used to predict structures of potential mimetopes by, for example, computer modelling, the predicted mimetope structures can then be

produced by, for example, chemical synthesis, recombinant DNA technology, or by isolating a mimetope from a natural source (e.g., plants, animals, bacteria and fungi).

In one embodiment, the β ARKnt mimetic is a small molecule. When the β ARKnt mimetic is a small molecule, a small molecule may be obtained using standard methods known to the skilled artisan. Such methods include chemical organic synthesis or biological means. Biological means include purification from a biological source, recombinant synthesis and in vitro translation systems, using methods well known in the art. In one embodiment, a small molecule β ARKnt mimetic of the invention comprises an organic molecule, inorganic molecule, biomolecule, synthetic molecule, and the like.

Nucleic Acids

In one embodiment, the present invention provides a composition comprising an isolated nucleic acid encoding a β ARKnt peptide, a fragment thereof or a derivative thereof. For example, in one embodiment, the composition comprises an isolated nucleic acid encoding SEQ ID NO:1, or a variant or fragment thereof.

Further, the invention encompasses an isolated nucleic acid encoding a peptide having substantial homology to SEQ ID NO:1. In certain embodiments, the isolated nucleic acid sequence encodes a peptide having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence homology with SEQ ID NO:1.

The isolated nucleic acid sequence encoding a β ARKnt peptide can be obtained using any of the many recombinant methods known in the art, such as, for example by screening libraries from cells expressing the gene, by deriving the gene from a vector known to include the same, or by isolating directly from cells and tissues containing the same, using standard techniques. Alternatively, the gene of interest can be produced synthetically, rather than cloned.

The isolated nucleic acid may comprise any type of nucleic acid, including, but not limited to DNA and RNA. For example, in one embodiment, the composition comprises an isolated DNA molecule, including for example, an isolated cDNA molecule, encoding a β ARKnt peptide, or functional variant thereof. In one

embodiment, the composition comprises an isolated RNA molecule encoding a β ARKnt peptide, or a functional variant thereof.

The nucleic acid molecules of the present invention can be modified to improve stability in serum or in growth medium for cell cultures. Modifications can be added to enhance stability, functionality, and/or specificity and to minimize immuno-stimulatory properties of the nucleic acid molecule of the invention. For example, in order to enhance the stability, the 3'-residues may be stabilized against degradation, e.g., they may be selected such that they consist of purine nucleotides, particularly adenosine or guanosine nucleotides. Alternatively, substitution of pyrimidine nucleotides by modified analogues, e.g., substitution of uridine by 2'-deoxythymidine is tolerated and does not affect function of the molecule.

In one embodiment of the present invention the nucleic acid molecule may contain at least one modified nucleotide analogue. For example, the ends may be stabilized by incorporating modified nucleotide analogues.

Non-limiting examples of nucleotide analogues include sugar- and/or backbone-modified ribonucleotides (i.e., include modifications to the phosphate-sugar backbone). For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. In preferred backbone-modified ribonucleotides the phosphoester group connecting to adjacent ribonucleotides is replaced by a modified group, e.g., of phosphothioate group. In preferred sugar-modified ribonucleotides, the 2' OH-group is replaced by a group selected from H, OR, R, halo, SH, SR, NH₂, NHR, NR₂ or ON, wherein R is C₁-C₆ alkyl, alkenyl or alkynyl and halo is F, Cl, Br or I.

Other examples of modifications are nucleobase-modified ribonucleotides, i.e., ribonucleotides, containing at least one non-naturally occurring nucleobase instead of a naturally occurring nucleobase. Bases may be modified to block the activity of adenosine deaminase. Exemplary modified nucleobases include, but are not limited to, uridine and/or cytidine modified at the 5-position, e.g., 5-(2-amino)propyl uridine, 5-bromo uridine; adenosine and/or guanosines modified at the 8 position, e.g., 8-bromo guanosine; deaza nucleotides, e.g., 7-deaza-adenosine; O- and N-alkylated nucleotides,

e.g., N6-methyl adenosine are suitable. It should be noted that the above modifications may be combined.

In some instances, the nucleic acid molecule comprises at least one of the following chemical modifications: 2'-H, 2'-O-methyl, or 2'-OH modification of one or more nucleotides. In certain embodiments, a nucleic acid molecule of the invention can have enhanced resistance to nucleases. For increased nuclease resistance, a nucleic acid molecule, can include, for example, 2'-modified ribose units and/or phosphorothioate linkages. For example, the 2' hydroxyl group (OH) can be modified or replaced with a number of different "oxy" or "deoxy" substituents. For increased nuclease resistance the nucleic acid molecules of the invention can include 2'-O-methyl, 2'-fluorine, 2'-O-methoxyethyl, 2'-O-aminopropyl, 2'-amino, and/or phosphorothioate linkages. Inclusion of locked nucleic acids (LNA), ethylene nucleic acids (ENA), e.g., 2'-4'-ethylene-bridged nucleic acids, and certain nucleobase modifications such as 2-amino-A, 2-thio (e.g., 2-thio-U), G-clamp modifications, can also increase binding affinity to a target.

In one embodiment, the nucleic acid molecule includes a 2'-modified nucleotide, e.g., a 2'-deoxy, 2'-deoxy-2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), 2'-O-dimethylaminoethoxyethyl (2'-O-DMAEOE), or 2'-O-N-methylacetamido (2'-O-NMA). In one embodiment, the nucleic acid molecule includes at least one 2'-O-methyl-modified nucleotide, and in some embodiments, all of the nucleotides of the nucleic acid molecule include a 2'-O-methyl modification.

Nucleic acid agents discussed herein include otherwise unmodified RNA and DNA as well as RNA and DNA that have been modified, e.g., to improve efficacy, and polymers of nucleoside surrogates. Unmodified RNA refers to a molecule in which the components of the nucleic acid, namely sugars, bases, and phosphate moieties, are the same or essentially the same as that which occur in nature, preferably as occur naturally in the human body. The art has referred to rare or unusual, but naturally occurring, RNAs as modified RNAs, see, e.g., Limbach et al. (Nucleic Acids Res., 1994, 22:2183-2196). Such rare or unusual RNAs, often termed modified RNAs, are typically the result of a post-transcriptional modification and are within the term unmodified RNA as used

herein. Modified RNA, as used herein, refers to a molecule in which one or more of the components of the nucleic acid, namely sugars, bases, and phosphate moieties, are different from that which occur in nature, preferably different from that which occurs in the human body. While they are referred to as “modified RNAs” they will of course, because of the modification, include molecules that are not, strictly speaking, RNAs. Nucleoside surrogates are molecules in which the ribophosphate backbone is replaced with a non-ribophosphate construct that allows the bases to be presented in the correct spatial relationship such that hybridization is substantially similar to what is seen with a ribophosphate backbone, e.g., non-charged mimics of the ribophosphate backbone.

10 Modifications of the nucleic acid of the invention may be present at one or more of, a phosphate group, a sugar group, backbone, N-terminus, C-terminus, or nucleobase.

 The present invention also includes a vector in which the isolated nucleic acid of the present invention is inserted. The art is replete with suitable vectors that are useful in the present invention.

15 In brief summary, the expression of natural or synthetic nucleic acids encoding a β ARKnt peptide is typically achieved by operably linking a nucleic acid encoding a β ARKnt peptide or portions thereof to a promoter, and incorporating the construct into an expression vector. The vectors to be used are suitable for replication and, optionally, integration in eukaryotic cells. Typical vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the desired nucleic acid sequence.

 The vectors of the present invention may also be used for nucleic acid immunization and gene therapy, using standard gene delivery protocols. Methods for gene delivery are known in the art. See, e.g., U.S. Pat. Nos. 5,399,346, 5,580,859, 5,589,466, incorporated by reference herein in their entireties. In another embodiment, the invention provides a gene therapy vector.

 The isolated nucleic acid of the invention can be cloned into a number of types of vectors. For example, the nucleic acid can be cloned into a vector including, but not limited to a plasmid, a phagemid, a phage derivative, an animal virus, and a cosmid.

Vectors of particular interest include expression vectors, replication vectors, probe generation vectors, and sequencing vectors.

Further, the vector may be provided to a cell in the form of a viral vector. Viral vector technology is well known in the art and is described, for example, in
5 Sambrook et al. (2012, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in other virology and molecular biology manuals. Viruses, which are useful as vectors include, but are not limited to, retroviruses, adenoviruses, adeno-associated viruses, herpes viruses, and lentiviruses. In general, a suitable vector contains an origin of replication functional in at least one organism, a promoter sequence,
10 convenient restriction endonuclease sites, and one or more selectable markers, (e.g., WO 01/96584; WO 01/29058; and U.S. Pat. No. 6,326,193).

A number of viral based systems have been developed for gene transfer into mammalian cells. For example, retroviruses provide a convenient platform for gene delivery systems. A selected gene can be inserted into a vector and packaged in retroviral
15 particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the subject either in vivo or ex vivo. A number of retroviral systems are known in the art. In some embodiments, adenovirus vectors are used. A number of adenovirus vectors are known in the art. In one embodiment, lentivirus vectors are used.

20 For example, vectors derived from retroviruses such as the lentivirus are suitable tools to achieve long-term gene transfer since they allow long-term, stable integration of a transgene and its propagation in daughter cells. Lentiviral vectors have the added advantage over vectors derived from onco-retroviruses such as murine leukemia viruses in that they can transduce non-proliferating cells, such as hepatocytes.
25 They also have the added advantage of low immunogenicity. In one embodiment, the composition includes a vector derived from an adeno-associated virus (AAV). Adeno-associated viral (AAV) vectors have become powerful gene delivery tools for the treatment of various disorders. AAV vectors possess a number of features that render them ideally suited for gene therapy, including a lack of pathogenicity, minimal
30 immunogenicity, and the ability to transduce postmitotic cells in a stable and efficient manner. Expression of a particular gene contained within an AAV vector can be

specifically targeted to one or more types of cells by choosing the appropriate combination of AAV serotype, promoter, and delivery method

In certain embodiments, the vector also includes conventional control elements which are operably linked to the transgene in a manner which permits its
5 transcription, translation and/or expression in a cell transfected with the plasmid vector or infected with the virus produced by the invention. As used herein, “operably linked” sequences include both expression control sequences that are contiguous with the gene of interest and expression control sequences that act in trans or at a distance to control the gene of interest. Expression control sequences include appropriate transcription initiation,
10 termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation (polyA) signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (i.e., Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product. A great number of expression control sequences,
15 including promoters which are native, constitutive, inducible and/or tissue-specific, are known in the art and may be utilized.

Additional promoter elements, e.g., enhancers, regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain
20 functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the thymidine kinase (tk) promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function
25 either cooperatively or independently to activate transcription.

One example of a suitable promoter is the immediate early cytomegalovirus (CMV) promoter sequence. This promoter sequence is a strong constitutive promoter sequence capable of driving high levels of expression of any polynucleotide sequence operatively linked thereto. Another example of a suitable
30 promoter is Elongation Growth Factor -1 α (EF-1 α). However, other constitutive promoter sequences may also be used, including, but not limited to the simian virus 40 (SV40)

early promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, MoMuLV promoter, an avian leukemia virus promoter, an Epstein-Barr virus immediate early promoter, a Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin
5 promoter, the myosin promoter, the hemoglobin promoter, and the creatine kinase promoter. Further, the invention should not be limited to the use of constitutive promoters. Inducible promoters are also contemplated as part of the invention. The use of an inducible promoter provides a molecular switch capable of turning on expression of the polynucleotide sequence which it is operatively linked when such expression is
10 desired, or turning off the expression when expression is not desired. Examples of inducible promoters include, but are not limited to a metallothioneine promoter, a glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter.

Enhancer sequences found on a vector also regulates expression of the gene contained therein. Typically, enhancers are bound with protein factors to enhance
15 the transcription of a gene. Enhancers may be located upstream or downstream of the gene it regulates. Enhancers may also be tissue-specific to enhance transcription in a specific cell or tissue type. In one embodiment, the vector of the present invention comprises one or more enhancers to boost transcription of the gene present within the vector.

20 In order to assess the expression of a β ARKnt peptide, the expression vector to be introduced into a cell can also contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of expressing cells from the population of cells sought to be transfected or infected through viral vectors. In other aspects, the selectable marker may be carried on a separate piece of DNA and used in a
25 co- transfection procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers include, for example, antibiotic-resistance genes, such as neo and the like.

Reporter genes are used for identifying potentially transfected cells and for
30 evaluating the functionality of regulatory sequences. In general, a reporter gene is a gene that is not present in or expressed by the recipient organism or tissue and that encodes a

polypeptide whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells. Suitable reporter genes may include genes encoding luciferase, beta-galactosidase, chloramphenicol acetyl transferase, secreted alkaline phosphatase, or the green fluorescent protein gene (e.g., Ui-Tei et al., 2000 FEBS Letters 479: 79-82). Suitable expression systems are well known and may be prepared using known techniques or obtained commercially. In general, the construct with the minimal 5' flanking region showing the highest level of expression of reporter gene is identified as the promoter. Such promoter regions may be linked to a reporter gene and used to evaluate agents for the ability to modulate promoter- driven transcription.

Methods of introducing and expressing genes into a cell are known in the art. In the context of an expression vector, the vector can be readily introduced into a host cell, e.g., mammalian, bacterial, yeast, or insect cell by any method in the art. For example, the expression vector can be transferred into a host cell by physical, chemical, or biological means.

Physical methods for introducing a polynucleotide into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Methods for producing cells comprising vectors and/or exogenous nucleic acids are well-known in the art. See, for example, Sambrook et al. (2012, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York). A preferred method for the introduction of a polynucleotide into a host cell is calcium phosphate transfection.

Biological methods for introducing a polynucleotide of interest into a host cell include the use of DNA and RNA vectors. Viral vectors, and especially retroviral vectors, have become the most widely used method for inserting genes into mammalian, e.g., human cells. Other viral vectors can be derived from lentivirus, poxviruses, herpes simplex virus I, adenoviruses and adeno-associated viruses, and the like. See, for example, U.S. Pat. Nos. 5,350,674 and 5,585,362.

Chemical means for introducing a polynucleotide into a host cell include colloidal dispersion systems, such as macromolecule complexes, nanocapsules,

microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. An exemplary colloidal system for use as a delivery vehicle in vitro and in vivo is a liposome (e.g., an artificial membrane vesicle).

In the case where a non-viral delivery system is utilized, an exemplary
5 delivery vehicle is a liposome. The use of lipid formulations is contemplated for the introduction of the nucleic acids into a host cell (in vitro, ex vivo or in vivo). In another aspect, the nucleic acid may be associated with a lipid. The nucleic acid associated with a lipid may be encapsulated in the aqueous interior of a liposome, interspersed within the lipid bilayer of a liposome, attached to a liposome via a linking molecule that is
10 associated with both the liposome and the oligonucleotide, entrapped in a liposome, complexed with a liposome, dispersed in a solution containing a lipid, mixed with a lipid, combined with a lipid, contained as a suspension in a lipid, contained or complexed with a micelle, or otherwise associated with a lipid. Lipid, lipid/DNA or lipid/expression vector associated compositions are not limited to any particular structure in solution. For
15 example, they may be present in a bilayer structure, as micelles, or with a “collapsed” structure. They may also simply be interspersed in a solution, possibly forming aggregates that are not uniform in size or shape. Lipids are fatty substances which may be naturally occurring or synthetic lipids. For example, lipids include the fatty droplets that naturally occur in the cytoplasm as well as the class of compounds which contain long-
20 chain aliphatic hydrocarbons and their derivatives, such as fatty acids, alcohols, amines, amino alcohols, and aldehydes.

Lipids suitable for use can be obtained from commercial sources. For example, dimyristyl phosphatidylcholine (“DMPC”) can be obtained from Sigma, St. Louis, MO; dicetyl phosphate (“DCP”) can be obtained from K & K Laboratories
25 (Plainview, NY); cholesterol (“Choi”) can be obtained from Calbiochem-Behring; dimyristyl phosphatidylglycerol (“DMPG”) and other lipids may be obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Stock solutions of lipids in chloroform or chloroform/methanol can be stored at about -20°C. Chloroform is used as the only solvent since it is more readily evaporated than methanol. “Liposome” is a generic term
30 encompassing a variety of single and multilamellar lipid vehicles formed by the generation of enclosed lipid bilayers or aggregates. Liposomes can be characterized as

having vesicular structures with a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh et al., 1991 *Glycobiology* 5: 505-10). However, compositions that have different structures in solution than the normal vesicular structure are also encompassed. For example, the lipids may assume a micellar structure or merely exist as nonuniform aggregates of lipid molecules. Also contemplated are lipofectamine-nucleic acid complexes.

Regardless of the method used to introduce exogenous nucleic acids into a host cell, in order to confirm the presence of the recombinant DNA sequence in the host cell, a variety of assays may be performed. Such assays include, for example, “molecular biological” assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR; “biochemical” assays, such as detecting the presence or absence of a particular peptide, e.g., by immunological means (ELISAs and Western blots) or by assays described herein to identify agents falling within the scope of the invention.

Adeno-Associated Virus Vectors

Adeno-associated virus (AAV) is a unique, non-pathogenic member of the Parvoviridae family of small, non-enveloped, single-stranded DNA animal viruses. AAV require helper virus (e.g., adenovirus) for replication and, thus, do not replicate upon administration to a subject. AAV can infect a relatively wide range of cell types and stimulate only a mild immune response, particularly as compared to a number of other viruses, such as adenovirus. Over 100 AAV isolates have been reported. Biochemical and molecular characterization of many suggests that some exhibit different tissue tropism, persistence, and transduction efficiency (see, for example, Kwon et al., *ibid.*). Examples of AAV include AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, and AAV12, which appear to be of simian or human origin. AAV have

also been found in other animals, including birds (e.g., avian AAV, or AAV), bovines (e.g., bovine AAV, or BAAV), canines, equines, ovines, and porcines.

Vectors and virions based upon AAV have advanced to the forefront of gene therapy, due to their ability to achieve long-term transgene expression in vivo and low immunogenicity (see, for example, Halbert et al., 2000, J Virol 74, 1524-1532; Sumner-Jones et al., *ibid.*; Stieger et al., *ibid.*; Niemeyer et al., *ibid.*). AAV virions have hitherto not been associated with any malignant disease. Furthermore, all viral protein genes can be deleted from AAV vectors and AAV virions contributing to their safety profile (see, for example, Daya et al., *ibid.*). Several Phase I/II clinical trials support a good overall safety profile for AAV virions and little associated toxicity in humans (see, for example, Moss et al., *ibid.*; Mandel et al., *ibid.*, Diaz-Nido et al., *ibid.*, Simonelli et al., *ibid.*; Bainbridge et al., *ibid.*).

An AAV vector is a recombinant nucleic acid molecule in which at least a portion of the AAV genome is replaced by a heterologous nucleic acid molecule. It is possible to replace about 4.7 kilobases (kb) of AAV genome DNA, e.g., by removing the viral replication and capsid genes. Often the heterologous nucleic acid molecule is simply flanked by AAV inverted terminal repeats (ITRs) on each terminus. The ITRs serve as origins of replication and contain cis acting elements required for rescue, integration, excision from cloning vectors, and packaging. Such vectors typically also include a promoter operatively linked to the heterologous nucleic acid molecule to control expression.

An AAV vector can be packaged into an AAV capsid in vitro with the assistance of a helper virus or helper functions expressed in cells to yield an AAV virion. The serotype and cell tropism of an AAV virion are conferred by the nature of the viral capsid proteins.

AAV vectors and AAV virions have been shown to transduce cells efficiently, including both dividing and non-dividing cells (see, for example, Lai et al., 2002, DNA Cell Biol 21, 895-913). Among AAVs, serotype 5 (AAV5) has demonstrated enhanced gene transfer activity in lung, eye and central nervous system (CNS) as well as rodent salivary glands (see, for example, Katano et al., *ibid.*). AAV vectors and virions

have been shown to be safe and to lead to long term in vivo persistence and expression in a variety of cell types.

As used herein, an AAV vector that encodes an β ARKnt peptide is a nucleic acid molecule that comprises a nucleic acid molecule that encodes an β ARKnt peptide of the invention, an ITR joined to 5' terminus of the β ARKnt encoding nucleic acid molecule, and an ITR joined to the 3' terminus of the β ARKnt encoding nucleic acid molecule. Examples of ITRs include, but are not limited, to AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV, BAAV, and other AAV ITRs known to those skilled in the art. In one embodiment, an AAV ITR is selected from an AAV2 ITR, an AAV5 ITR, an AAV6 ITR, and a BAAV ITR. In one embodiment, an AAV ITR is an AAV2 ITR. In one embodiment, an AAV ITR is an AAV5 ITR. In one embodiment, an AAV ITR is an AAV6 ITR. In one embodiment, an AAV ITR is a BAAV ITR.

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

The disclosure provides AAV vectors of different serotypes (as determined by the serotype of the ITRs within such vector) that encode an β ARKnt peptide. Such an AAV vector can be selected from an AAV1 vector, an AAV2 vector, an

AAV3 vector, an AAV4 vector, an AAV5 vector, an AAV6 vector, an AAV7 vector, an AAV8 vector, an AAV9 vector, an AAV10 vector, an AAV11 vector, an AAV12 vector, an AAV vector, and a BAAV vector, and other AAV vectors known to those skilled in the art, wherein any of such vectors encode an β ARKnt peptide. One embodiment is an AAV2 vector, an AAV5 vector, an AAV6 vector or a BAAV vector, wherein the respective vector encodes an β ARKnt peptide. One embodiment is an AAV2 vector that encodes an β ARKnt peptide. One embodiment is an AAV5 vector that encodes an β ARKnt peptide. One embodiment is an AAV6 vector that encodes an β ARKnt peptide. One embodiment is a BAAV vector that encodes an β ARKnt peptide.

One embodiment is an AAV vector that comprises AAV ITRs and a CMV promoter operatively linked to a nucleic acid molecule encoding a β ARKnt peptide. One embodiment is an AAV5 vector that comprises AAV5 ITRs and a CMV promoter operatively linked to a nucleic acid molecule encoding β ARKnt peptide.

In one embodiment, the AAV vector provides cardio-specific expression of the encoded β ARKnt peptide. In one embodiment, the EF1alpha promoter comprises mir122. In one embodiment, the AAV vector is an AAV serotype 6 vector. In one embodiment, the AAV vector is an AAV serotype 9 vector. In one embodiment, the AAV vector comprises a cardiac-specific promoter operably linked to the nucleic acid sequence encoding the β ARKnt peptide. For example, in one embodiment, the AAV vector comprises an alpha cardiac actin enhancer. In one embodiment, the AAV vector comprises an EF1alpha promoter. In one embodiment the AAV vector comprises a nucleic acid that reduces liver retention. For example, in one embodiment, the AAV vector comprises a nucleic acid encoding mir122. In one embodiment, the EF1alpha promoter comprises mir122. In one embodiment, mir122 comprises the nucleic acid sequence of

CCTTAGCAGAGCTGTGGAGTGTGACAATGGTGTGTTGTGTCTAAACTATCAAACGCCATTATCACACTAAATAGCTACTGCTAGGC (SEQ ID NO:2), or a functional equivalent sequence having at least 80%, more preferably 90%, 95%, 96%, 97%, 98%, 99%, sequence identity thereto.

Treatment Methods

The present invention provides a method for the treatment or prevention of a cardiovascular disease or disorder, or a condition associated with a cardiovascular disease or disorder, in a subject in need thereof. The present invention also provides a method for altering the β -adrenergic receptor (β AR) density, in a subject in need thereof.

5 For example, chronic β AR agonist administration may downregulate the density of β AR. Accordingly, in one embodiment, the method comprises increasing β AR density in a patient with decreased β AR density.

Exemplary conditions treated or prevented by way of the present invention include, but are not limited to, heart failure and cardiac infarction, stroke, hypertension, and diseases associated with altered vascular or other smooth muscle responsiveness. In one embodiment, the condition is heart failure. In one embodiment, the methods of the invention prevent or treat a disease or disorder associated with cardiac or vascular dysfunction. For example, in one embodiment, the methods of the invention prevent or treat diabetes.

15 In one embodiment, the invention comprises a method of treating or preventing a cardiovascular disease or disorder. In one embodiment, the invention comprises a method of treating or preventing heart failure. In one embodiment, the method comprises administering an effective amount of a peptide or nucleic acid described herein to a subject diagnosed with, suspected of having, or at risk for a cardiovascular disease or disorder. Thus, the present invention relates to the prevention and treatment of a disease or disorder by administration of a therapeutically effective amount of a β ARKnt peptide, a composition comprising said peptide, a nucleic acid encoding a β ARKnt peptide, or a composition comprising a nucleic acid encoding a β ARKnt peptide to a subject in need thereof, for the treatment or prevention of a cardiovascular disease or disorder, including but not limited to heart failure.

The following are non-limiting examples of cardiovascular diseases that can be treated by the disclosed methods and compositions: arterial cardiovascular thromboembolic disorders, venous cardiovascular thromboembolic disorders, and thromboembolic disorders in the chambers of the heart; atherosclerosis; restenosis; peripheral arterial disease; coronary bypass grafting surgery; carotid artery disease; arteritis; myocarditis; cardiovascular inflammation; vascular inflammation; coronary

heart disease (CHD); unstable angina (UA); unstable refractory angina; stable angina (SA); chronic stable angina; acute coronary syndrome (ACS); first or recurrent myocardial infarction; acute myocardial infarction (AMI); myocardial infarction; non-Q wave myocardial infarction; non-STE myocardial infarction; coronary artery disease; cardiac ischemia; ischemia; ischemic sudden death; transient ischemic attack; stroke; atherosclerosis; peripheral occlusive arterial disease; venous thrombosis; deep vein thrombosis; thrombophlebitis; arterial embolism; coronary arterial thrombosis; cerebral arterial thrombosis; cerebral embolism; kidney embolism; pulmonary embolism; thrombosis resulting from (a) prosthetic valves or other implants, (b) indwelling catheters, (c) stents, (d) cardiopulmonary bypass, (e) hemodialysis, or (f) other procedures in which blood is exposed to an artificial surface that promotes thrombosis; thrombosis resulting from atherosclerosis, surgery or surgical complications, prolonged immobilization, arterial fibrillation, congenital thrombophilia, cancer, diabetes, effects of medications or hormones, and complications of pregnancy; cardiac arrhythmias including supraventricular arrhythmias, atrial arrhythmias, atrial flutter, and atrial fibrillation.

In one embodiment, the invention comprises a method of altering β AR density, in a subject in need thereof. In one embodiment, the invention provides a method of increasing β AR density, in a subject in need thereof. In one embodiment, the method comprises administering an effective amount of a peptide or nucleic acid described herein to a subject diagnosed with, suspected of having, or at risk for a downregulated β AR density. Thus, the present invention relates to the prevention and treatment of decreased β AR density by administration of a therapeutically effective amount of a β ARKnt peptide, a composition comprising said peptide, a nucleic acid encoding a β ARKnt peptide, or a composition comprising a nucleic acid encoding a β ARKnt peptide to a subject in need thereof, to increase β AR density.

In one embodiment, the subject has low β AR density. For example, chronic β AR agonist administration may downregulate the density of β AR. Accordingly, in one embodiment, the subject has taken a β AR agonist. In one embodiment, the method comprises administering a comprises administering an effective amount of a peptide or nucleic acid described herein with a second therapeutic. In one embodiment, the second therapeutic is a β AR agonist.

Exemplary β AR agonist include, but are not limited to, selected from isoproterenol, metaproterenol, formoterol, salmeterol, salbutamol, albuterol, terbutaline, fenoterol, and vilanterol.

In one embodiment, the method comprises administering to the subject a
5 AAV vector comprising a nucleic acid encoding a β ARKnt peptide. In some
embodiments, the AAV vector is a serotype 6 or serotype 9 AAV. In one embodiment,
the AAV vector comprises a comprises an alpha cardiac actin enhancer. In one
embodiment, the AAV vector comprises an EF1alpha promoter. In one embodiment the
AAV vector comprises a nucleic acid that reduces liver retention. For example, in one
10 embodiment, the AAV vector comprises a nucleic acid encoding mir122. In one
embodiment, the EF1alpha promoter comprises mir122.

Subjects to which administration of a peptide, nucleic acid or composition
of the invention is contemplated include, but are not limited to, humans and other
primates, mammals including commercially relevant mammals such as non-human
15 primates, cattle, pigs, horses, sheep, cats, and dogs.

The peptide, nucleic acid or composition of the present invention may be
administered in a manner appropriate to the disease to be treated (or prevented). The
quantity and frequency of administration will be determined by such factors as the
condition of the subject, and the type and severity of the subject's disease, although
20 appropriate dosages may be determined by clinical trials.

When "therapeutic amount" is indicated, the precise amount of the
compositions of the present invention to be administered can be determined by a
physician with consideration of individual differences in age, weight, disease type, extent
of disease, and condition of the patient (subject).

25 The administration of the peptide or the composition may be carried out in
any convenient manner, including by aerosol inhalation, injection, ingestion, transfusion,
implantation or transplantation. The compositions described herein may be administered
to a patient subcutaneously, intradermally, intratumorally, intranodally, intramedullary,
intramuscularly, by intravenous (i.v.) injection, or intraperitoneally. In one embodiment,
30 the compositions of the present invention are administered to a patient by intradermal or

subcutaneous injection. In another embodiment, the compositions of the present invention are preferably administered by i.v. injection.

Methods of treatment of the diseases encompassed by the invention can comprise the transplantation of single cells, cell lines, compositions, or cell populations of the invention into a subject in need thereof. In certain embodiments, the subject is a human.

Pharmaceutical Composition and Administration

The peptides, nucleic acids and compositions of the invention can be formulated and administered to a subject. For example, a β ARKnt peptide or nucleic acids encoding a β ARKnt peptide can be formulated and administered to a subject for the treatment and/or prevention of a disease or disorder.

The present invention also provides pharmaceutical compositions comprising one or more of the compositions described herein. In some aspects, the invention encompasses the preparation and use of pharmaceutical compositions comprising a β ARKnt peptide or nucleic acid encoding a β ARKnt peptide as an active ingredient for the treatment or prevention of a disease or disorder, including but not limited to, diabetes. Formulations may be employed in admixtures with conventional excipients, i.e., pharmaceutically acceptable organic or inorganic carrier substances suitable for administration to the wound or treatment site. The pharmaceutical compositions may be sterilized and if desired mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure buffers, coloring, and/or aromatic substances and the like. They may also be combined where desired with other active agents, e.g., other analgesic agents.

As used herein, "additional ingredients" include, but are not limited to, one or more of the following: excipients; surface active agents; dispersing agents; inert diluents; granulating and disintegrating agents; binding agents; lubricating agents; coloring agents; preservatives; physiologically degradable compositions such as gelatin; aqueous vehicles and solvents; oily vehicles and solvents; suspending agents; dispersing or wetting agents; emulsifying agents, demulcents; buffers; salts; thickening agents; fillers; emulsifying agents; antioxidants; antibiotics; antifungal agents; stabilizing agents;

and pharmaceutically acceptable polymeric or hydrophobic materials. Other “additional ingredients” that may be included in the pharmaceutical compositions of the invention are known in the art and described, for example in Genaro, ed. (1985, Remington’s Pharmaceutical Sciences, Mack Publishing Co., Easton, PA), which is incorporated
5 herein by reference.

The composition of the invention may comprise a preservative from about 0.005% to 2.0% by total weight of the composition. The preservative is used to prevent spoilage in the case of exposure to contaminants in the environment. Examples of preservatives useful in accordance with the invention included but are not limited to those
10 selected from the group consisting of benzyl alcohol, sorbic acid, parabens, imidurea and combinations thereof. A particularly preferred preservative is a combination of about 0.5% to 2.0% benzyl alcohol and 0.05% to 0.5% sorbic acid.

In an embodiment, the composition includes an anti-oxidant and a chelating agent that inhibits the degradation of one or more components of the
15 composition. Preferred antioxidants for some compounds are BHT, BHA, alpha-tocopherol and ascorbic acid in the preferred range of about 0.01% to 0.3% and more preferably BHT in the range of 0.03% to 0.1% by weight by total weight of the composition. Preferably, the chelating agent is present in an amount of from 0.01% to 0.5% by weight by total weight of the composition. Particularly preferred chelating
20 agents include edetate salts (e.g. disodium edetate) and citric acid in the weight range of about 0.01% to 0.20% and more preferably in the range of 0.02% to 0.10% by weight by total weight of the composition. The chelating agent is useful for chelating metal ions in the composition that may be detrimental to the shelf life of the formulation. While BHT and disodium edetate are the particularly preferred antioxidant and chelating agent
25 respectively for some compounds, other suitable and equivalent antioxidants and chelating agents may be substituted therefore as would be known to those skilled in the art.

Liquid suspensions may be prepared using conventional methods to achieve suspension of the HMW-HA or other composition of the invention in an aqueous
30 or oily vehicle. Aqueous vehicles include, for example, water, and isotonic saline. Oily vehicles include, for example, almond oil, oily esters, ethyl alcohol, vegetable oils such

as arachis, olive, sesame, or coconut oil, fractionated vegetable oils, and mineral oils such as liquid paraffin. Liquid suspensions may further comprise one or more additional ingredients including, but not limited to, suspending agents, dispersing or wetting agents, emulsifying agents, demulcents, preservatives, buffers, salts, flavorings, coloring agents, and sweetening agents. Oily suspensions may further comprise a thickening agent.

Known suspending agents include, but are not limited to, sorbitol syrup, hydrogenated edible fats, sodium alginate, polyvinylpyrrolidone, gum tragacanth, gum acacia, and cellulose derivatives such as sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose. Known dispersing or wetting agents include, but are not limited to, naturally occurring phosphatides such as lecithin, condensation products of an alkylene oxide with a fatty acid, with a long chain aliphatic alcohol, with a partial ester derived from a fatty acid and a hexitol, or with a partial ester derived from a fatty acid and a hexitol anhydride (e.g., polyoxyethylene stearate, heptadecaethyleneoxycetanol, polyoxyethylene sorbitol monooleate, and polyoxyethylene sorbitan monooleate, respectively). Known emulsifying agents include, but are not limited to, lecithin, and acacia. Known preservatives include, but are not limited to, methyl, ethyl, or n-propyl para hydroxybenzoates, ascorbic acid, and sorbic acid.

Powdered and granular formulations of a pharmaceutical preparation of the invention may be prepared using known methods. Such formulations may be administered directly to a subject, used, for example, to form tablets, to fill capsules, or to prepare an aqueous or oily suspension or solution by addition of an aqueous or oily vehicle thereto. Each of these formulations may further comprise one or more of dispersing or wetting agent, a suspending agent, and a preservative. Additional excipients, such as fillers and sweetening, flavoring, or coloring agents, may also be included in these formulations.

A pharmaceutical composition of the invention may also be prepared, packaged, or sold in the form of oil-in-water emulsion or a water-in-oil emulsion. The oily phase may be a vegetable oil such as olive or arachis oil, a mineral oil such as liquid paraffin, or a combination of these. Such compositions may further comprise one or more emulsifying agents such as naturally occurring gums such as gum acacia or gum tragacanth, naturally-occurring phosphatides such as soybean or lecithin phosphatide,

esters or partial esters derived from combinations of fatty acids and hexitol anhydrides such as sorbitan monooleate, and condensation products of such partial esters with ethylene oxide such as polyoxyethylene sorbitan monooleate. These emulsions may also contain additional ingredients including, for example, sweetening or flavoring agents.

5 Methods for impregnating or coating a material with a chemical composition are known in the art, and include, but are not limited to methods of depositing or binding a chemical composition onto a surface, methods of incorporating a chemical composition into the structure of a material during the synthesis of the material (i.e., such as with a physiologically degradable material), and methods of absorbing an
10 aqueous or oily solution or suspension into an absorbent material, with or without subsequent drying.

 As used herein, “parenteral administration” of a pharmaceutical composition includes any route of administration characterized by physical breaching of a tissue of a subject and administration of the pharmaceutical composition through the
15 breach in the tissue. Parenteral administration thus includes, but is not limited to, administration of a pharmaceutical composition by injection of the composition, by application of the composition through a surgical incision, by application of the composition through a tissue-penetrating non-surgical wound, and the like. In particular, parenteral administration is contemplated to include, but is not limited to, cutaneous,
20 subcutaneous, intraperitoneal, intravenous, intramuscular, intracisternal injection, and kidney dialytic infusion techniques.

 Formulations of a pharmaceutical composition suitable for parenteral administration comprise the active ingredient combined with a pharmaceutically acceptable carrier, such as sterile water or sterile isotonic saline. Such formulations may
25 be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable formulations may be prepared, packaged, or sold in unit dosage form, such as in ampules or in multi-dose containers containing a preservative. Formulations for parenteral administration include, but are not limited to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and implantable
30 sustained-release or biodegradable formulations. Such formulations may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing,

or dispersing agents. In one embodiment of a formulation for parenteral administration, the active ingredient is provided in dry (i.e., powder or granular) form for reconstitution with a suitable vehicle (e.g., sterile pyrogen-free water) prior to parenteral administration of the reconstituted composition.

5 The pharmaceutical compositions may be prepared, packaged, or sold in the form of a sterile injectable aqueous or oily suspension or solution. This suspension or solution may be formulated according to the known art, and may comprise, in addition to the active ingredient, additional ingredients such as the dispersing agents, wetting agents, or suspending agents described herein. Such sterile injectable formulations may be
10 prepared using a non-toxic parenterally-acceptable diluent or solvent, such as water or 1,3-butane diol, for example. Other acceptable diluents and solvents include, but are not limited to, Ringer's solution, isotonic sodium chloride solution, and fixed oils such as synthetic mono- or di-glycerides. Other parentally-administrable formulations which are useful include those which comprise the active ingredient in microcrystalline form, in a
15 liposomal preparation, or as a component of a biodegradable polymer systems. Compositions for sustained release or implantation may comprise pharmaceutically acceptable polymeric or hydrophobic materials such as an emulsion, an ion exchange resin, a sparingly soluble polymer, or a sparingly soluble salt.

 Pharmaceutical compositions of the invention formulated for pulmonary
20 delivery may also provide the active ingredient in the form of droplets of a solution or suspension. Such formulations may be prepared, packaged, or sold as aqueous or dilute alcoholic solutions or suspensions, optionally sterile, comprising the active ingredient, and may conveniently be administered using any nebulization or atomization device. Such formulations may further comprise one or more additional ingredients including,
25 but not limited to, a flavoring agent such as saccharin sodium, a volatile oil, a buffering agent, a surface active agent, or a preservative such as methylhydroxybenzoate. The droplets provided by this route of administration preferably have an average diameter in the range from about 0.1 to about 200 nanometers. The formulations described herein as being useful for pulmonary delivery are also useful for intranasal delivery of a
30 pharmaceutical composition of the invention. Another formulation suitable for intranasal

administration is a coarse powder comprising the active ingredient and having an average particle from about 0.2 to 500 micrometers.

Pharmaceutical compositions that are useful in the methods of the invention may be prepared, packaged, or sold in formulations suitable for oral, rectal, vaginal, parenteral, topical, pulmonary, intranasal, buccal, intravenous, transdermal, subcutaneous, intramuscular, ophthalmic, intrathecal and other known routes of administration. Other contemplated formulations include projected nanoparticles, liposomal preparations, resealed erythrocytes containing the active ingredient, and immunologically-based formulations.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in bulk, as a single unit dose, or as a plurality of single unit doses. As used herein, a “unit dose” is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

Typically dosages of the compound of the invention which may be administered to an animal, preferably a human, range in amount from about 0.01 mg to about 1000 mg per kilogram of body weight of the animal. The precise dosage administered will vary depending upon any number of factors, including, but not limited to, the type of animal and type of disease or disorder being treated, the age of the animal and the route of administration. Preferably, the dosage of the compound will vary from about 1 mg to about 100 mg per kilogram of body weight of the animal. The compound can be administered to an animal as frequently as several times daily, or it can be administered less frequently, such as once a day, once a week, once every two weeks, once a month, or even less frequently, such as once every several months or even once a year or less. The frequency of the dose will be readily apparent to the skilled artisan and will depend upon any number of factors, such as, but not limited to, the type and severity of the disease or disorder being treated, the type and age of the animal, etc.

EXPERIMENTAL EXAMPLES

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be
5 construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The
10 following working examples therefore, specifically point out the preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

Example 1: Domain-specific interactions with the amino-terminus of GRK2 may reveal
15 novel roles in cardiac signaling and function

During heart failure (HF), cardiac levels and activity of the G protein-coupled receptor (GPCR) kinase (GRK) GRK2 are elevated and contribute to adverse remodeling and contractile dysfunction, while inhibition via a carboxyl-terminal peptide, β ARKct, enhances heart function and can prevent HF development. Mounting evidence
20 supports the idea of a dynamic “interactome” in which GRK2 can uncouple GPCRs via novel protein-protein interactions. Several GRK2 interacting partners are important for adaptive and maladaptive myocyte growth; therefore, an understanding of domain-specific interactions with signaling and regulatory molecules could lead to novel targets for HF therapy. For instance, GRK2 contains a putative amino-terminal Regulator of G
25 protein Signaling (RGS) domain (β ARK-RGS) that directly interacts with Gq and appears to inhibit signaling without altering Gq enzymatic activity. Cardiac-specific transgenic (Tg) expression of a fragment of this RGS domain (β ARKnt) did not alter acute hypertrophy after pressure overload or demonstrate RGS activity in vivo against Gq-mediated signaling. In contrast, β ARKnt induced hypertrophy and elevated β -
30 adrenergic receptor (β AR) density without altering agonist-induced contractility or adenylyl cyclase activity, due to a compensatory increase in GRK2 activity. Importantly,

though, β AR downregulation in response to chronic agonist administration was attenuated by β ARKnt expression, indicating a novel regulation of β AR receptor density. Given these findings we have recently investigated the effect of β ARKnt expression during chronic pressure overload post trans-aortic constriction (TAC). Echocardiographic analysis revealed increased posterior wall thickness and left-ventricular mass 4 weeks post-TAC compared to non-transgenic littermate controls (NLC). Importantly, despite enhanced hypertrophy, the progression to HF was inhibited in β ARKnt mice 14 weeks post-TAC (%LV Ejection Fraction of 36.1 ± 0.2 in NLC versus 56.6 ± 0.9 in Tg mice). Histological analysis of interstitial fibrosis and cross-sectional areas determines any alterations in maladaptive remodeling. Further, cardiomyocyte signaling and β ARKnt protein-binding partners are important as these data indicate that β ARKnt-mediated regulation of β AR density may provide a novel means of cardioprotection during pressure-overload induced HF. Further, AAV6- and AAV9- β ARKnt constructs are developed for cardiac selective expression of the peptide following systemic injection. This would all for the definition of the dose range capable of preventing the progression of cardiovascular injury and the therapeutic window of when during disease progression this therapeutic intervention can be administered and maintain a beneficial effect. The adeno-associated viral vector of serotypes 6 and 9 are expressed under an alpha cardiac actin enhancer and EF1alpha promoter containing mir122 inserts that reduce liver retention. This construct allows the virus to be injected systemically through intravenous injection yet be predominantly expressed in the myocardium. This system allows delivery of the virus at any time point prior to or after disease development to investigate the disease states that are positively affected by β ARKnt expression. Further, this method of delivery facilitates a detailed determination of the dose-response relationship to determine the optimal dose range for patient therapy. This is particularly important given the vast array of cardiovascular disease etiologies and contributing factors including lifestyle and medical history, wherein intravenous viral delivery could be titrated up to each patient's individual effective dose.

Example 2: β ARKnt mice exhibit an improved response in an experimental model of heart failure

With approximately 550,000 new cases of heart failure (HF) diagnosed in the U.S. alone each year this disease represents a growing healthcare concern. Importantly, despite substantial improvements in its management, including improved mechanical and pharmacotherapy, outcomes in HF remain poor. Thus, there is an urgent
5 need to develop new therapeutic strategies, including cell and gene-based therapies, and recent research has been aimed at the underlying mechanisms of HF progression. Developing new approaches to HF therapy has included development and characterization of transgenic animals with α MHC promoter driven expression of the cDNA that encodes bovine GRK2 residues 50-145 (β ARKnt) with a carboxy-terminal
10 flag-tag. In this model, proof-of-concept has been demonstrated for inhibiting decompensation to HF in a murine model of trans-aortic constriction-mediated chronic pressure overload hypertrophy and heart failure in transgenic animals with continuous expression of the peptide beginning with post-natal activation of the promoter. While this is an in vivo beneficial effect, a limitation of this invention was that it did not allow
15 thorough investigation into whether β ARKnt can reverse or attenuate established left-ventricular remodeling in various disease models, but is limited to prophylactic or protective applications.

Importantly, current pharmacotherapy targeting the sympathetic overdrive and GRK2-mediated decrease in β AR signaling and density uses β -blocking agents to
20 shield the receptors from agonist binding. This remains the first line of defense in the heart and a standard of care for HF patients. The present data indicate that the β ARKnt peptide enhances β AR signaling by a different mechanism wherein it restores β AR density and signaling in the presence of enhanced GRK2 activity, potentially by facilitating internalization and recycling of agonist bound receptors. This process of
25 internalization and recycling restores the β AR receptors to the membrane where they may again be bound by agonist for signaling, and preserves them from the degradation pathway. Thus, rather than shielding the heart from overstimulation, the present invention enhances its natural ability to manage the stimulation and regulate it in a sustainable manner. Further, the ability to target cardiovascular expression via the tropism of the
30 AAV6- and AAV9- β ARKnt constructs can provide a significant advance over a main

limitation of β -blocker therapy by removing the side-effects resulting from inhibition of β AR signaling in other tissues throughout the body.

Therefore, the present invention includes the development of adeno-associated viral vectors that can be utilized as gene therapy agents, and the development of a small molecule mimic of the peptide is being performed, both of which can allow study of the protective effects of β ARKnt in large animals models of disease. Furthermore, these new compounds can allow thorough investigation into whether β ARKnt can reverse or attenuate established left-ventricular remodeling in various disease models. The AAV6- and AAV9- β ARKnt constructs would provide the means necessary to achieve effective gene delivery of β ARKnt to its desired site of action, the heart, in animals and eventually in humans.

The invention comprises an adeno-associated viral vector of serotypes 6 and 9 expressed under an alpha cardiac actin enhancer and EF1alpha promoter comprising mir122 inserts that reduce liver retention. This construct allows the virus to be injected systemically through intravenous injection yet be predominantly expressed in the myocardium. This system allows delivery of the virus at any time point prior to or after disease development to investigate the disease states that are positively affected by β ARKnt expression. Further, this method of delivery facilitates a detailed determination of the dose-response relationship to determine the optimal dose range for patient therapy. This is particularly important given the vast array of cardiovascular disease etiologies and contributing factors including lifestyle and medical history, wherein intravenous viral delivery could be titrated up to each patients individual effective dose.

Introduction

There is no cure for heart failure (HF) except cardiac transplantation, and that can only occur in ~3,000 patients worldwide. This is significant because HF currently affects more than 5 million Americans and contributes to 300,000 deaths per year, a rising number due to increased survival rates post-myocardial infarction (MI). While MI is the major cause of HF, several other co-morbid conditions, including chronic hypertension or ischemic and valvular heart disease, can reduce myocardial efficiency and ultimately lead to HF. The present data demonstrate that a peptide can prevent

decompensation to HF in a murine model of pressure overload-induced hypertrophy and HF despite significant left-ventricular remodeling. This peptide is unique to the present invention of AAV6- and AAV9- β ARKnt constructs, which can allow the investigation of dosing, timing of administration during disease progression, and side effect profiles in multiple disease models representing the various etiologies that cause human HF.

During heart failure (HF), cardiac levels and activity of the G protein-coupled receptor (GPCR) kinase (GRK) GRK2 are elevated and contribute to adverse remodeling and contractile dysfunction, while inhibition via a carboxyl-terminal peptide, β ARKct, enhances heart function and can prevent HF development. Mounting evidence supports the idea of a dynamic “interactome” in which GRK2 can uncouple GPCRs via novel protein-protein interactions. Several GRK2 interacting partners are important for adaptive and maladaptive myocyte growth; therefore, an understanding of domain-specific interactions with signaling and regulatory molecules can lead to novel targets for HF therapy. For instance, GRK2 has a putative amino-terminal Regulator of G protein Signaling (RGS) domain (β ARK-RGS) that directly interacts with Gq and appears to inhibit signaling without altering Gq enzymatic activity. Previously, cardiac-specific transgenic (Tg) expression of a fragment of this RGS domain (β ARKnt) was investigated. This fragment did not alter acute hypertrophy after pressure overload or demonstrate RGS activity in vivo against Gq-mediated signaling. In contrast, β ARKnt induced hypertrophy and elevated β -adrenergic receptor (β AR) density without altering agonist-induced contractility or adenylyl cyclase activity, due to a compensatory increase in GRK2 activity. Importantly, though, β AR downregulation in response to chronic agonist administration was attenuated by β ARKnt expression, indicating a novel regulation of β AR receptor density. Given these findings the effect of β ARKnt expression during chronic pressure overload post trans-aortic constriction (TAC) has been investigated. Echocardiographic analysis revealed increased posterior wall thickness and left-ventricular mass 4 weeks post-TAC compared to non-transgenic littermate controls (NLC). Importantly, despite enhanced hypertrophy, the progression to HF was inhibited in β ARKnt mice 14 weeks post-TAC (%LV Ejection Fraction of 36.1 ± 0.2 in NLC versus 56.6 ± 0.9 in Tg mice). These data indicate that β ARKnt-mediated regulation of

β AR density may provide a novel means of cardioprotection during pressure-overload induced HF. (Schumacher, SM et al., 2015, Circulation Research, 117:A365).

GRK2 comprises a putative amino-terminal Regulator of G protein Signaling (RGS) domain (β ARKrgs) that directly interacts with Gq and appears to inhibit signaling without altering Gq enzymatic activity. It is presently demonstrated that cardiac-specific transgenic (Tg) expression of both the RGS domain of GRK2 and a shorter N-terminal peptide (β ARKnt) can alter cardiac physiology when expressed in myocytes. Of note, they both halt HF progression in mice after pressure overload but have differential effects on the initial hypertrophic response.

Previously, it was demonstrated that cardiac expression of the β ARKnt peptide did not alter acute hypertrophy 7 days after pressure overload or demonstrate RGS activity in vivo against Gq-mediated signaling. In contrast, β ARKnt induced hypertrophy and elevated β -adrenergic receptor (β AR) density without altering agonist-induced contractility or adenylyl cyclase activity, due to a compensatory increase in GRK2 activity. Importantly, though, β AR downregulation in response to 7 days of agonist administration was attenuated by β ARKnt expression, indicating a novel regulation of β AR receptor density by an unknown mechanism. Unfortunately, this original line was lost before the mechanism could be investigated. A new variation of this line has therefore been generated. While the baseline phenotype was milder (no significant increase in β AR density) the effect of β ARKnt expression during chronic pressure overload after transaortic constriction (TAC) was investigated. Echocardiographic analysis revealed increased posterior wall thickness and left ventricular mass 4 weeks post-TAC compared to non-transgenic littermate controls (NLC). Importantly, despite enhanced hypertrophy, the progression to HF was inhibited in β ARKnt mice 14 weeks post-TAC (%LV Ejection Fraction of 34.8 ± 3.2 in NLC versus 57.3 ± 0.65 in Tg mice). These data indicate that β ARKnt-mediated regulation of β AR density may provide a novel means of cardioprotection during pressure-overload induced HF. Based on these data, AAV6-and AAV9- β ARKnt constructs for cardiac selective expression of the peptide following systemic injection can be used for HF therapy. This would allow one to define the dose range capable of preventing the progression of cardiovascular injury and the therapeutic window of when during disease

progression this therapeutic intervention can be administered and maintain a beneficial effect.

Systemic injection of AAV6- or AAV9- β ARKnt as a therapeutic intervention in patients is envisioned, with chronic hypertension or vascular disease prior to or during the deterioration of cardiovascular function to prevent decompensation to HF. Projected doses for large animal studies are as follows: Cohort 1: 7.5×10^{11} vg per subject ($\sim 1 \times 10^{10}$ vg/kg); Cohort 2: 3.75×10^{12} vg per subject ($\sim 5 \times 10^{10}$ vg/kg); Cohort 3: 7.5×10^{12} vg per subject ($\sim 1 \times 10^{11}$ vg/kg); Cohort 4: 3.75×10^{13} vg per subject ($\sim 5 \times 10^{11}$ vg/kg). Two lower doses are included because no data are available on the efficacy of β ARKnt in humans, whereas Cohorts 3 and 4 are in the range where it is expected to see some efficacy and are at or near what has been delivered through the coronary arteries to human HF patients in other trials. In fact, the dose of AAV-SERCA2a in the CUPID Trial that appeared to have some efficacy was the highest dose (1×10^{13} vg), so the planned Cohort 3 is just short of this and Cohort 4 is above so there should be sufficient dose coverage to potentially see some efficacy and certainly, the doses will cover the range needed for seeing any adverse events.

Results

During initial characterization of the β ARKnt line, cardiovascular function and adrenergic responsiveness were analyzed via terminal hemodynamics at baseline and upon challenge with increasing doses of the β -adrenergic receptor agonist isoproterenol (ISO) in Tg and NLC mice. No difference was observed in mean systemic pressure, the heart rate (HR) response to isoproterenol, or cardiac contractility and relaxation (dP/dt maximum and minimum) at baseline or in response to ISO, demonstrating that cardiac function and β AR responsiveness were not altered in the transgenic animals (Figure 36).

To determine whether β ARKrgs expression could alter hypertrophy in an animal model of pressure-overload, transgenic and NLC mice underwent trans-aortic constriction (TAC) or sham surgery. TAC was confirmed by pulsed-wave Doppler of the aortic arch 24 hr post-surgery to measure the systolic pressure gradient across the point of constriction, and this was important to confirm the level of cardiac stress was the same between TAC groups. Echocardiography was performed at baseline, 2, and 4 weeks to

monitor cardiac function and dimensions. Over this time course no effect of TAC on LVEF was typically found (Figure 3).

To further elucidate whether the observed effects were a selective interaction of β ARKrgs with Gq and not a GRK2-mediated effect, mice with cardiac-restricted GRK2 overexpression were studied, wherein the levels of GRK2 are similar to those observed in human HF. Unlike the β ARKrgs and GqI mice, cardiac hypertrophy was unaltered in GRK2 overexpressing mice, with the significant increase in LVPWs and mass nearly identical between transgenic and their respective NLCs 4 weeks post-TAC. Similarly, HW and LAW were elevated to the same degree. These data demonstrate that gain-of-function of full-length GRK2 is not sufficient to alter cardiac hypertrophic responses, and suggests that the RGS domain of GRK2 embodies distinct functional interactions that prevent hypertrophic signaling in the heart (Figure 7).

To further elucidate whether there was a selective interaction of β ARKrgs with Gq and not a GRK2-mediated effect, cardiac-targeted bARKct transgenic mice (TgbARKct) were utilized, which express the c-terminal 194 amino acids of GRK2 that competes for binding to G $\beta\gamma$ and membrane translocation, acting as a peptide inhibitor of GRK2 activity on GPCRs. Unlike β ARKrgs and GqI Tg mice, cardiac hypertrophy was unaltered in TgbARKct mice, with the significant increase in LVPWs and LV mass nearly identical between TgbARKct and their respective NLCs 4 weeks post-TAC. Similarly, heart weight and left atrial weight were elevated to the same degree (Figure 8).

One hallmark of human HF is the induction of the fetal gene program; therefore atrial natriuretic factor, brain natriuretic factor, and beta myosin heavy chain mRNA expression was analyzed via RT-PCR in the myocardial samples. Induction of the fetal gene program was observed. Further, hypertrophic gene expression was measured via RT-PCR of cardiac mRNA and fetal gene induction was similar between these animals. Together, these data demonstrate proportional hypertrophic growth in the β ARKnt transgenic and NLC mice in response to cardiac stress (Figure 14).

In contrast to the cardioprotection provided by β ARKrgs, GRK2 overexpression demonstrated the opposite effect, hastening the decompensation to HF with a more rapid deterioration in function and expansion of the LV, and a more progressive reduction in wall thickness, further suggesting that the β ARKrgs peptide

alone encompasses functional interactions that do not occur within the full-length enzyme in vivo. To investigate the underlying mechanisms of action of the β ARKrgs peptide, tissues were taken for biochemical and histological analysis 4 weeks post-TAC (Figure 15).

5 To elucidate the underlying mechanisms of action of the β ARKnt peptide, tissues were taken for histological and biochemical analysis 4 weeks after TAC and immunoprecipitation reactions were performed on NLC and Tg β ARKnt mice 4 weeks after Sham or TAC surgery to confirm that unlike the β ARKrgs peptide, IP of flag-tagged β ARKnt did not co-immunoprecipitate with G α q, demonstrating that they do interact in
10 complex with each other in vivo. Further, it was also found that the β ARKnt did not co-IP with full-length GRK2. Western blot of cardiac lysates prior to and remaining after IP demonstrated that the flag IP was efficient and there was no difference in the amount of β ARKnt IP'd from sham or TAC animals, confirming that β ARKnt does not co-IP with G α q (Figure 18).

15 In addition, Gq-mediated activation of PLC via IP₃ production was measured and it was found that IP₃ levels were elevated to the same degree in NLC and Tg β ARKnt mice after TAC, suggesting that the physiological effects of β ARKnt are not due to altered Gq-coupled GPCR activation (Figure 19).

20 To visualize the effect of β ARKnt peptide expression on myocardial structure, wheat germ agglutinin staining was performed to measure myocyte cross-sectional area in these mice and a significant increase in myocyte size was observed in transgenic sham animals and an even greater, but equal, increase in NLC and β ARKnt mice after TAC, demonstrating an alteration in cardiomyocyte size prior to cardiac stress (Figure 37).

25 Furthermore, Masson Trichrome staining was performed on paraffin sections taken at the level of the aortic outflow tract. Four chamber images of the myocardium revealed a proportional increase in cardiac size in NLC and β ARKnt hearts 4 weeks after TAC. To further investigate the integrity of the myocardium, higher magnification images of the LV were collected and a potential decrease in interstitial
30 fibrosis in the β ARKnt transgenic mice was observed. Together, these data suggest that the β ARKnt peptide may limit LV remodeling after TAC (Figure 38).

To further elucidate the mechanism of action of the β ARKnt peptide, β AR density was measured by radioligand binding and it was found that receptor density was preserved by β ARKnt expression after 4 weeks of pressure overload. Further, there was a trend towards an increase in GRK2 levels in these animals. These data are consistent with
5 the original line, though milder, suggesting that there may be a dose-dependent effect of β ARKnt peptide expression (Figure 22, Figure 32).

In addition, a trend towards an increase in clathrin was observed in the transgenic Sham and TAC mice, and it was found that in contrast to the potential decrease in NLC mice, phosphorylated Akt was significantly increased in β ARKnt mice
10 after TAC. Together, these data suggest that β AR regulation and cardiac signaling pathways are altered in the β ARKnt transgenic hearts (Figure 40).

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.
15 While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

CLAIMS

1. A composition comprising a β ARKnt peptide, a variant thereof, or a fragment thereof; or a nucleic acid encoding β ARKnt, a variant thereof or a fragment thereof.
2. The composition of claim 1, wherein the β ARKnt peptide comprises an amino acid sequence at least 90% homologous to SEQ ID NO:1.
3. The composition of claim 1, wherein the β ARKnt peptide comprises an amino acid sequence of SEQ ID NO:1.
4. The composition of claim 1, wherein the nucleic acid encoding β ARKnt, a variant thereof or a fragment thereof is contained within a vector.
5. The composition of claim 4, wherein the vector is an AAV vector.
6. The composition of claim 5, wherein the AAV vector is an AAV6 or AAV9 vector.
7. The composition of claim 6, wherein the AAV vector comprises a EF1alpha promoter.
8. The composition of claim 7, wherein the EF1alpha promoter comprises mir122.
9. A method for treating or preventing heart failure in a subject, the method comprising administering an effective amount of a composition comprising a β ARKnt peptide, a variant thereof, or a fragment thereof; or a nucleic acid encoding a β ARKnt peptide, a variant thereof or a fragment thereof.
10. The method of claim 9, wherein the β ARKnt peptide comprises an amino acid sequence at least 90% homologous to SEQ ID NO:1.
11. The method of claim 9, wherein the nucleic acid encoding a β ARKnt peptide, a variant thereof or a fragment thereof is contained within a vector.
12. The method of claim 9, wherein the subject is human.
13. A method for altering β -adrenergic receptor (β AR) density in a subject, the method comprising administering an effective amount of a composition comprising a β ARKnt peptide, a variant thereof, or a fragment thereof; or a nucleic acid encoding a β ARKnt peptide, a variant thereof or a fragment thereof.

14. The method of claim 13, wherein the β ARKnt peptide comprises an amino acid sequence at least 90% homologous to SEQ ID NO:1.
15. The method of claim 13, wherein the nucleic acid encoding a β ARKnt peptide, a variant thereof or a fragment thereof is contained within a vector.
16. The method of claim 13, wherein the β AR density is increased.
17. The method of claim 13, wherein the subject has low β AR density.
18. The method of claim 13, wherein the method further comprises administering a second therapeutic.
19. The method of claim 18, wherein the second therapeutic is a β AR agonist.

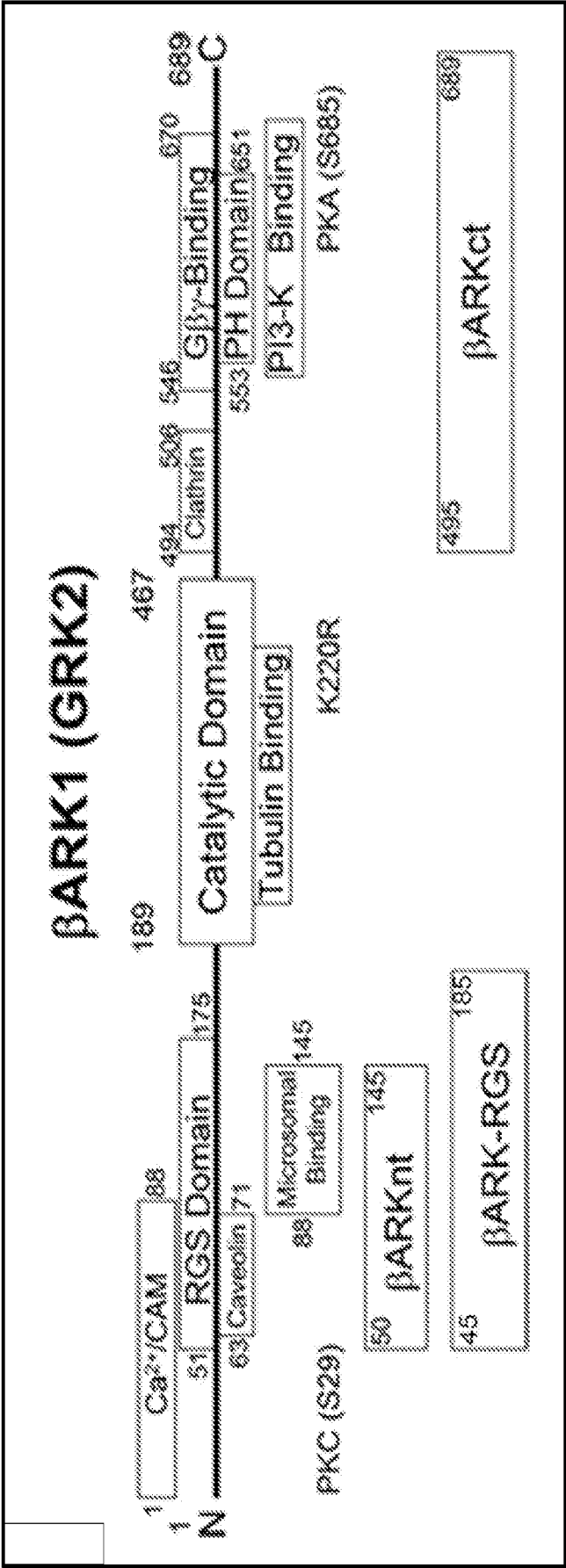


Figure 1A

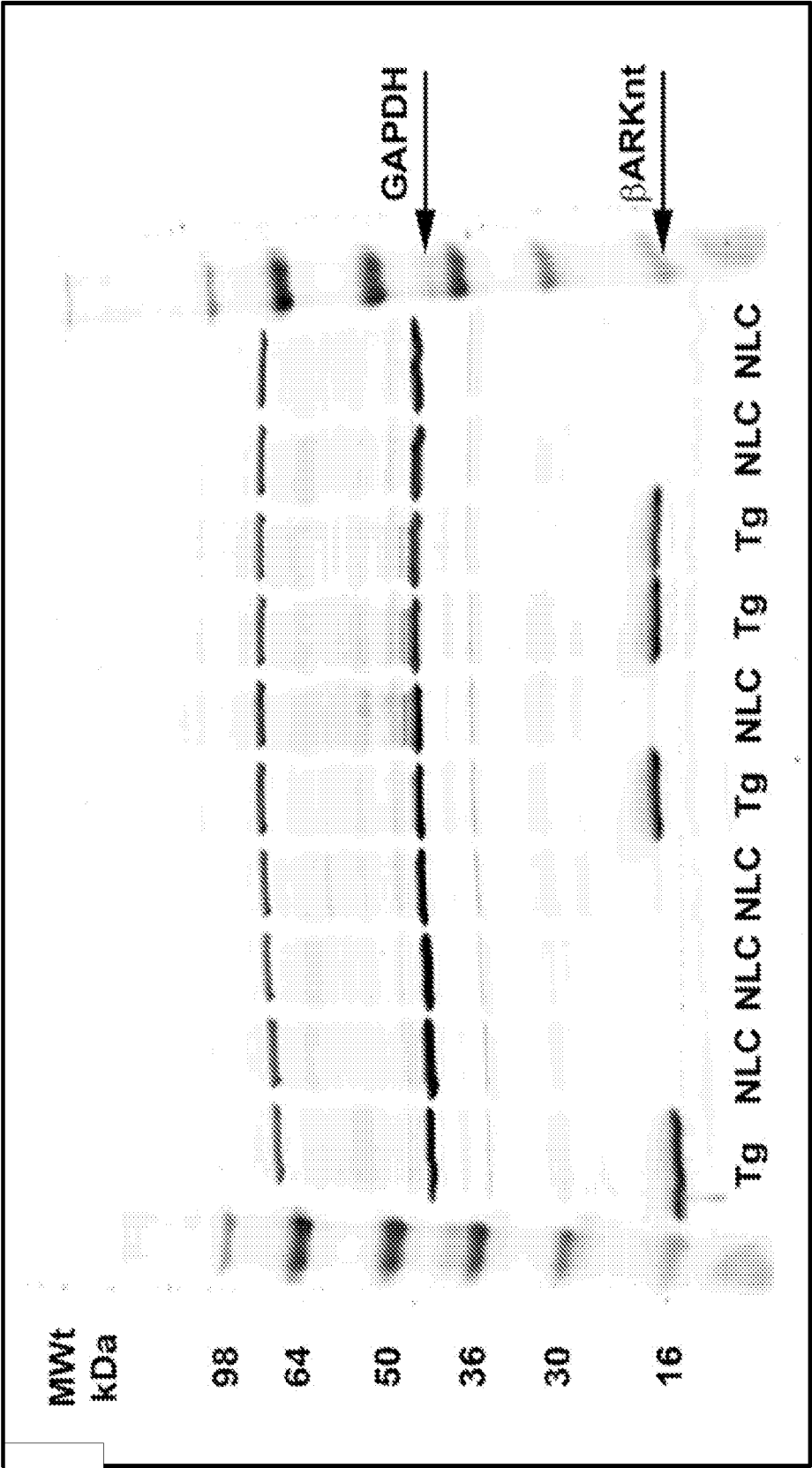
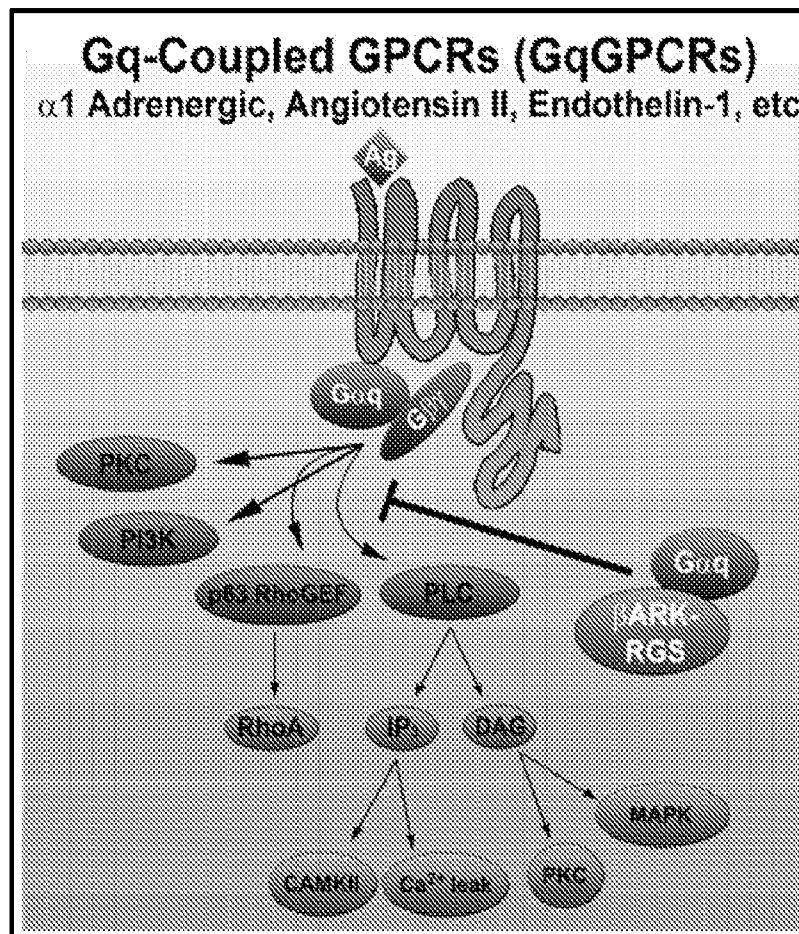


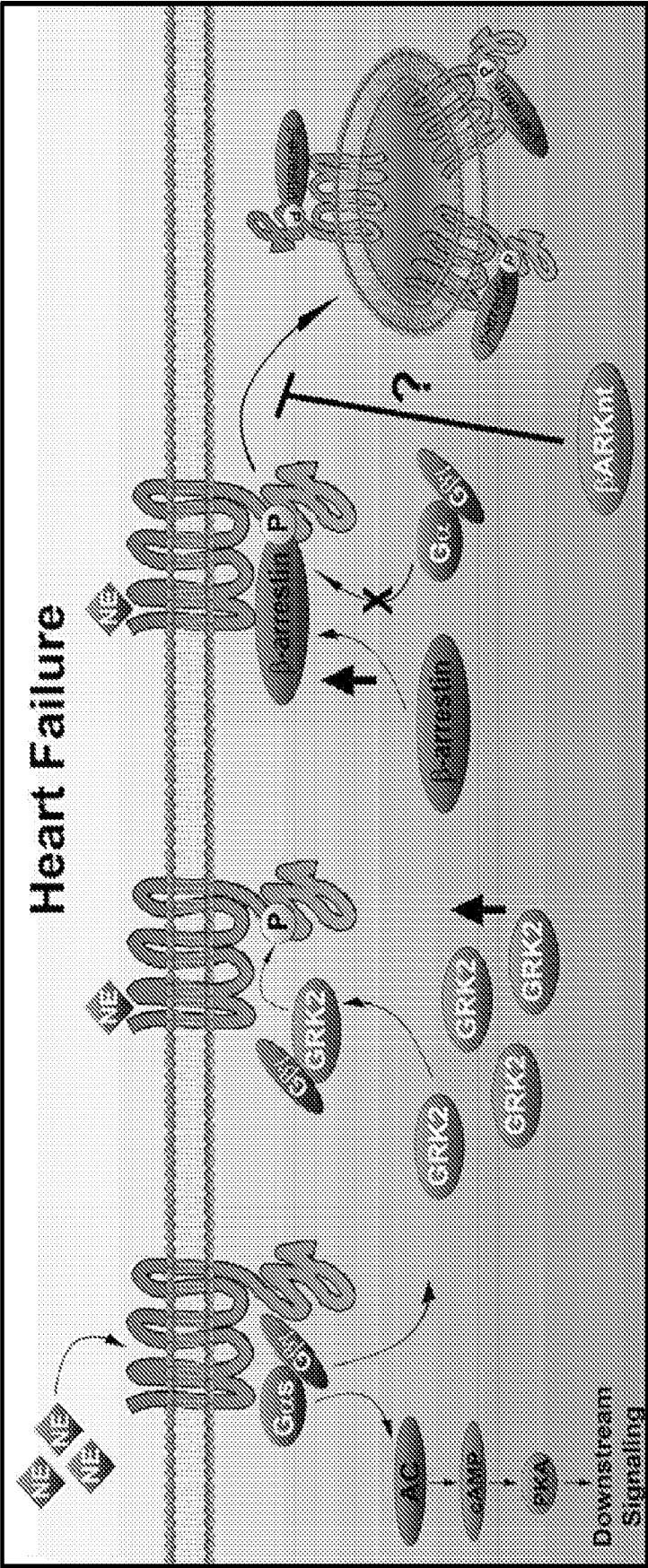
Figure 1B



Pro-Hypertrophic Line: Cardiac-specific
 β ARK-NT – GRK2 nt expression

Pro Heart Failure Line: Cardiac-specific
BK12 – GRK2 expression

Figure 2



Anti-Hypertrophic Line: Cardiac-specific
Gq10 – Gαq Inhibitor expression

Anti Heart Failure Line: Cardiac-specific
M27 – GRK2ct (βARKct) expression

Figure 2 (continued)

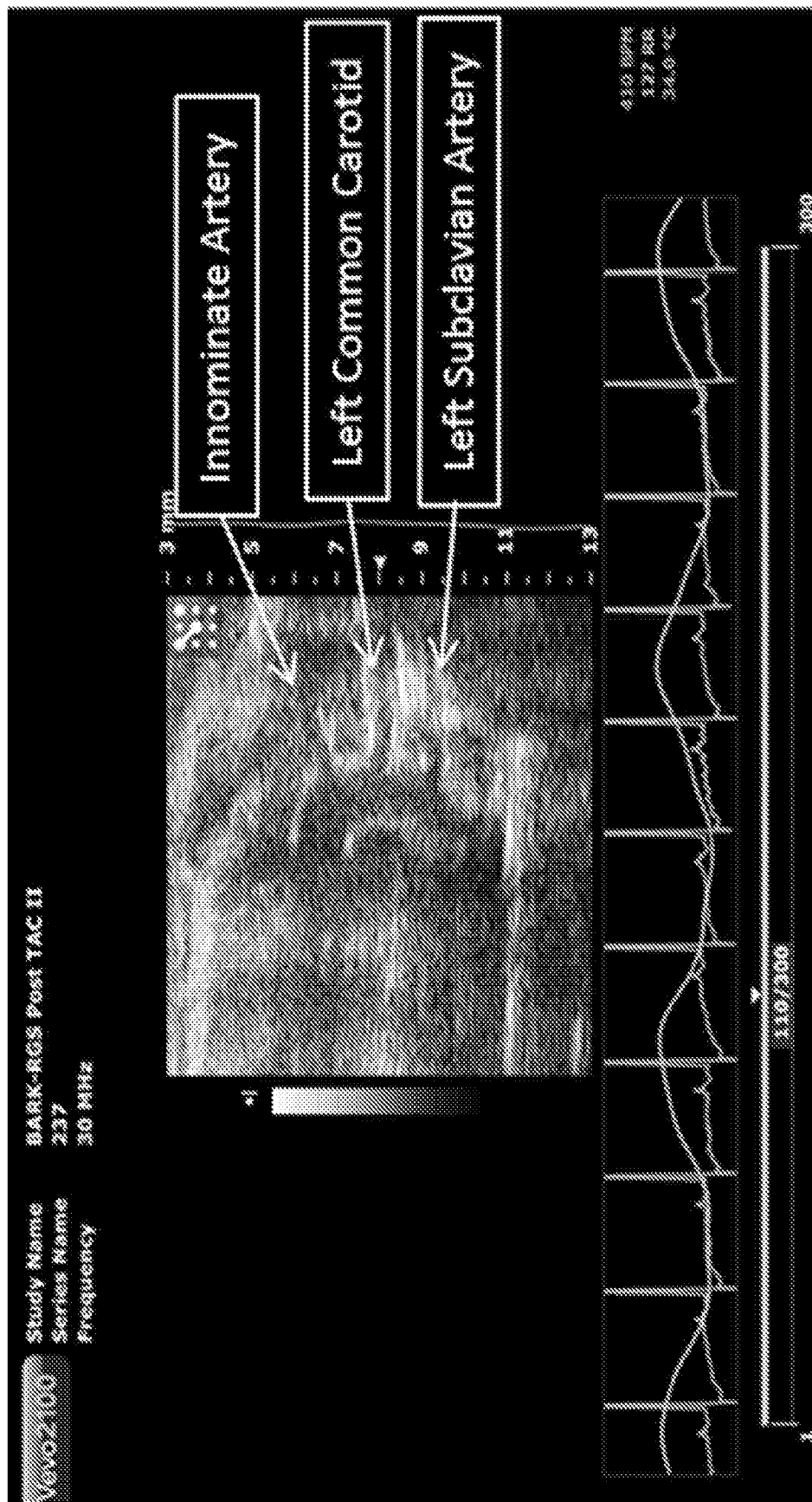


Figure 3

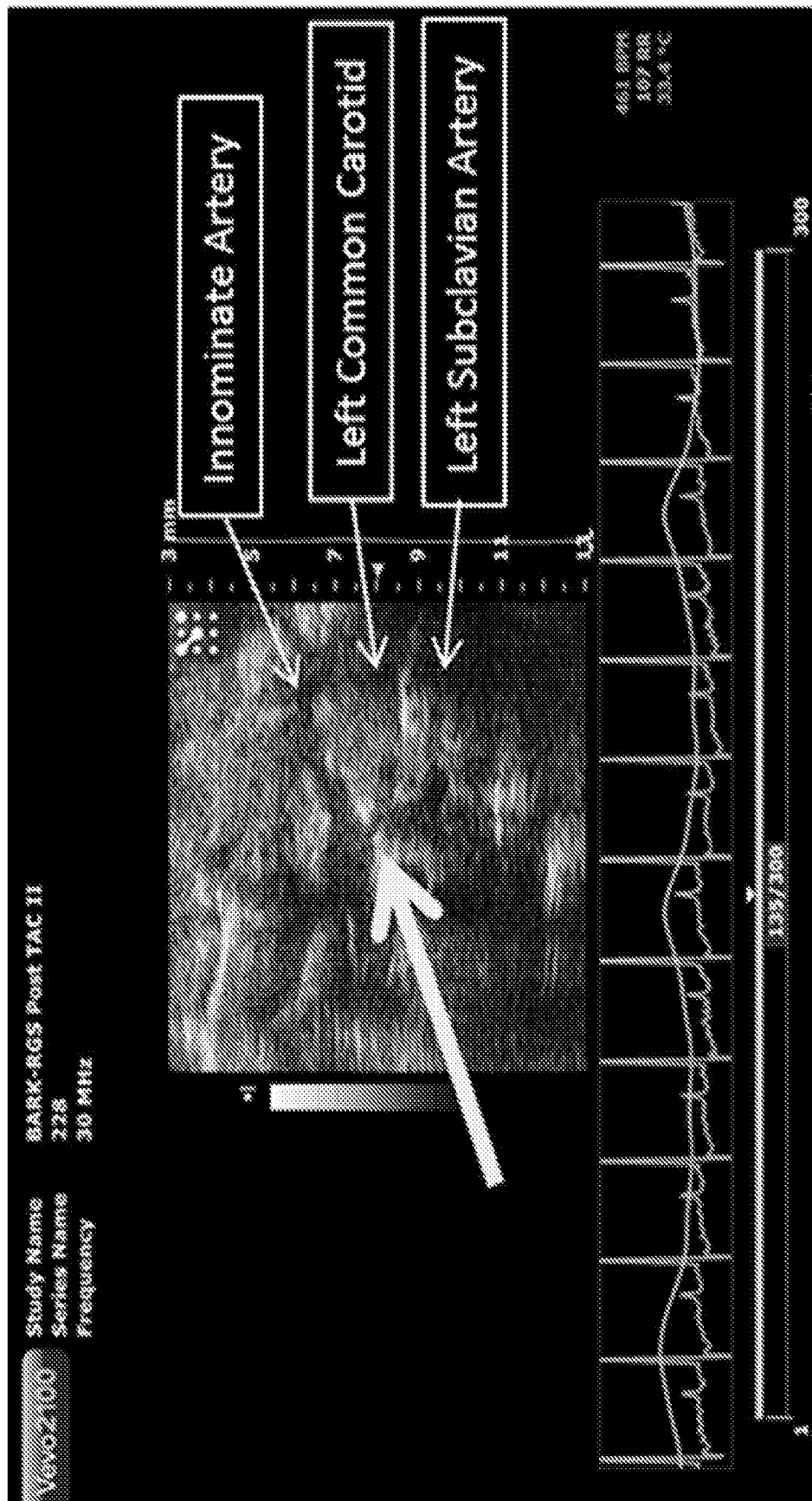


Figure 3 (continued)

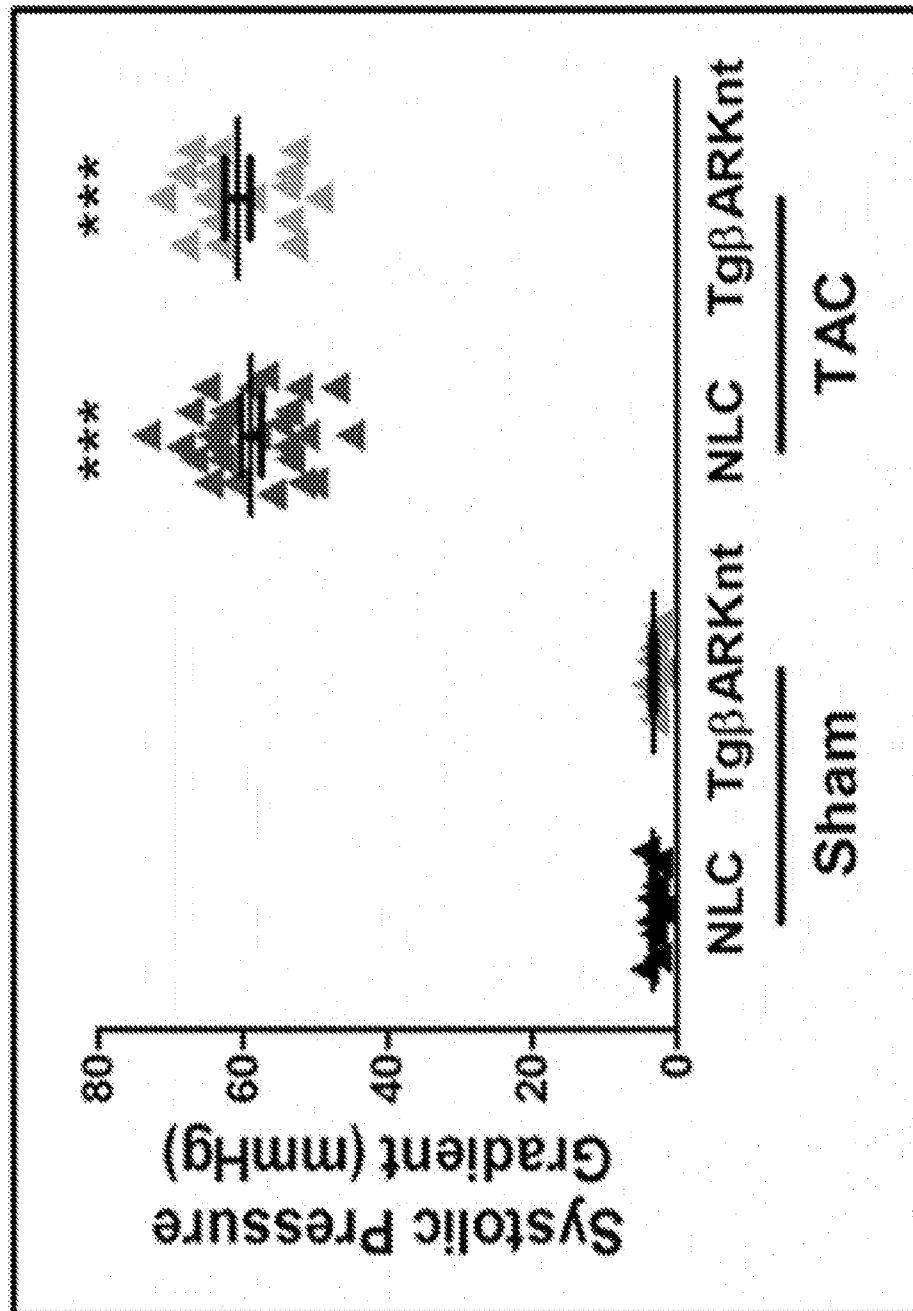


Figure 3 (continued)

Figure 4A

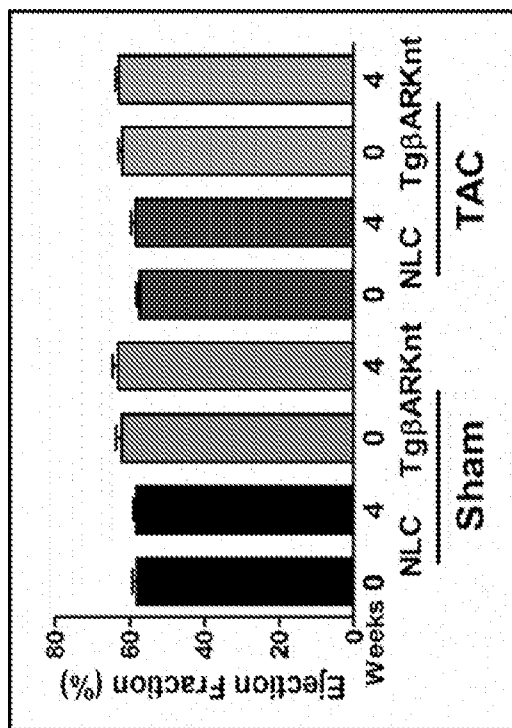


Figure 4B

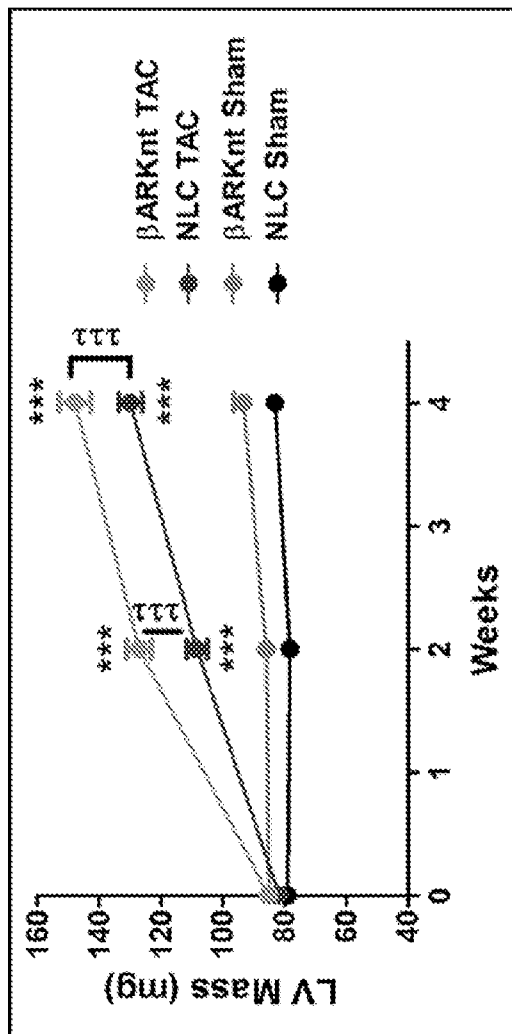


Figure 4C

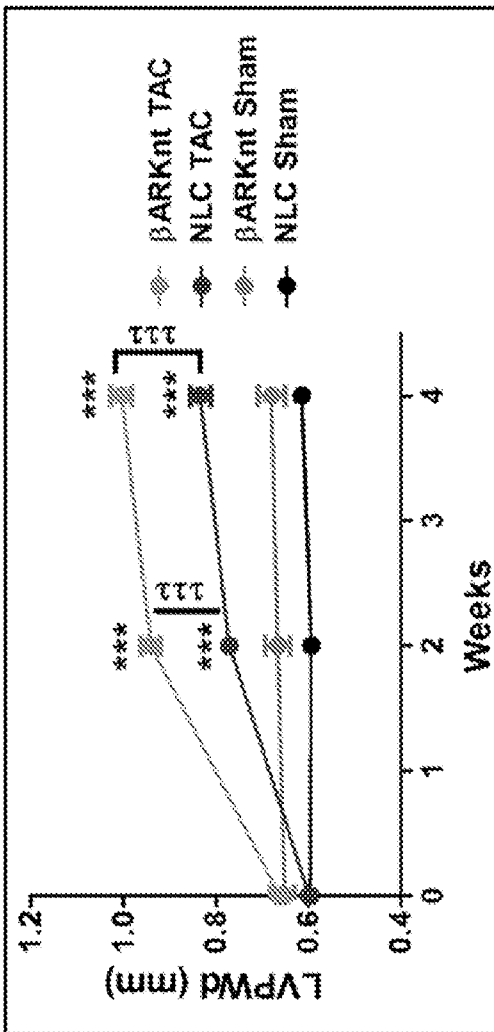


Figure 4D

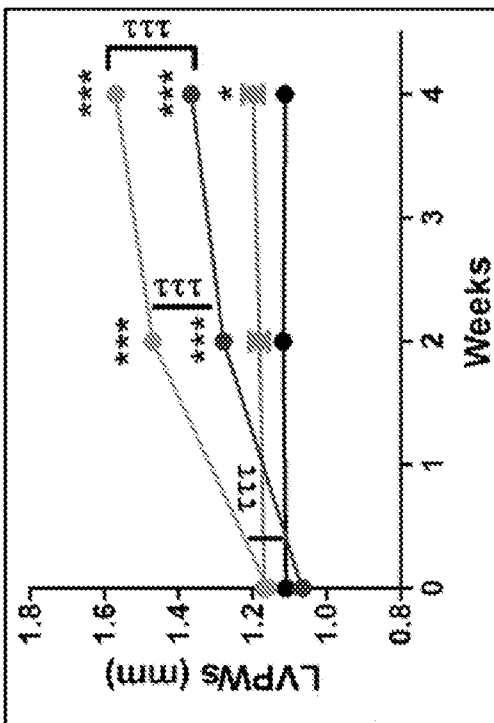


Figure 4

Figure 5B

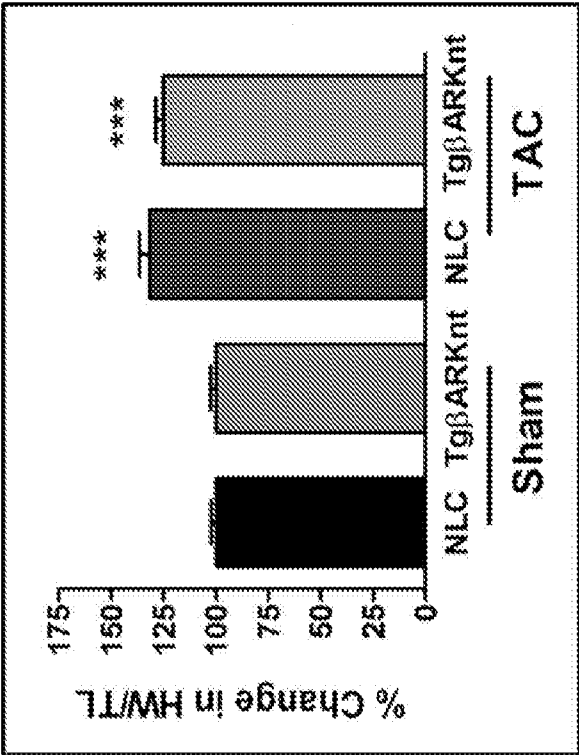


Figure 5A

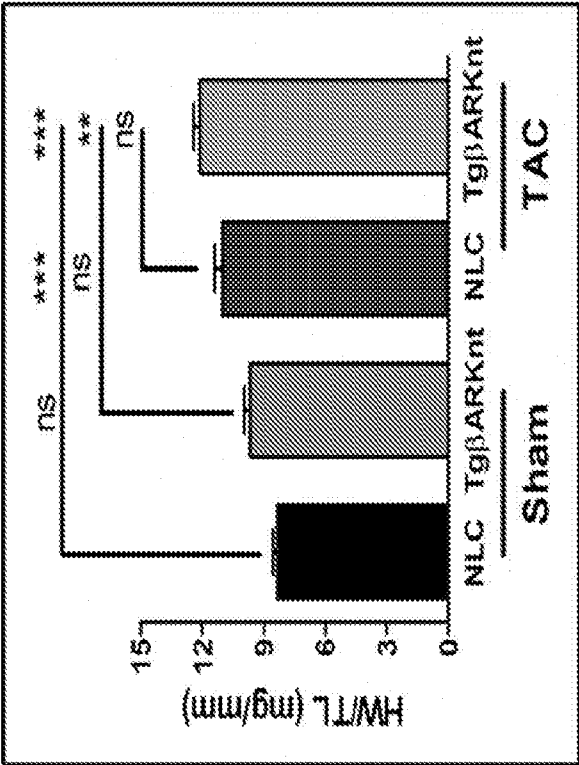


Figure 5D

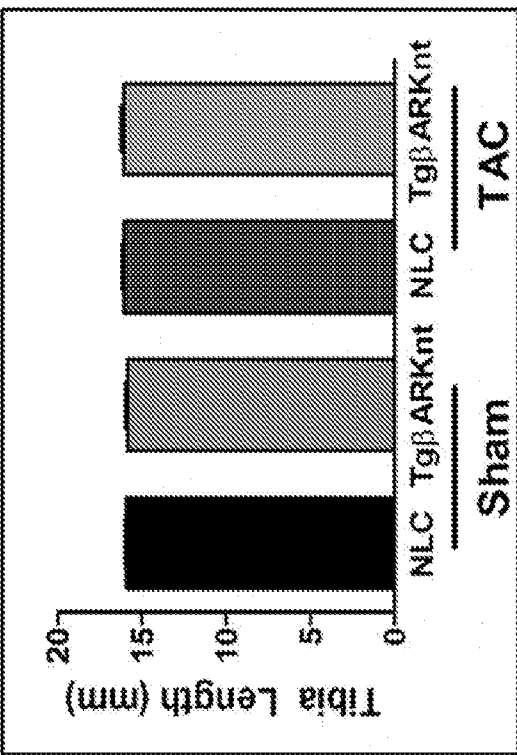


Figure 5C

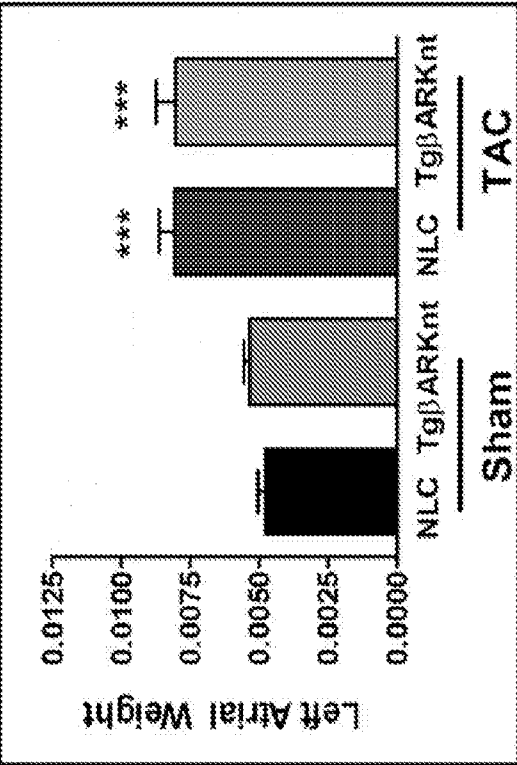


Figure 5

Figure 6C

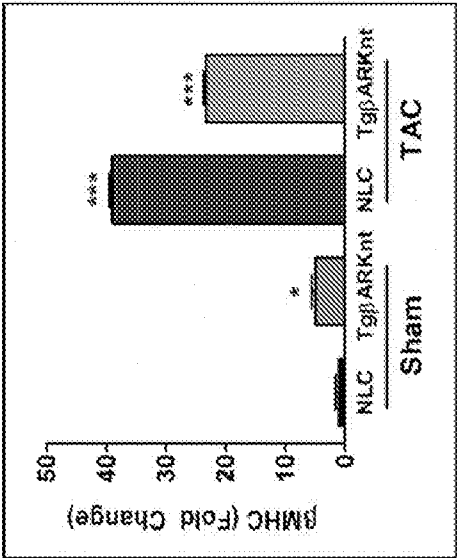


Figure 6B

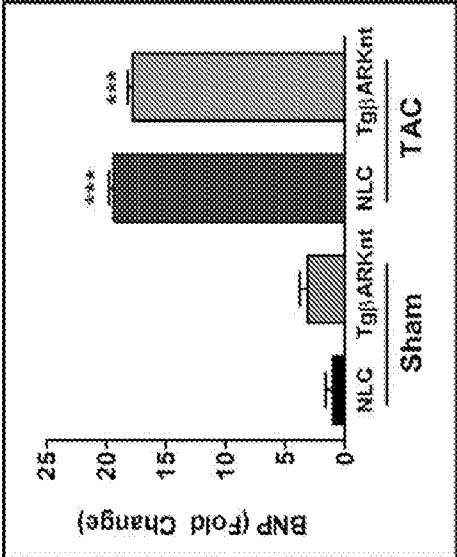


Figure 6A

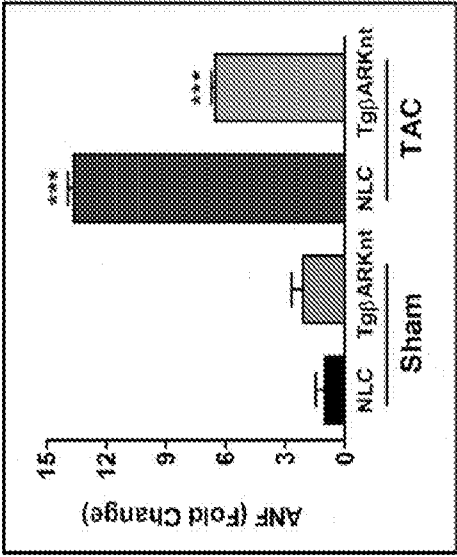


Figure 6

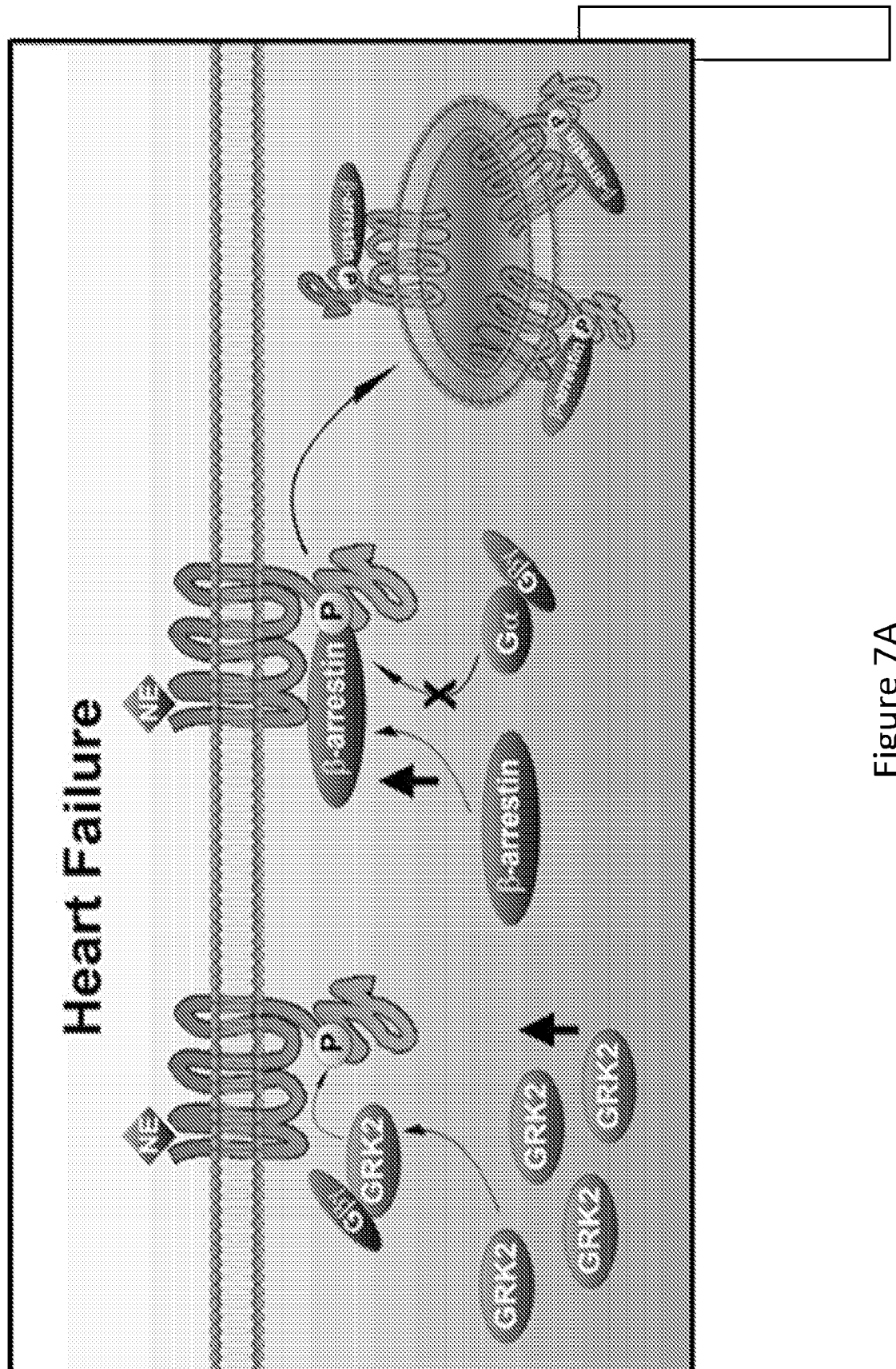


Figure 7A

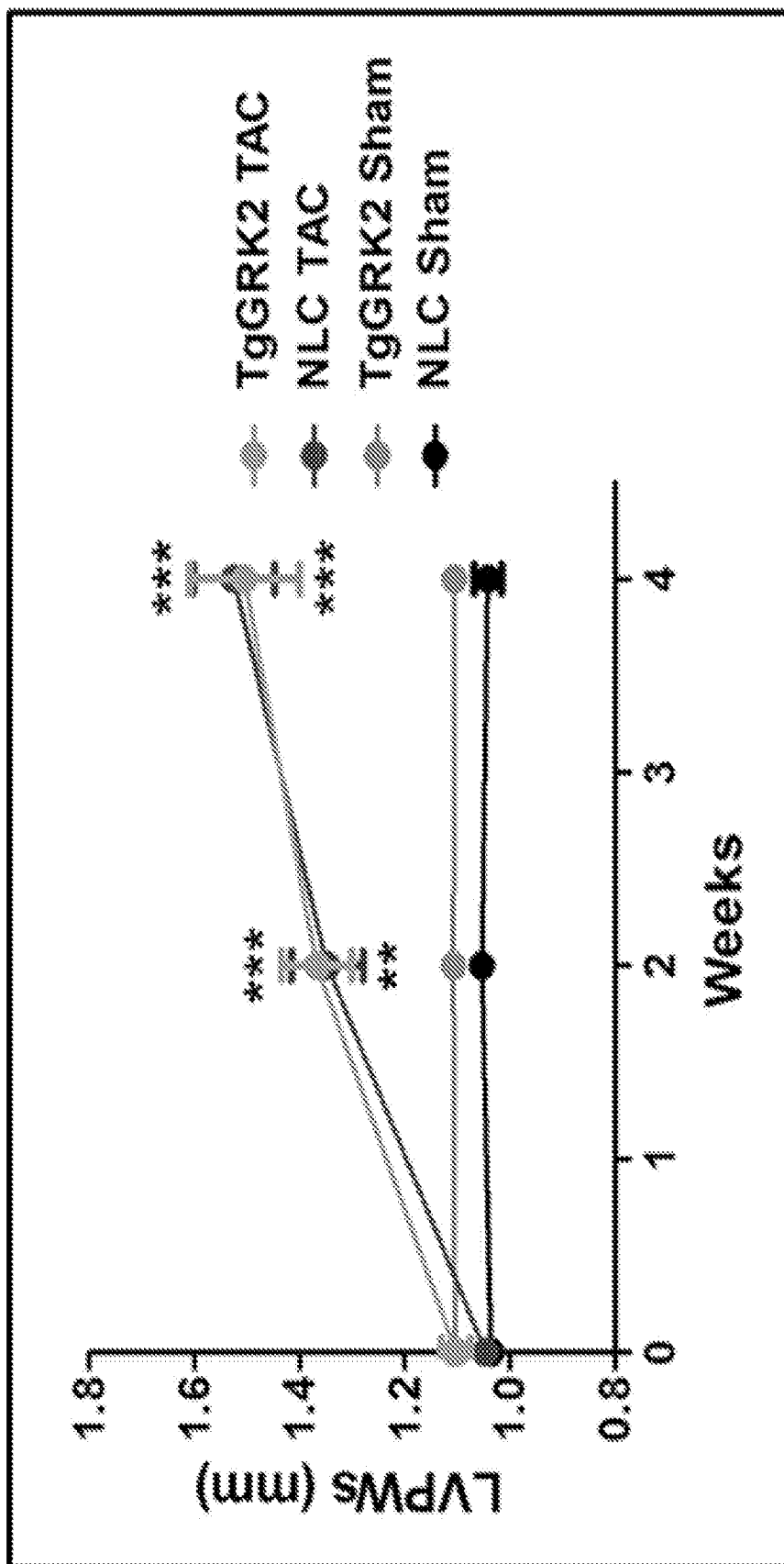


Figure 7B

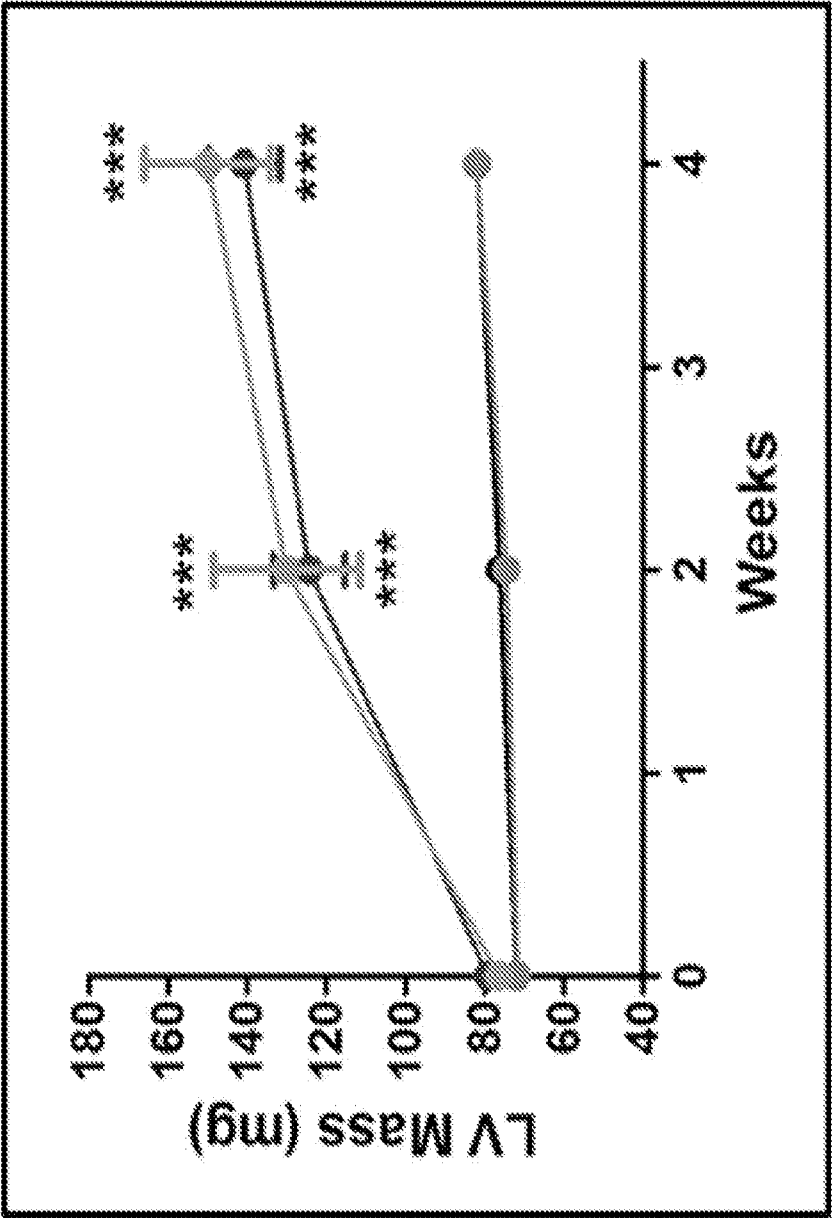


Figure 7C

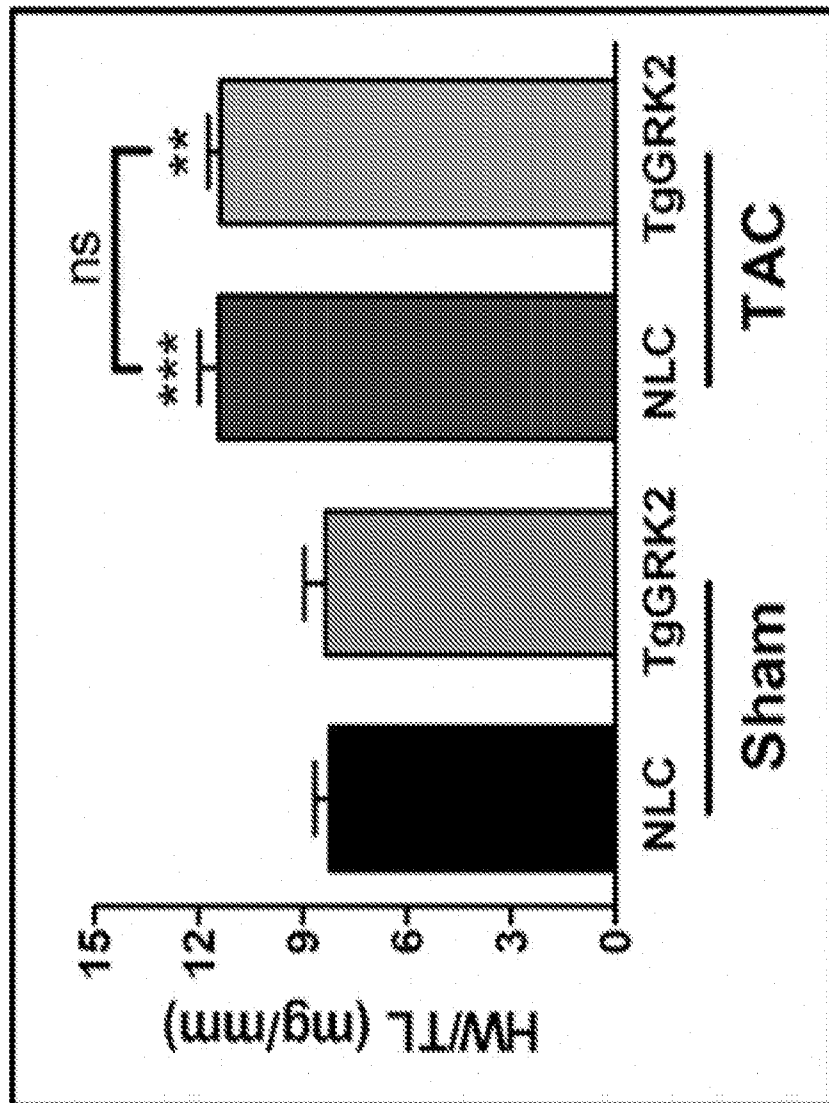


Figure 7D

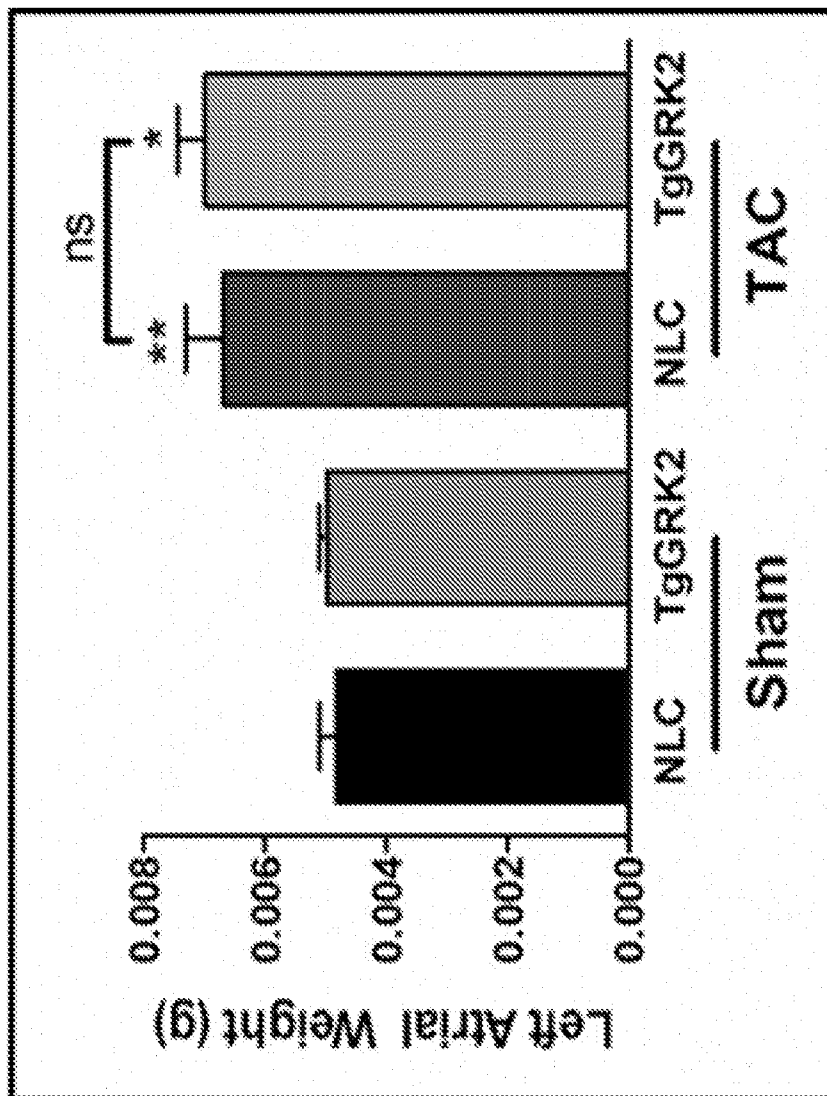


Figure 7E

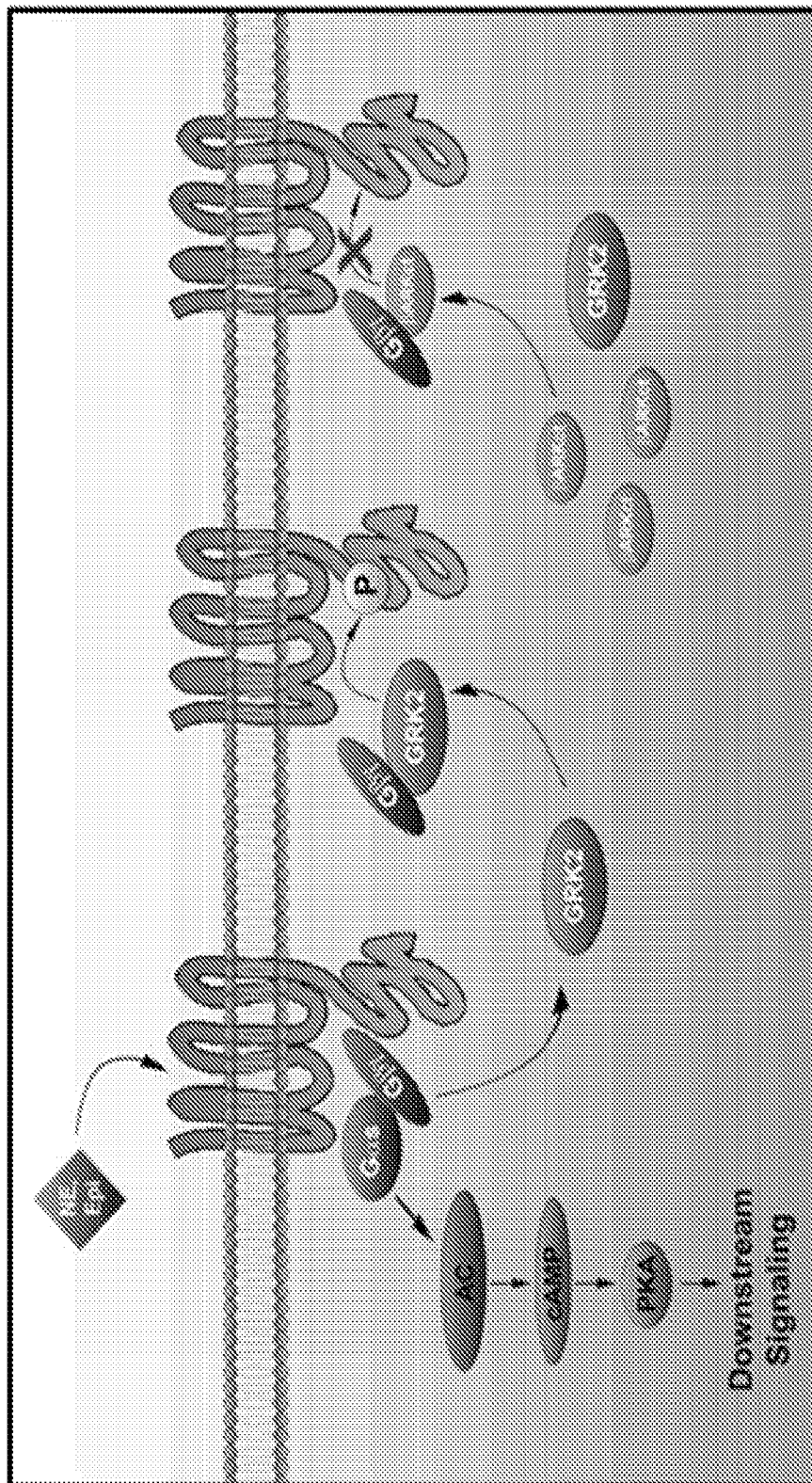


Figure 8A

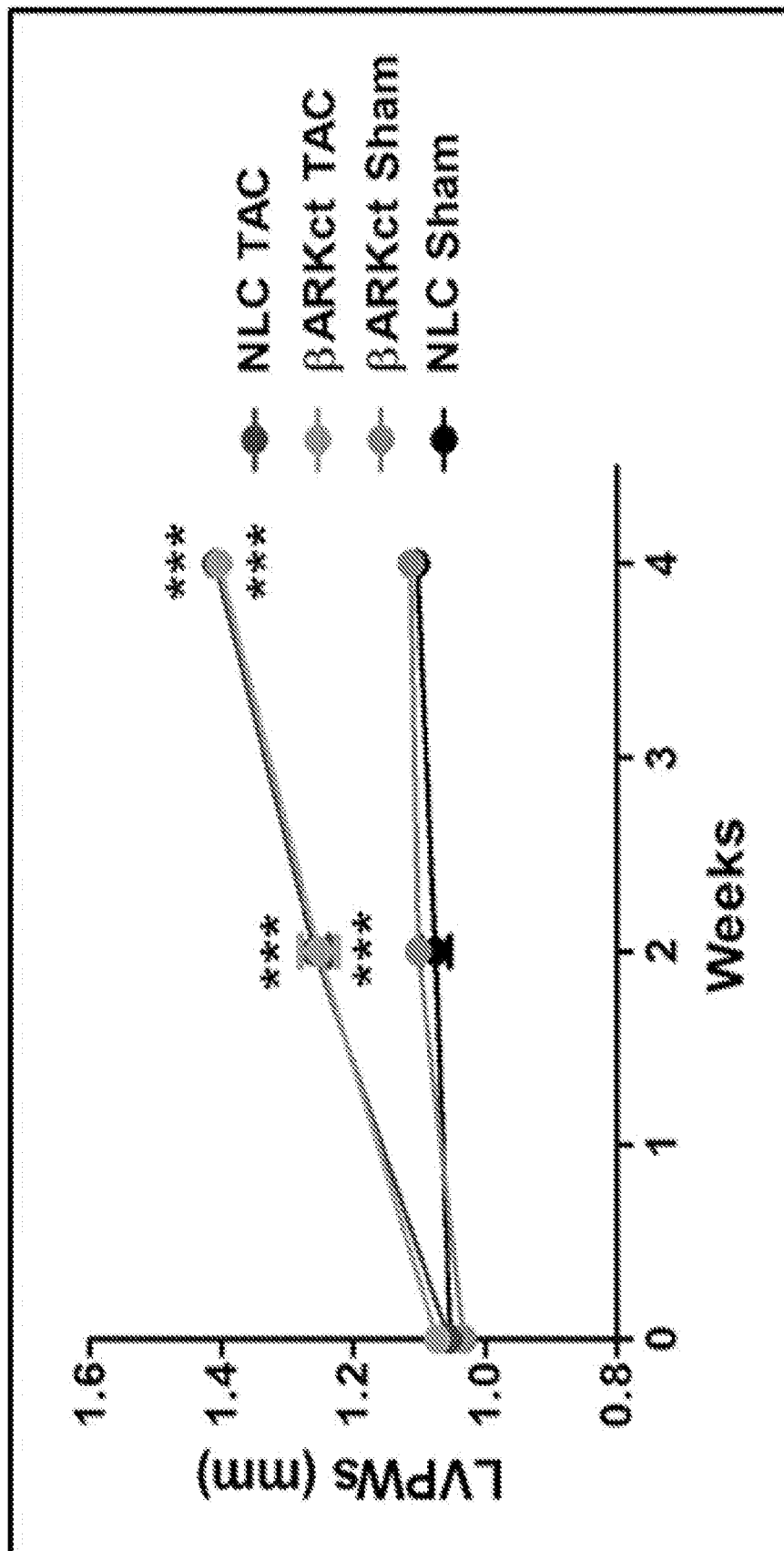


Figure 8B

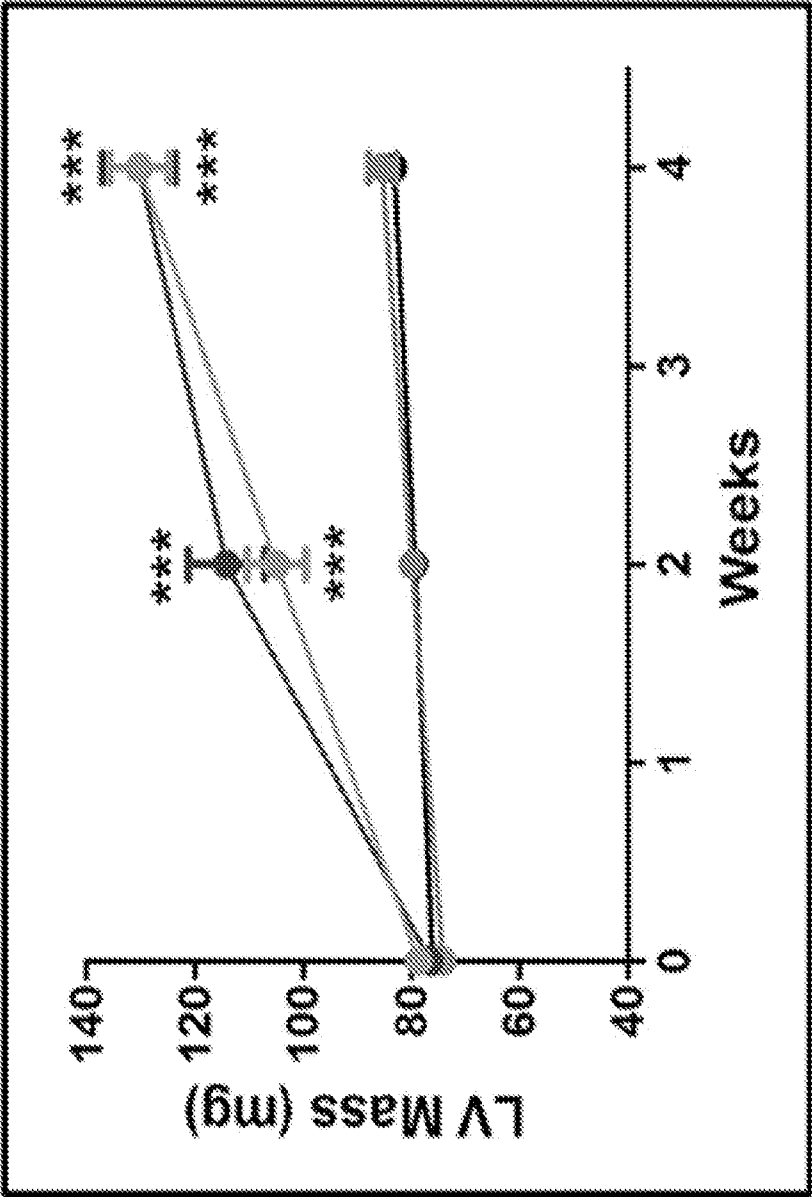


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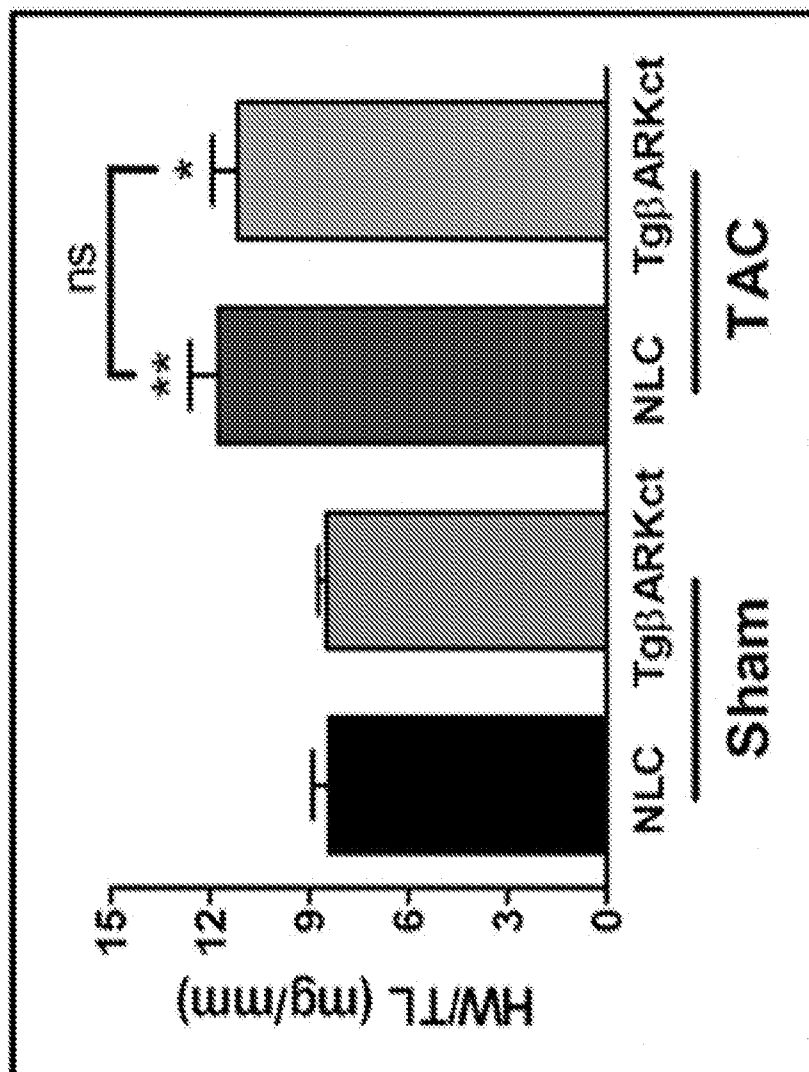


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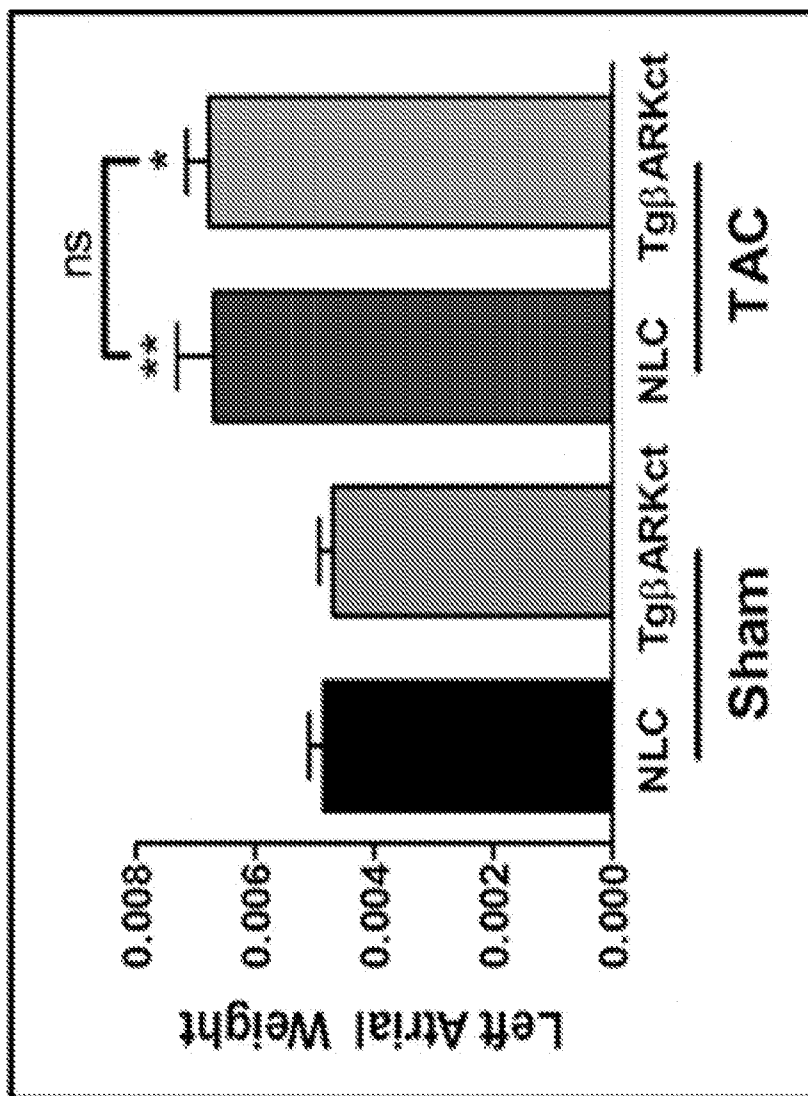


Figure 8E

Figure 9A

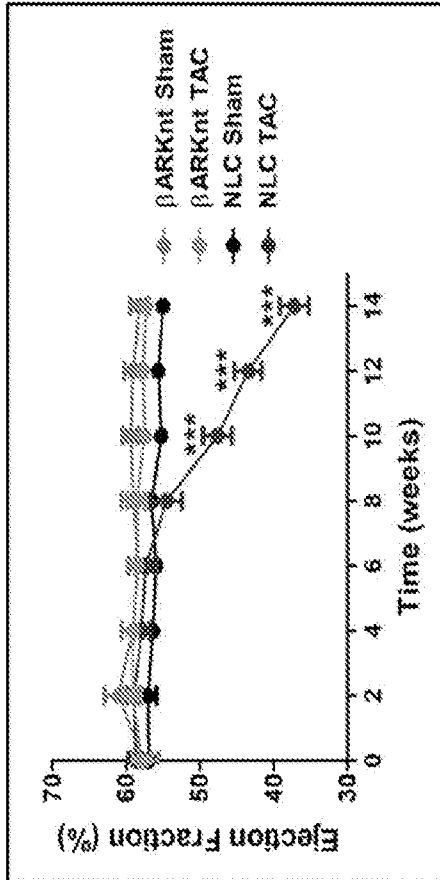


Figure 9B

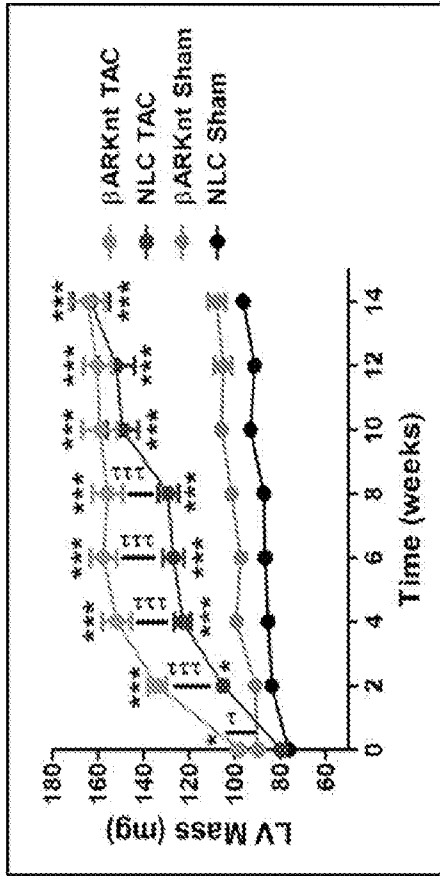


Figure 9C

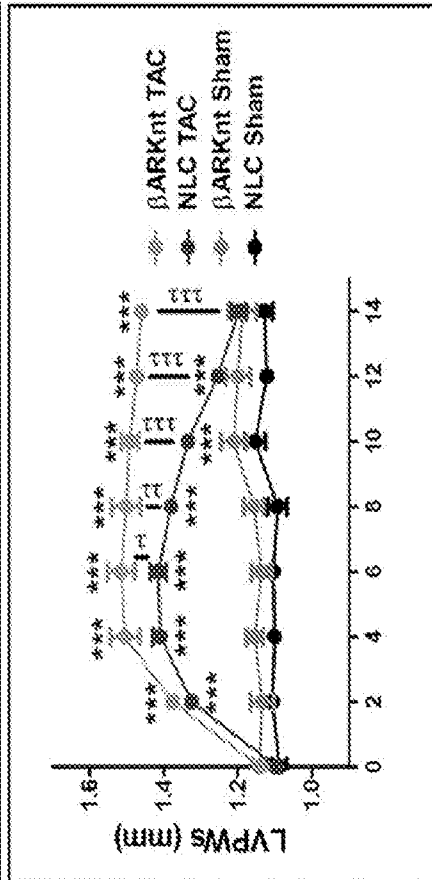


Figure 9D

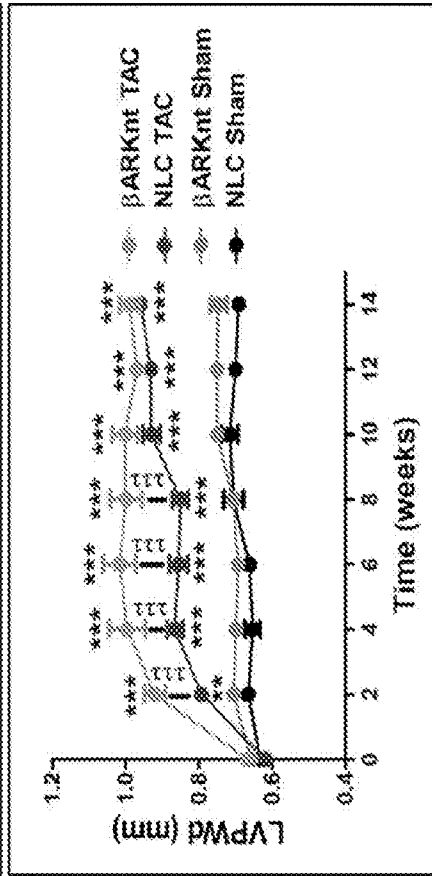


Figure 9

Figure 10B

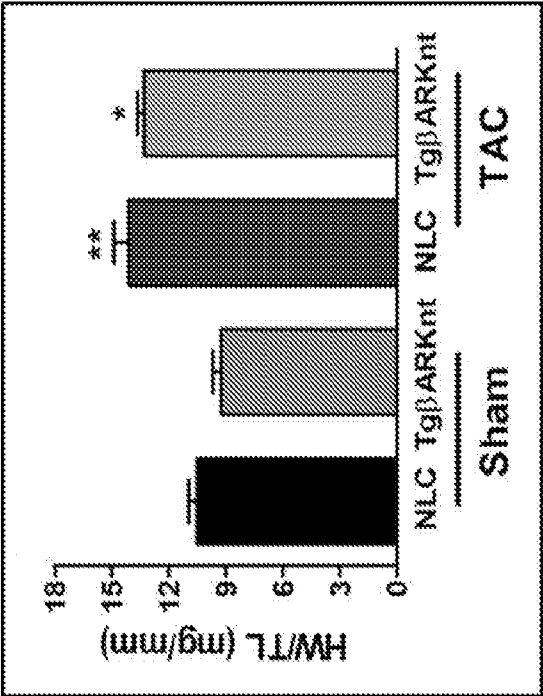


Figure 10A

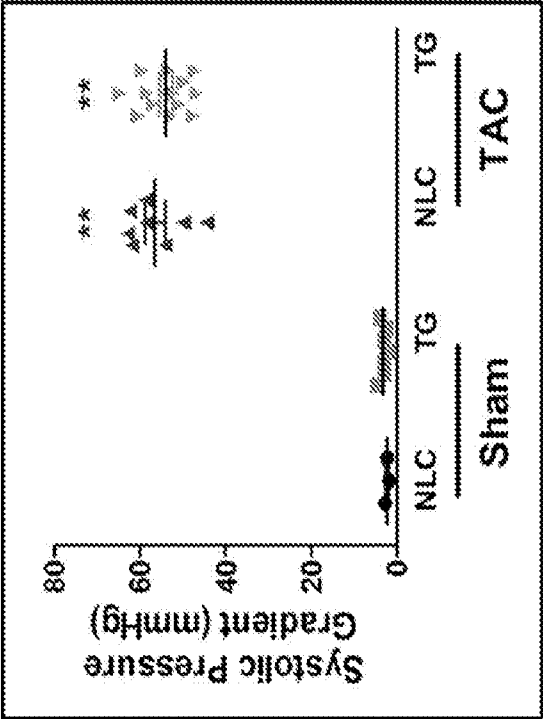


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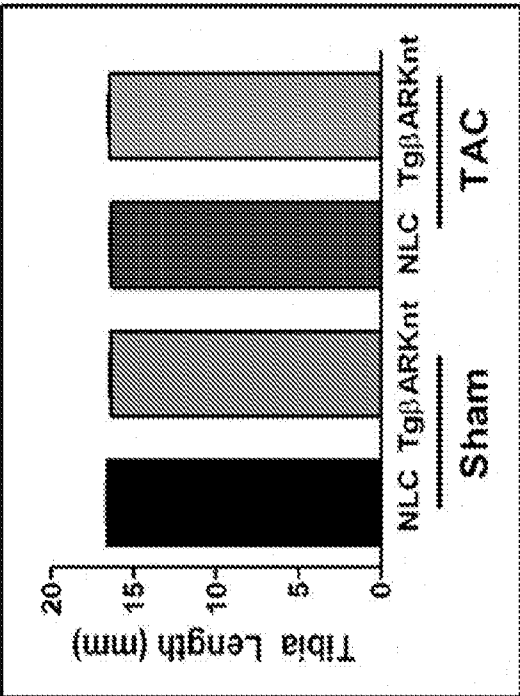


Figure 10C

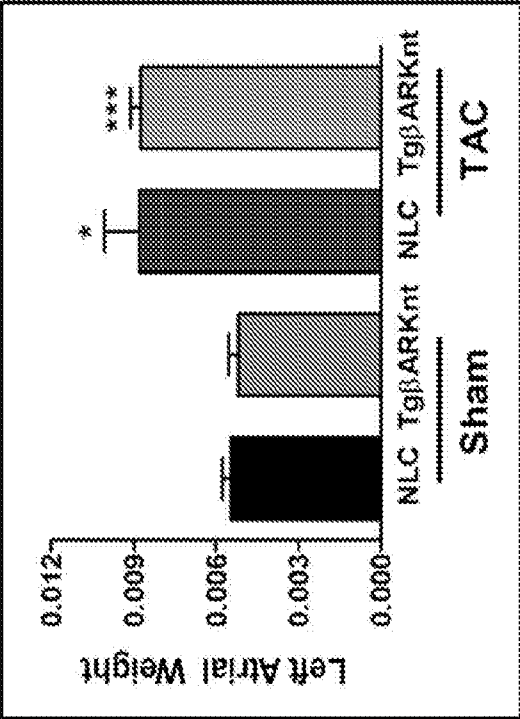


Figure 10

Figure 11C

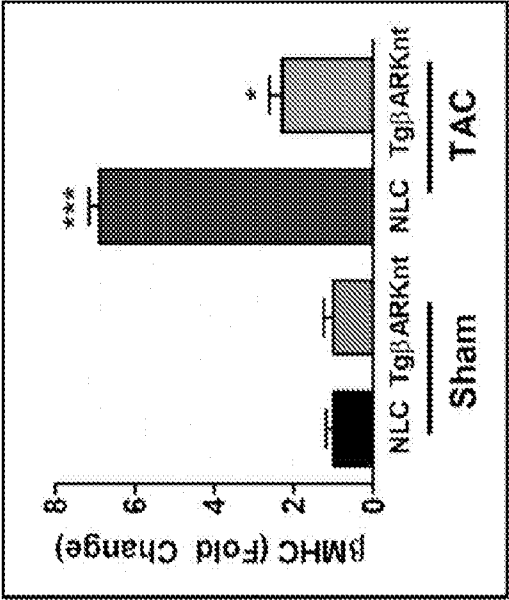


Figure 11B

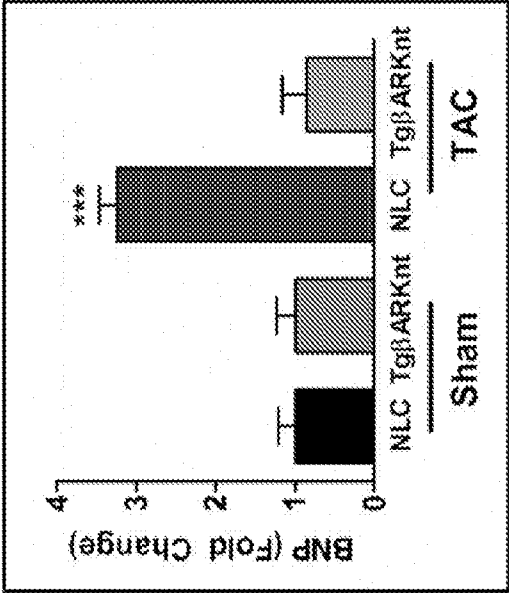


Figure 11A

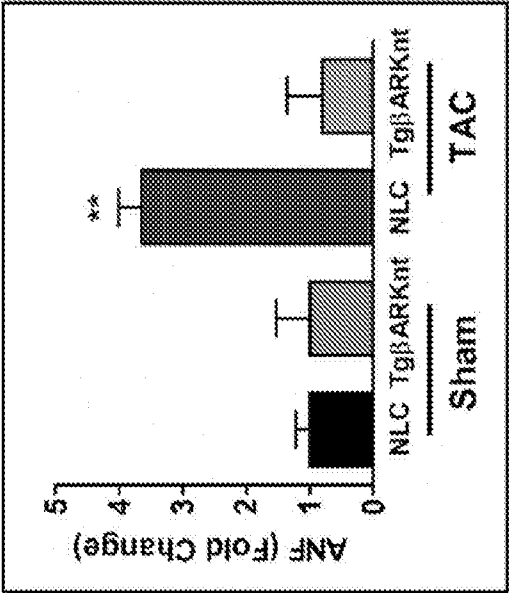


Figure 11

Figure 12B

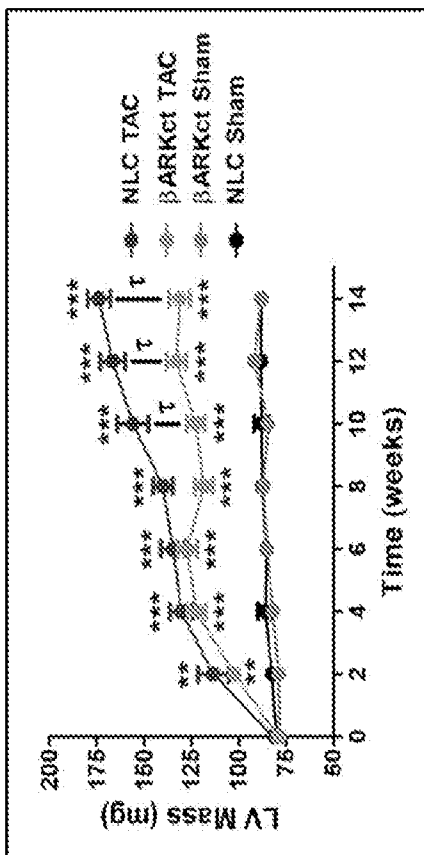


Figure 12A

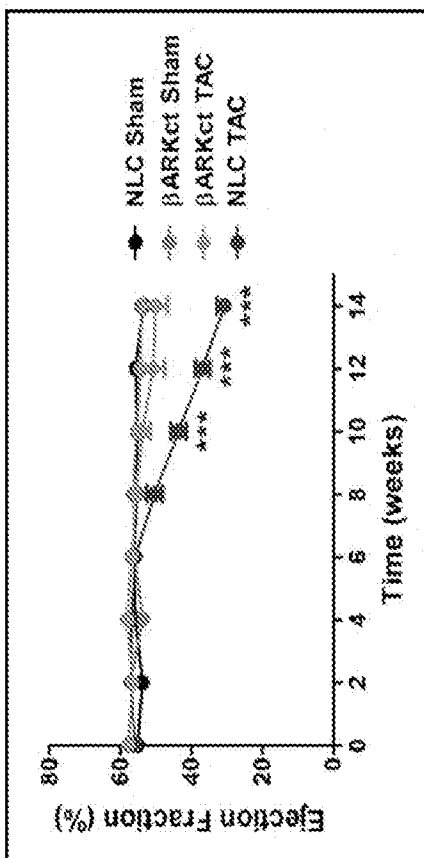


Figure 12D

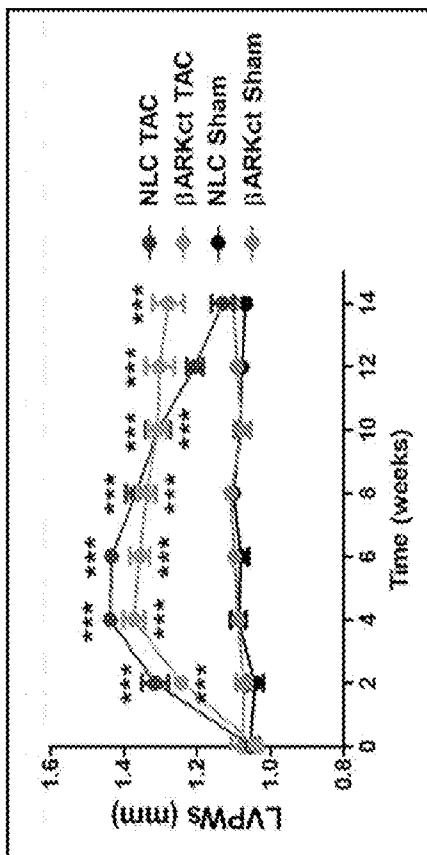


Figure 12C

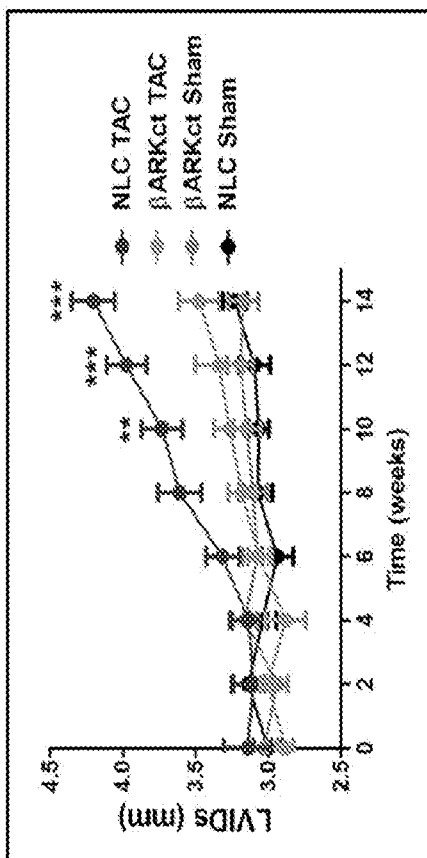


Figure 12

Figure 13A

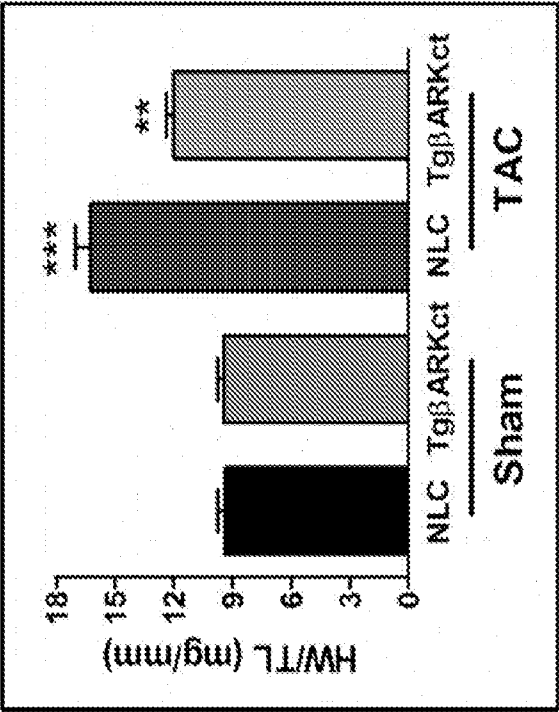


Figure 13B

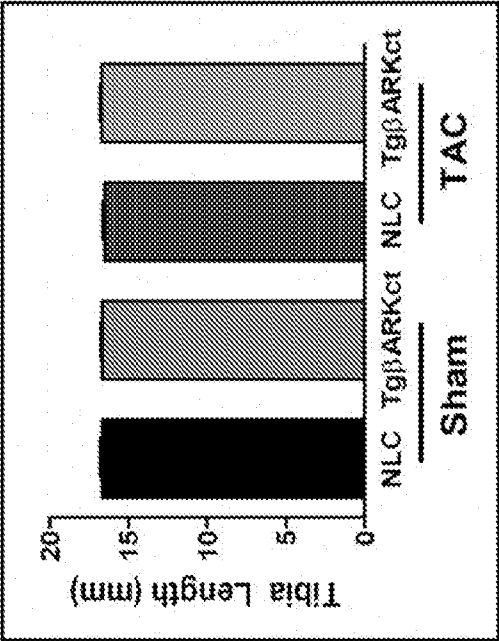
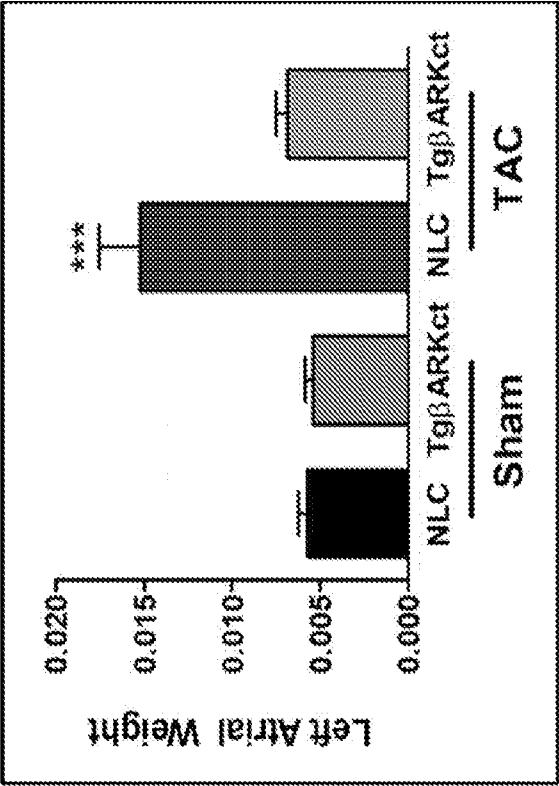


Figure 13C

Figure 13

Figure 14C

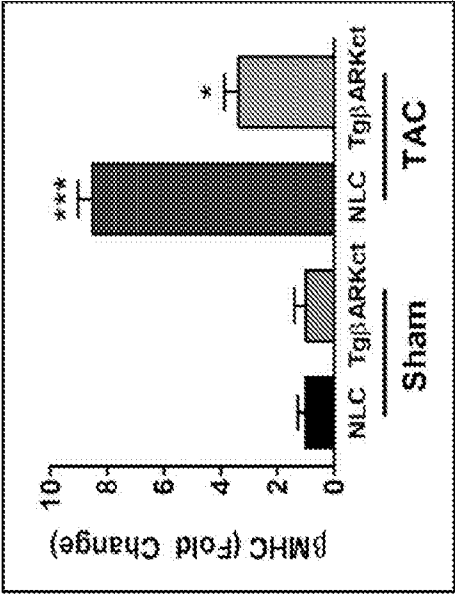


Figure 14B

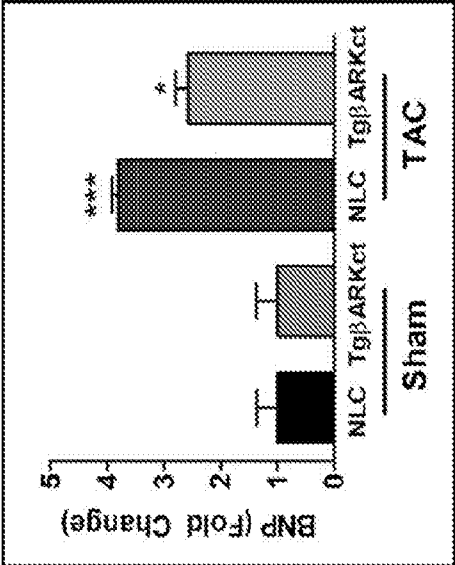


Figure 14A

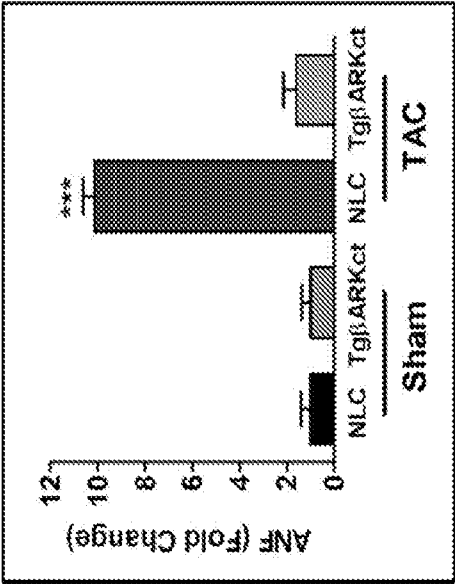


Figure 14

Figure 15A

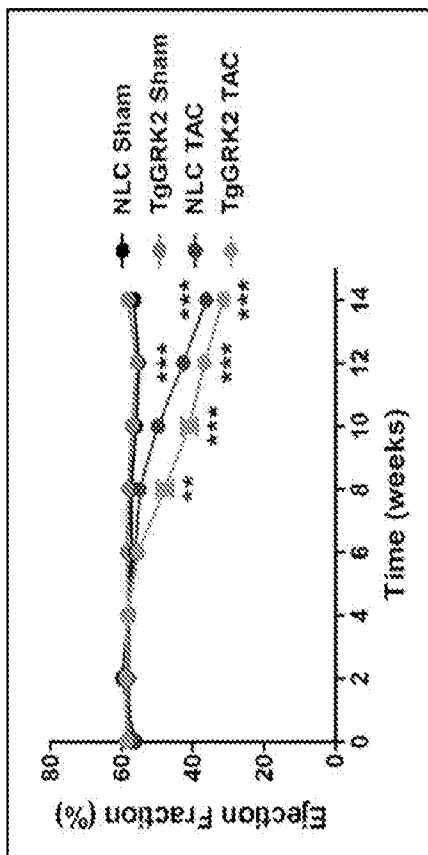


Figure 15B

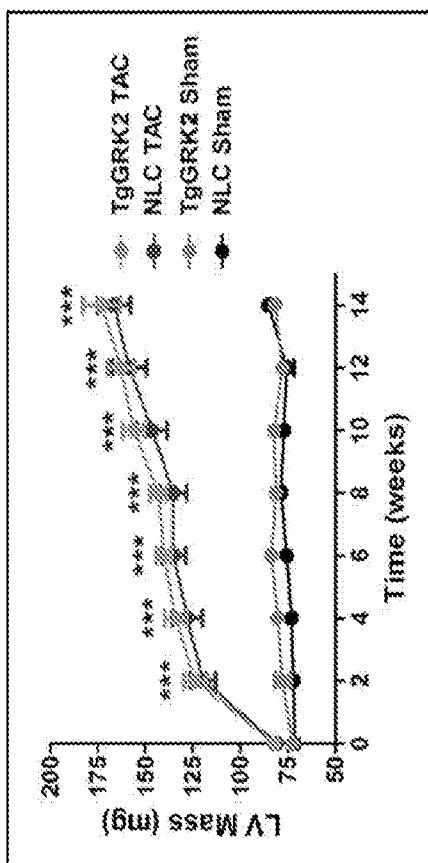


Figure 15C

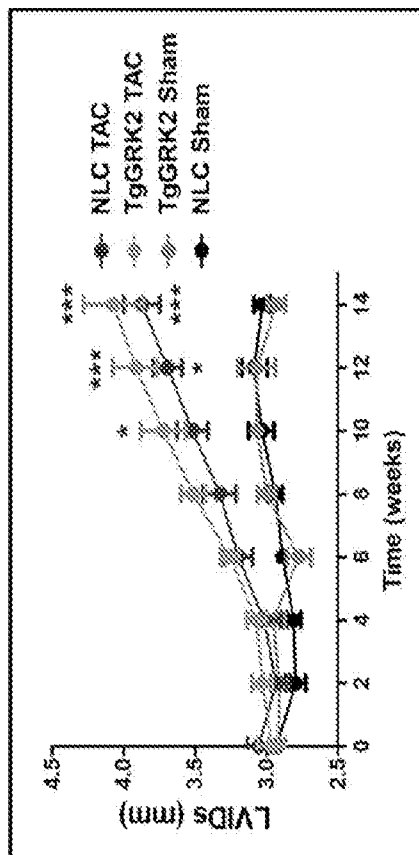


Figure 15D

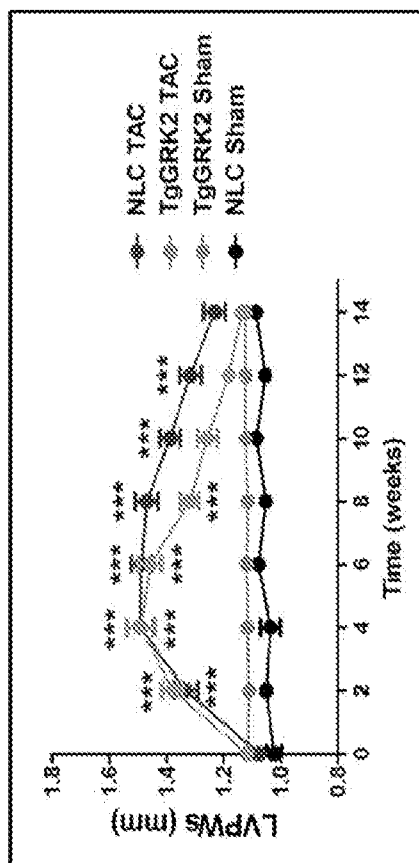


Figure 15

Figure 16A

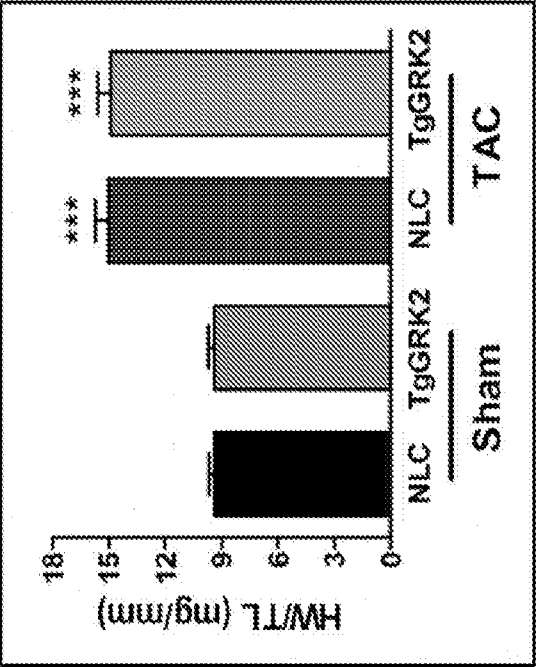


Figure 16B

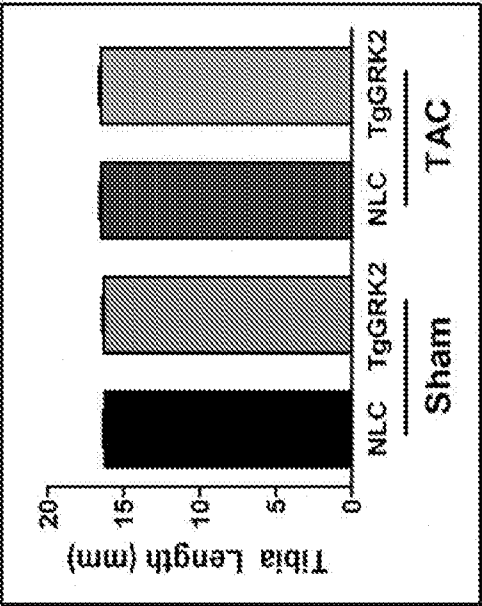
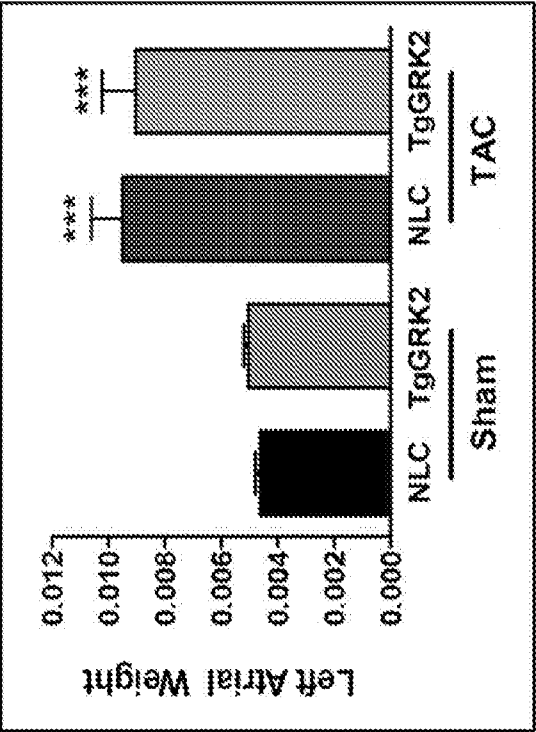


Figure 16C

Figure 16

Figure 17C

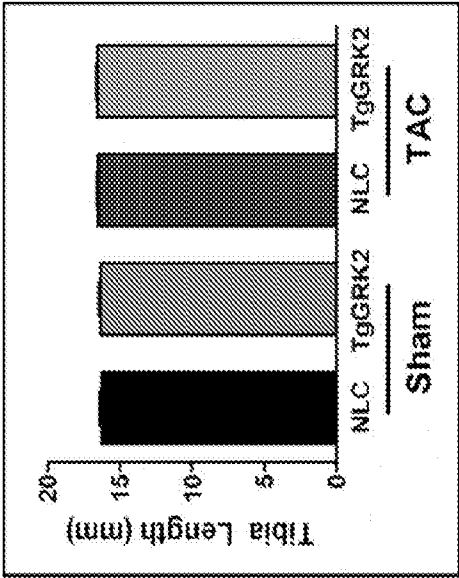


Figure 17B

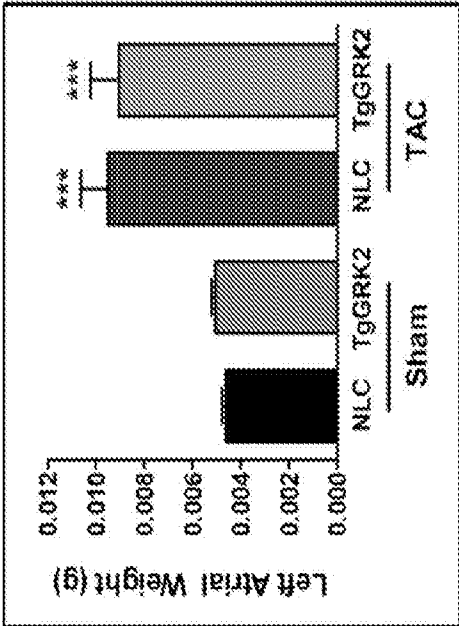


Figure 17A

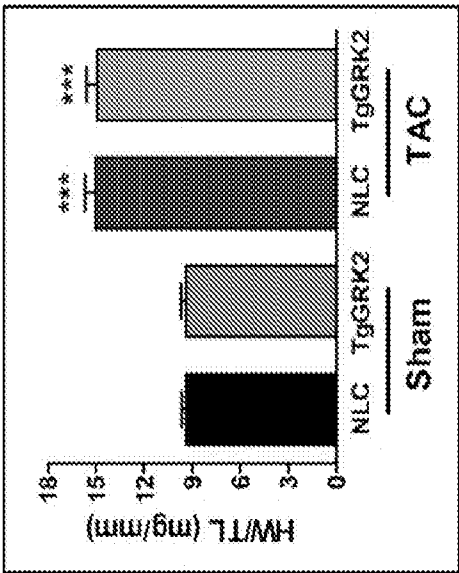


Figure 17

Figure 18A

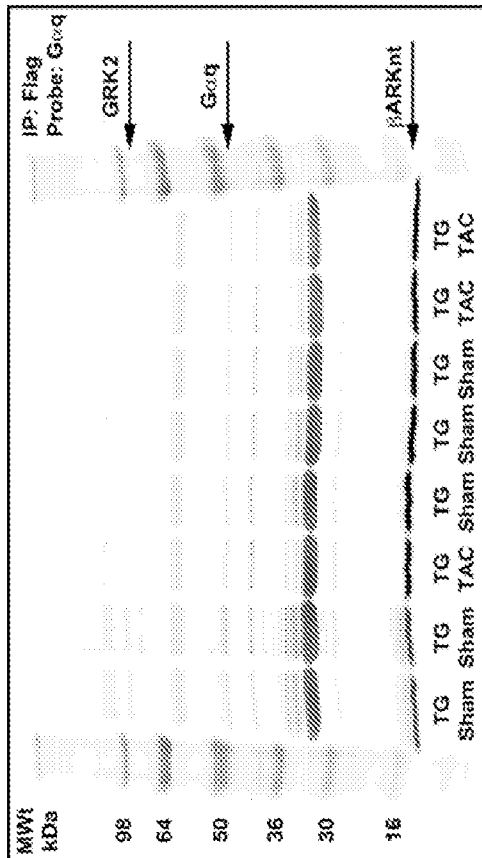


Figure 18B

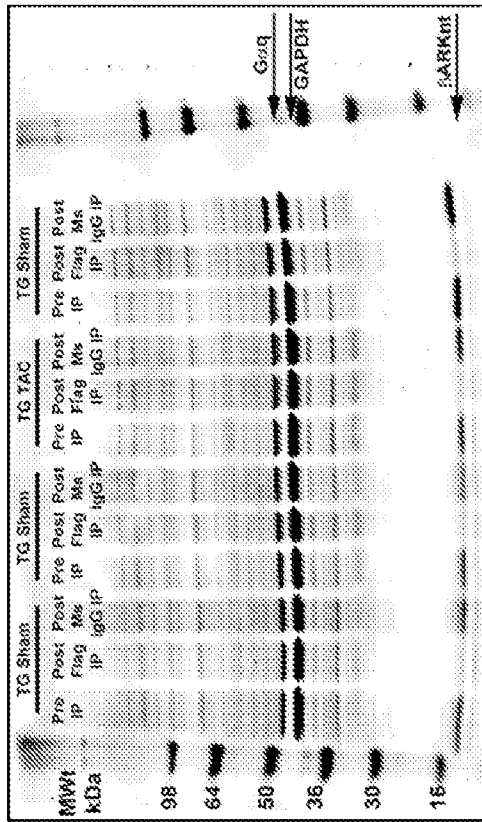


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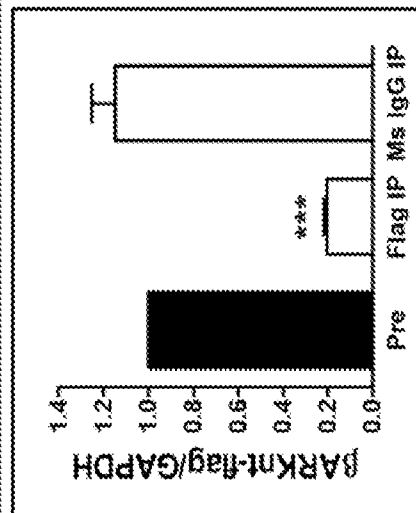


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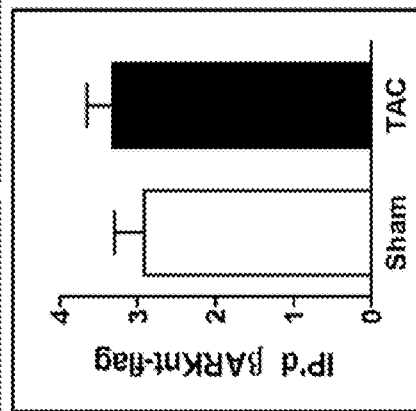


Figure 18E

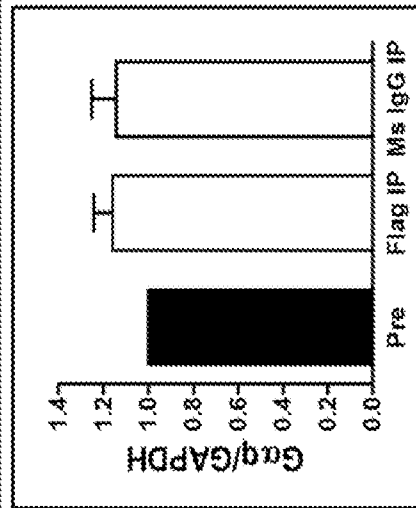


Figure 18

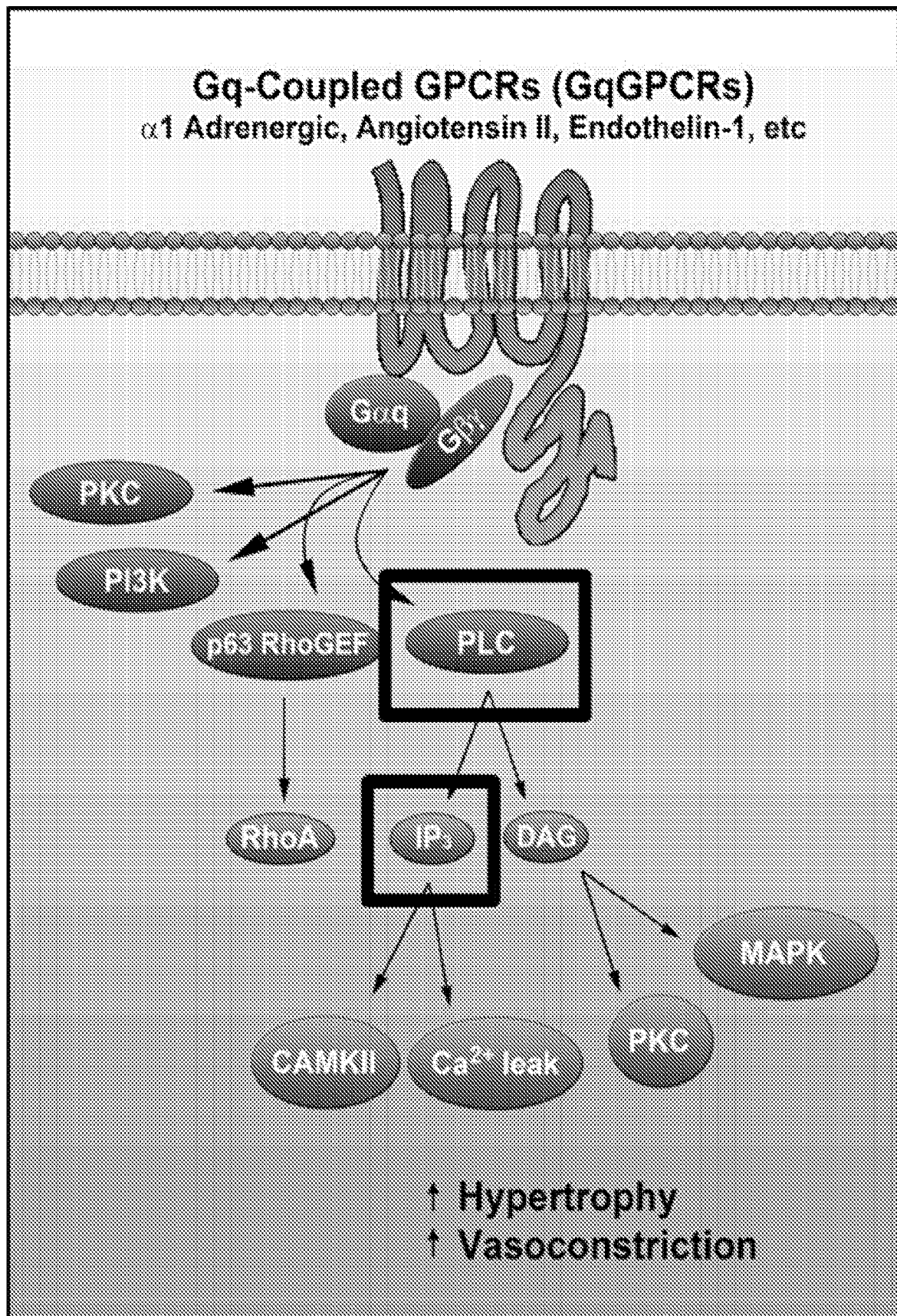


Figure 19A

SUBSTITUTE SHEET (RULE 26)

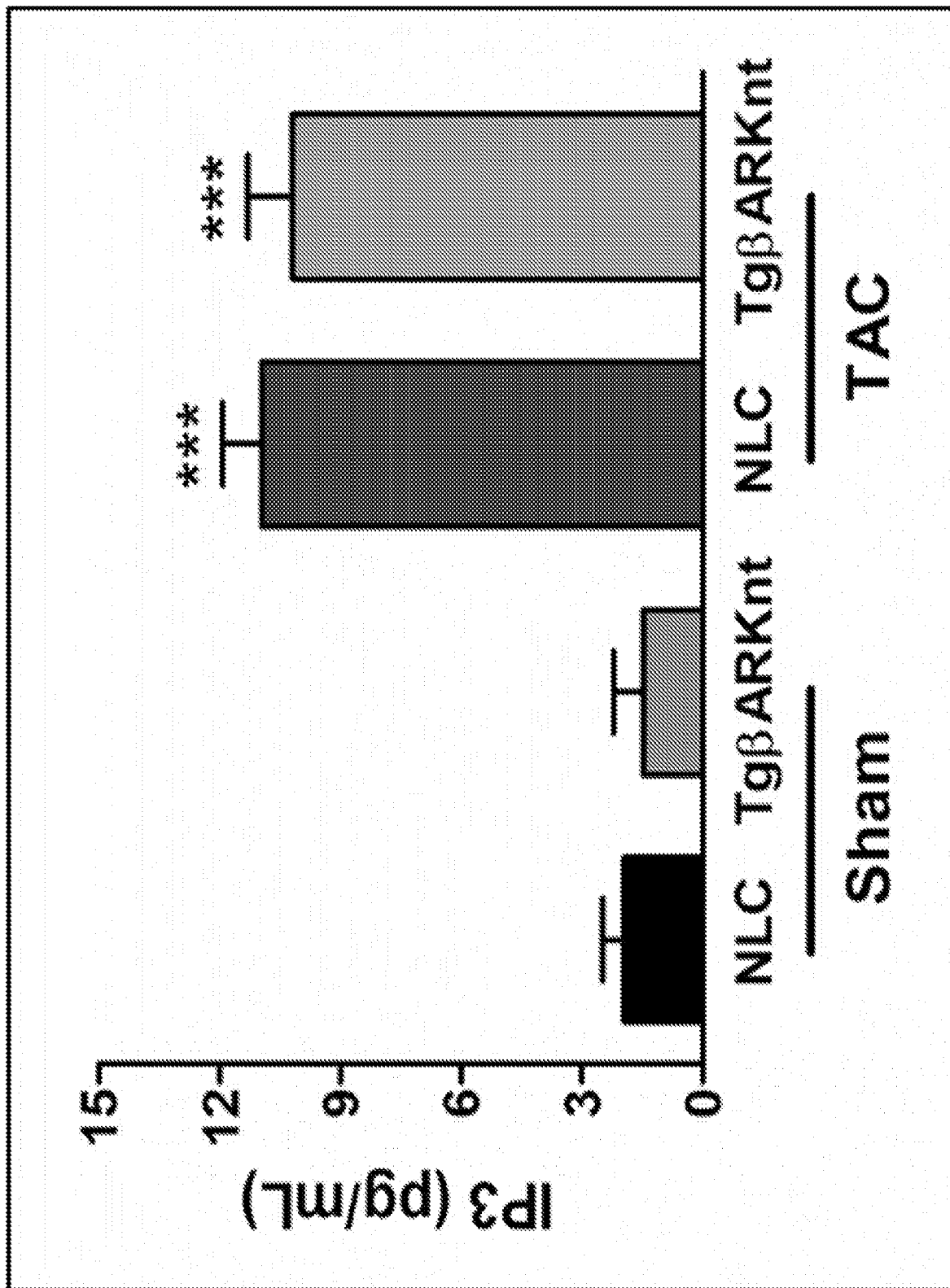


Figure 19B

Figure 20B

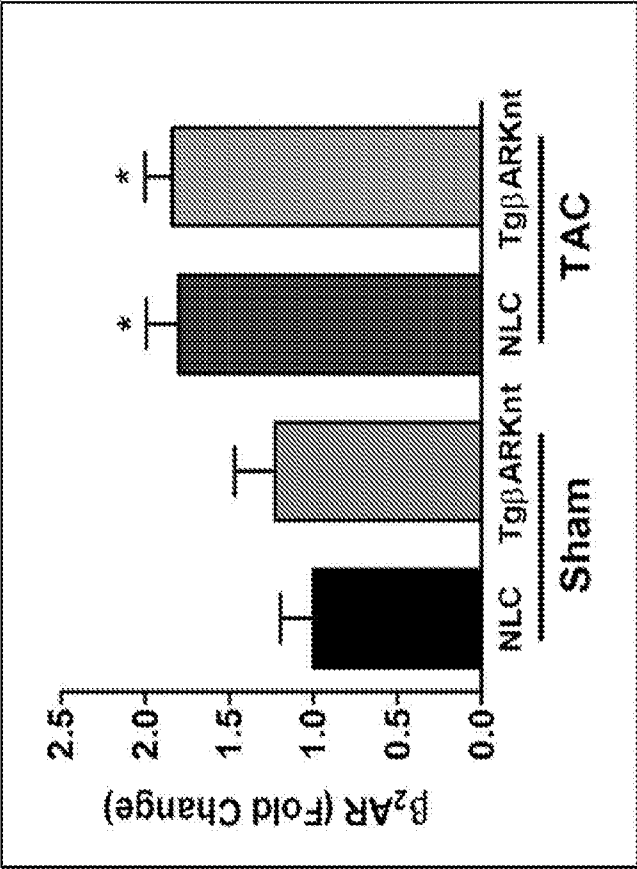


Figure 20A

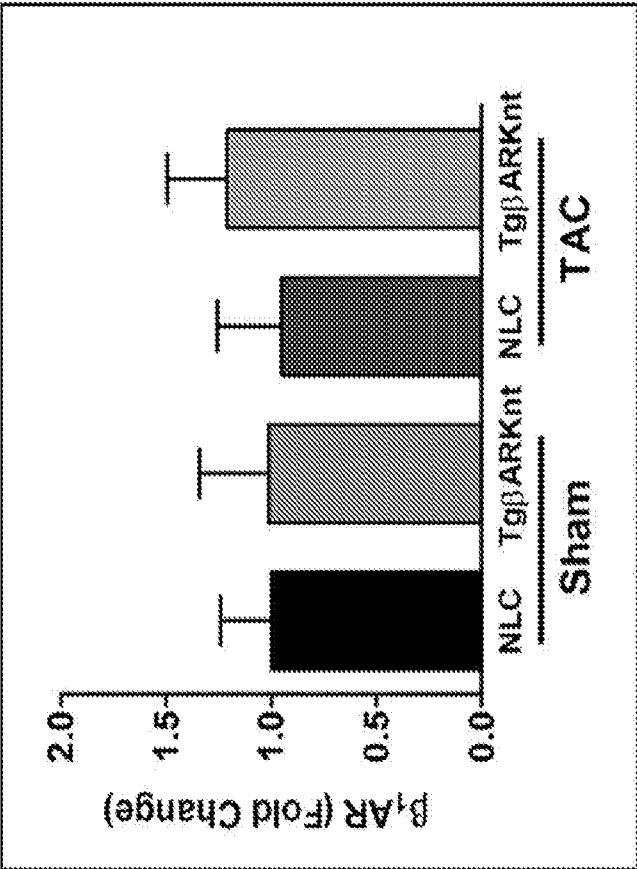


Figure 20

Figure 21B

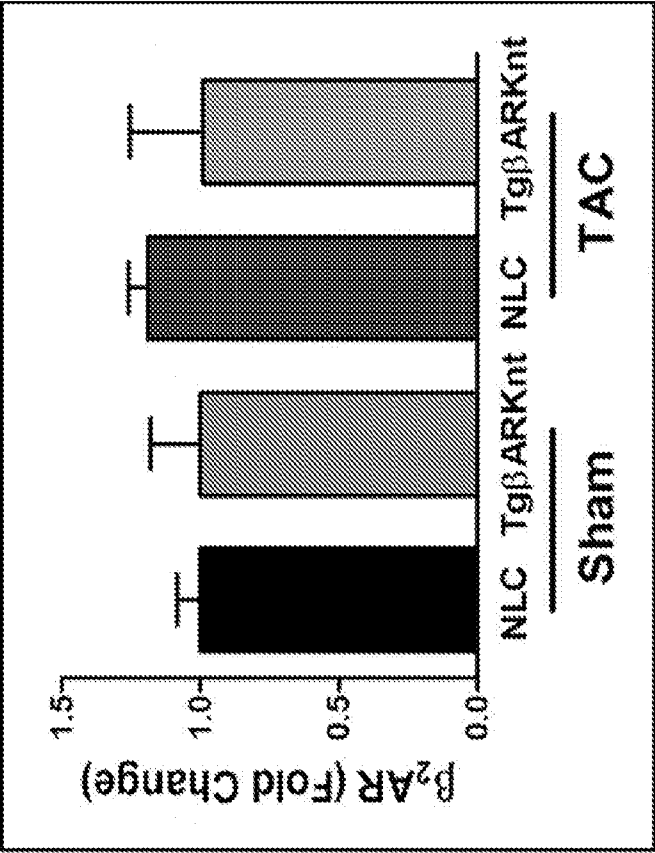


Figure 21A

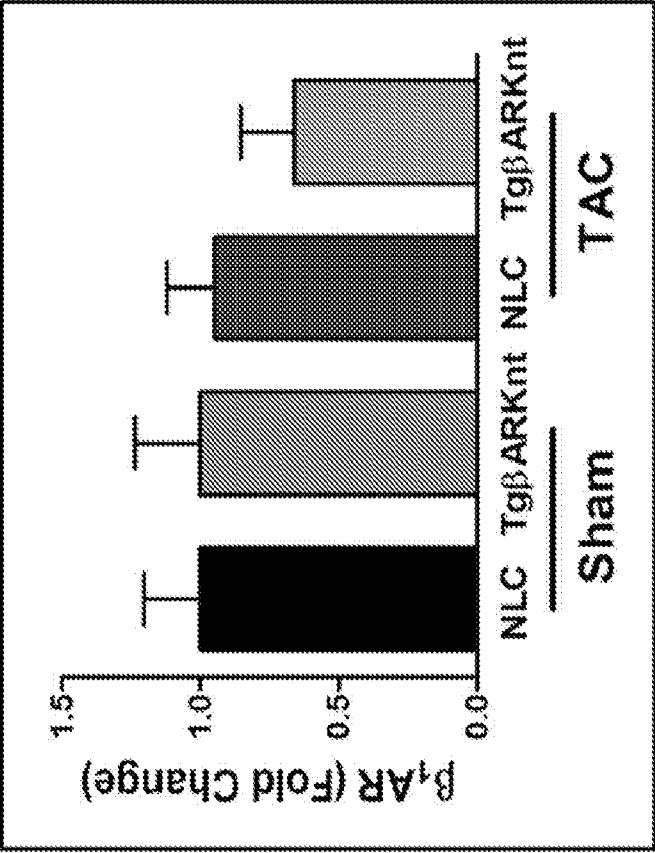


Figure 21

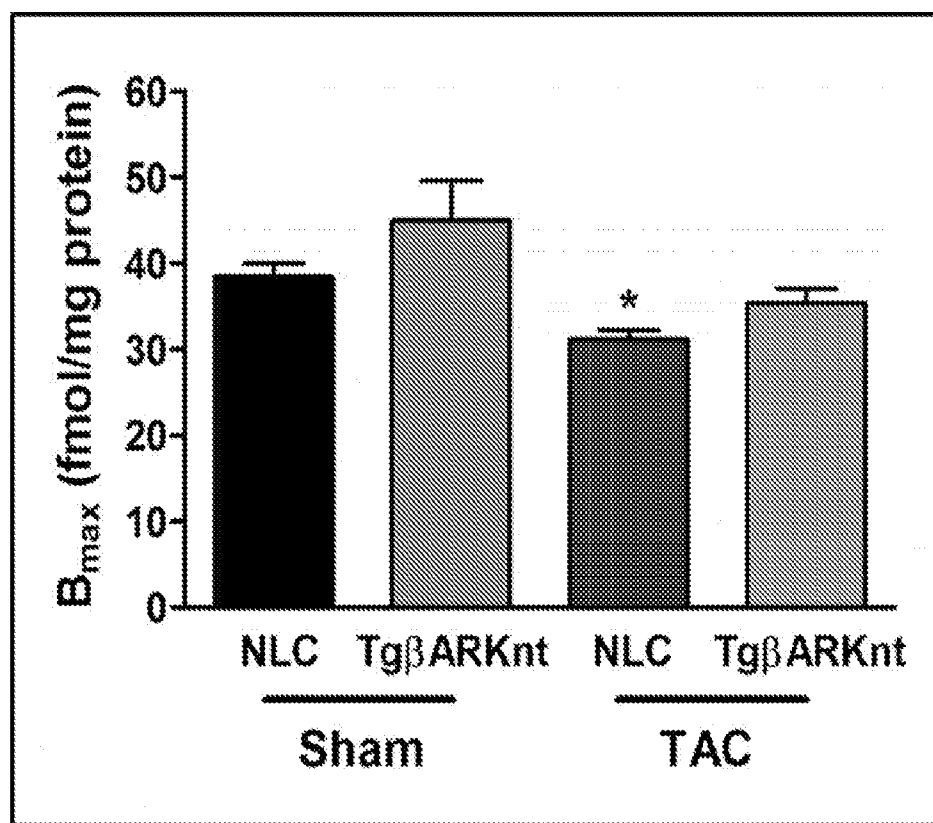


Figure 22
SUBSTITUTE SHEET (RULE 26)

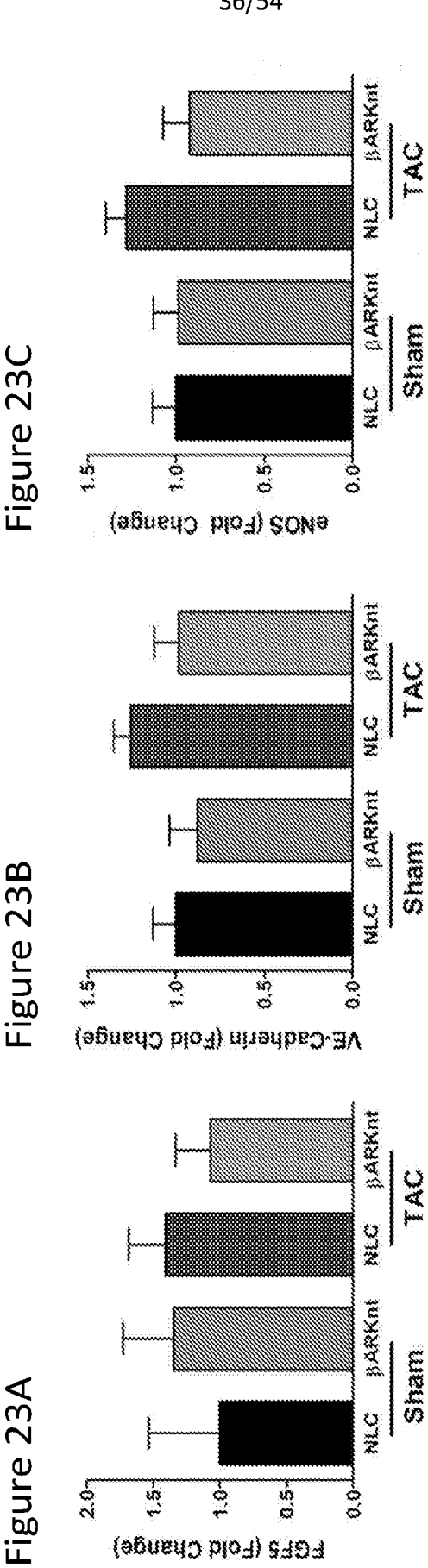


Figure 23

Figure 24A

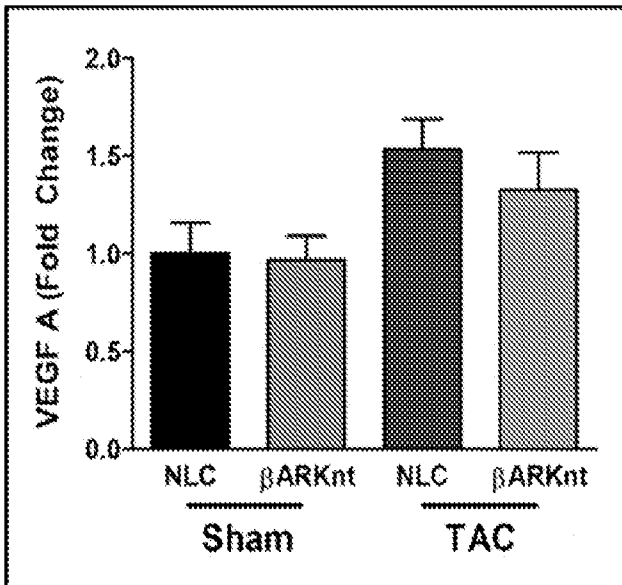


Figure 24B

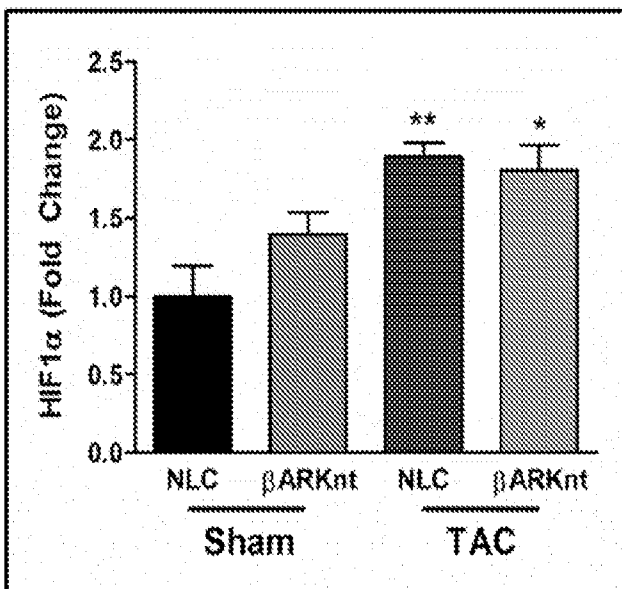
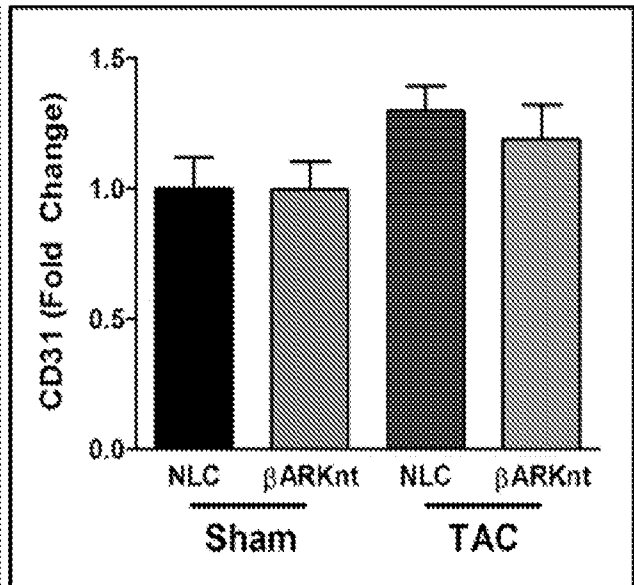


Figure 24C

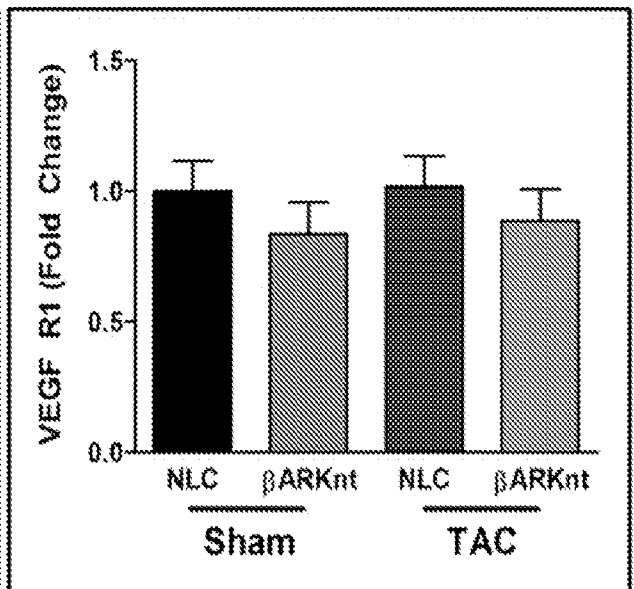


Figure 24D

Figure 25C

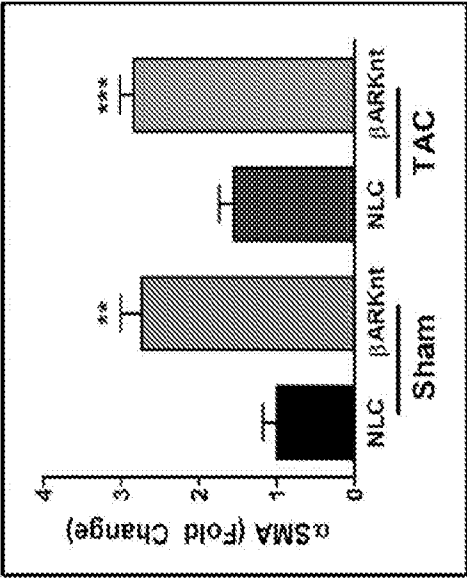


Figure 25B

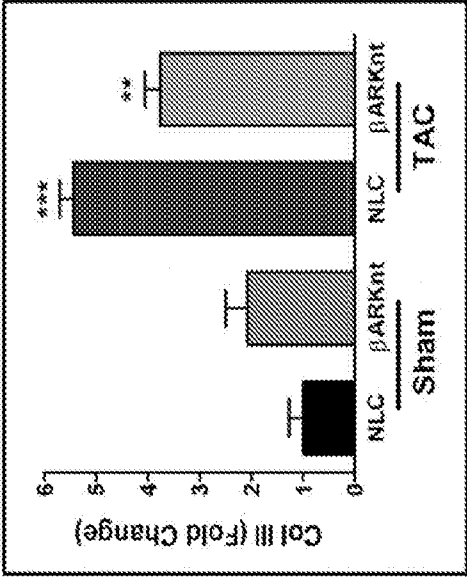


Figure 25A

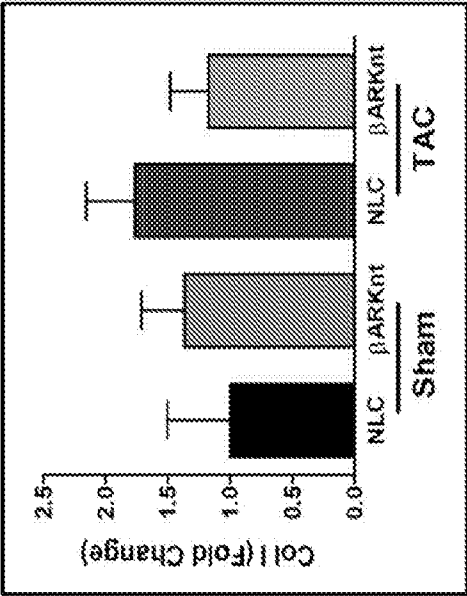


Figure 25

Figure 26A

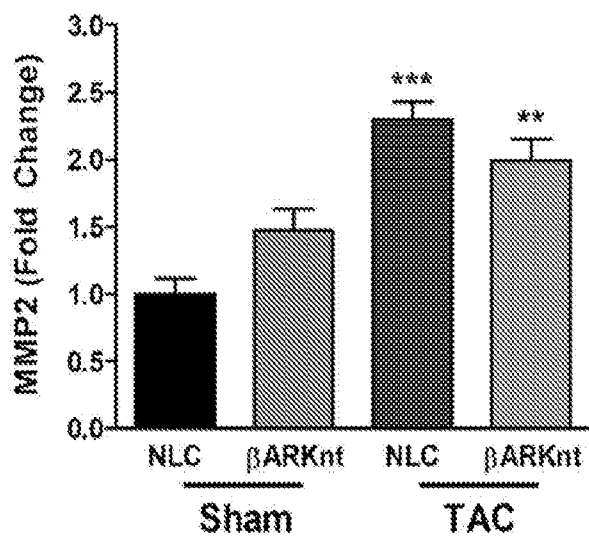


Figure 26B

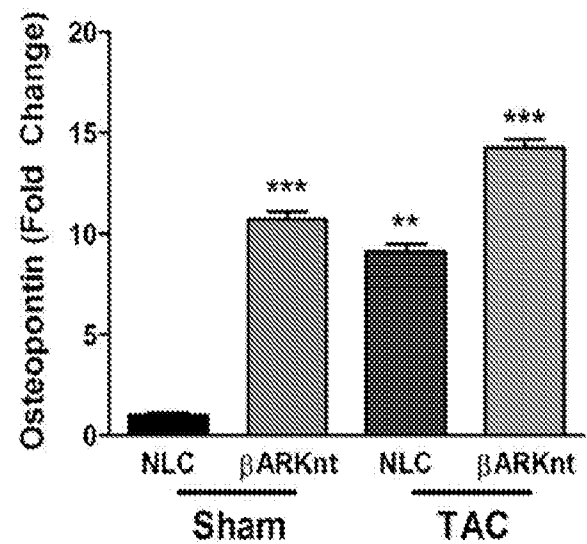
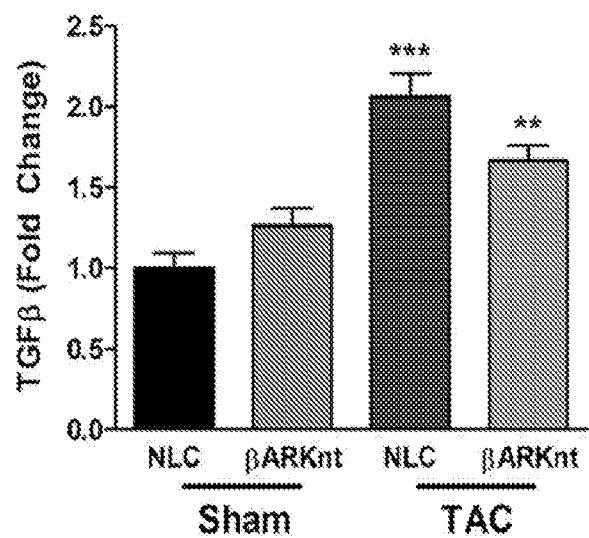
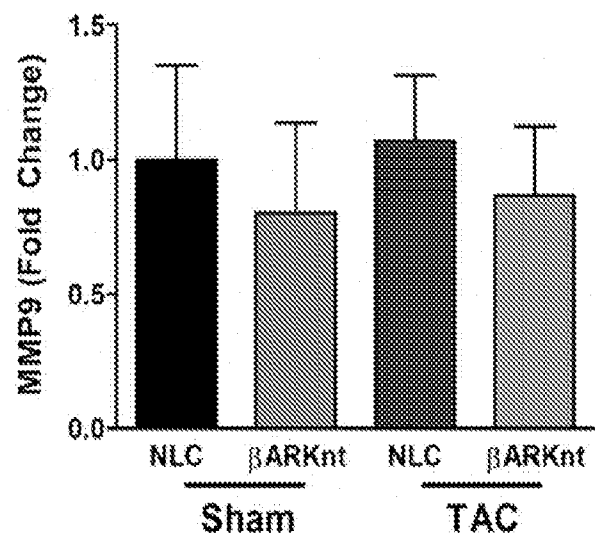


Figure 26C

Figure 26D

Figure 27A

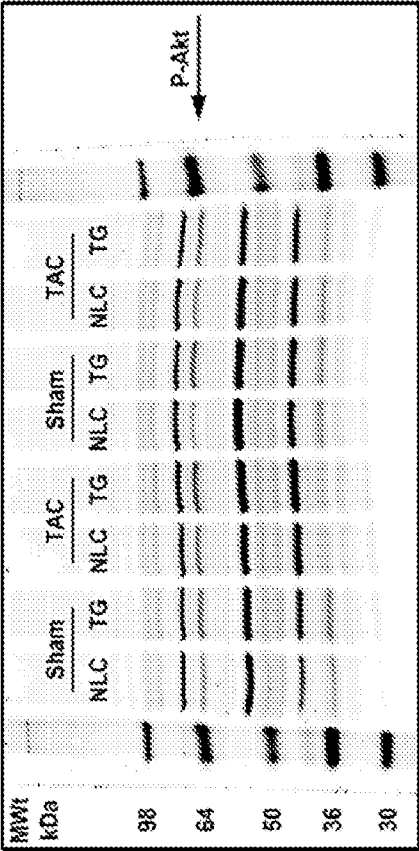


Figure 27B

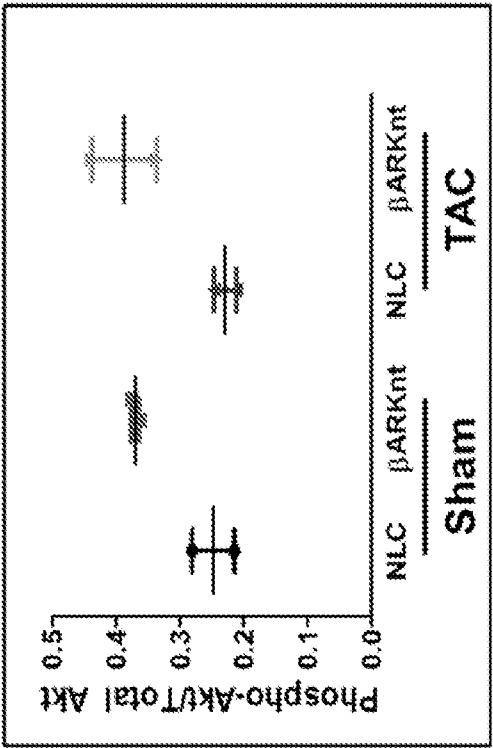
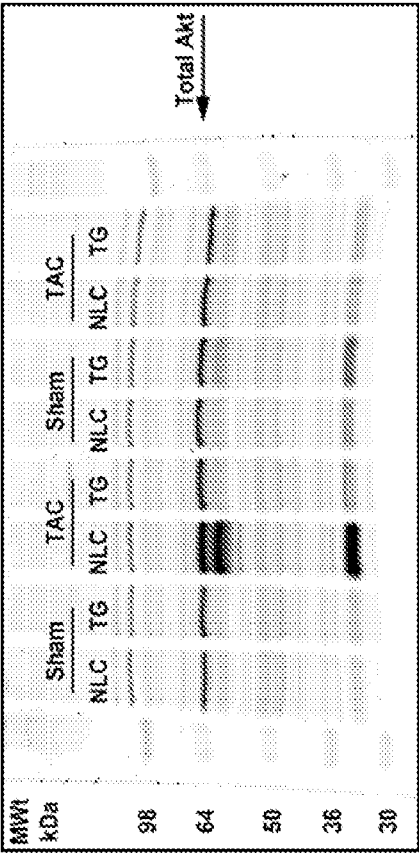


Figure 27C

Figure 27

Figure 28B

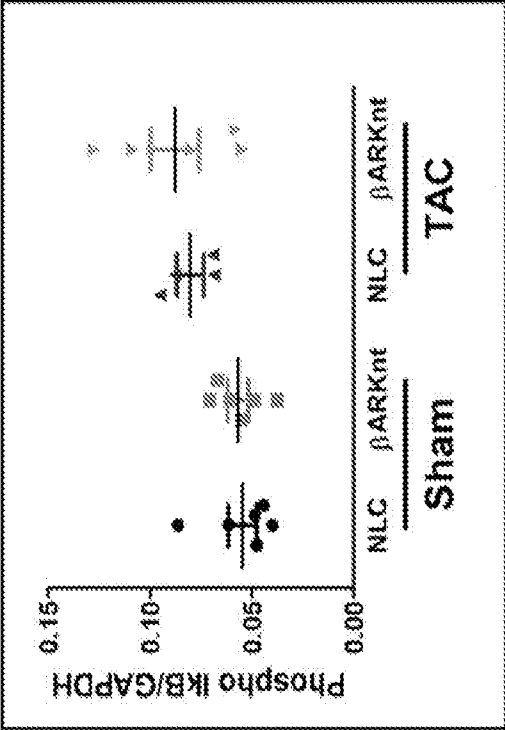


Figure 28A

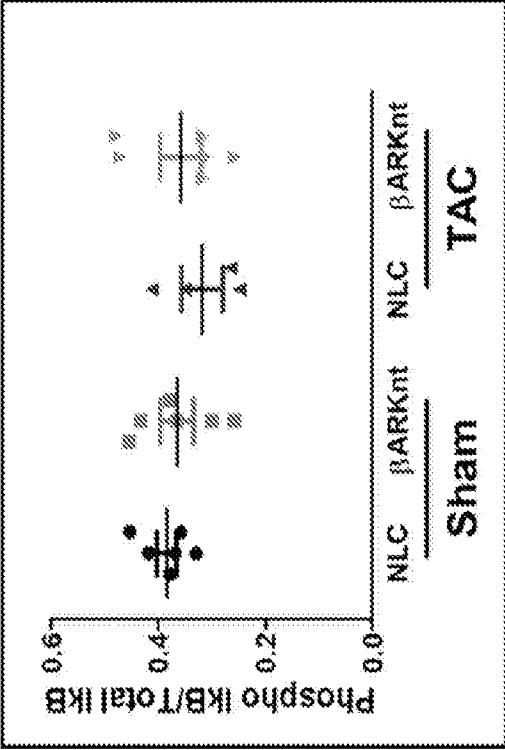


Figure 28D

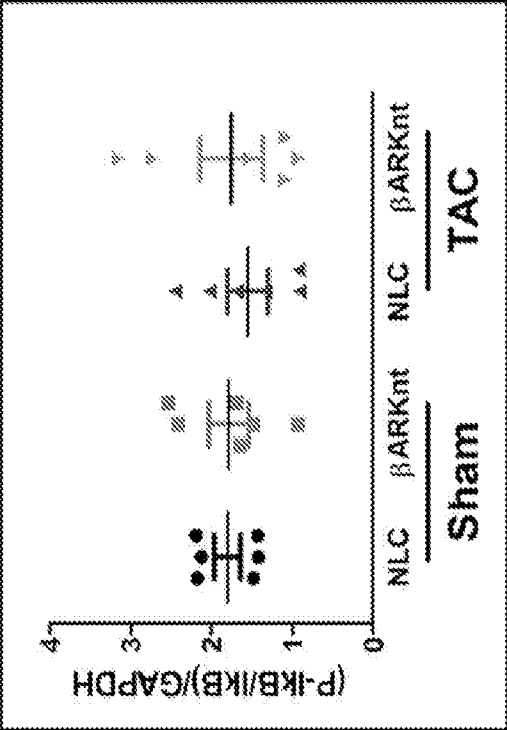


Figure 28C

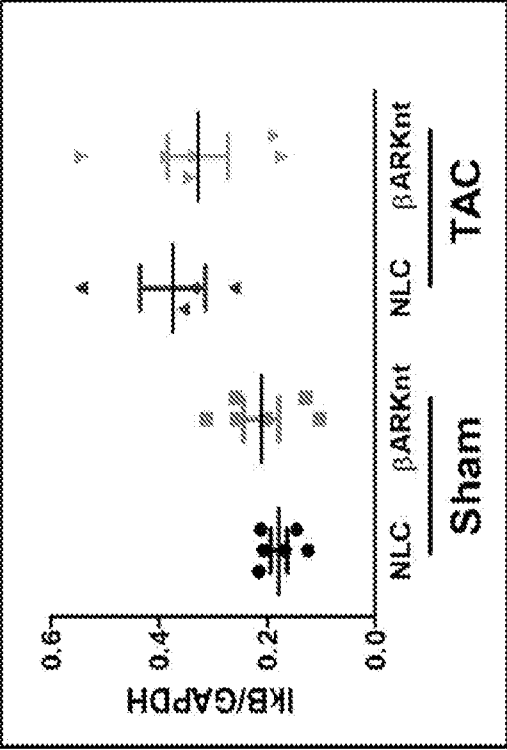


Figure 28

Figure 29A

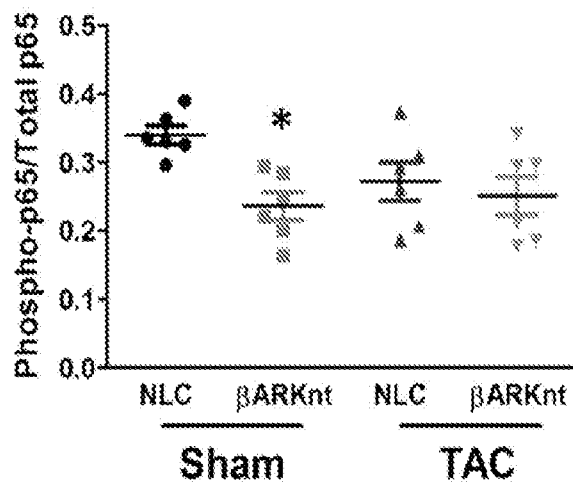


Figure 29B

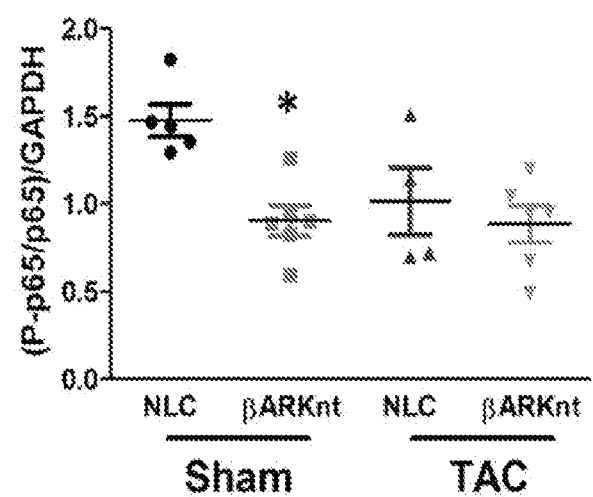
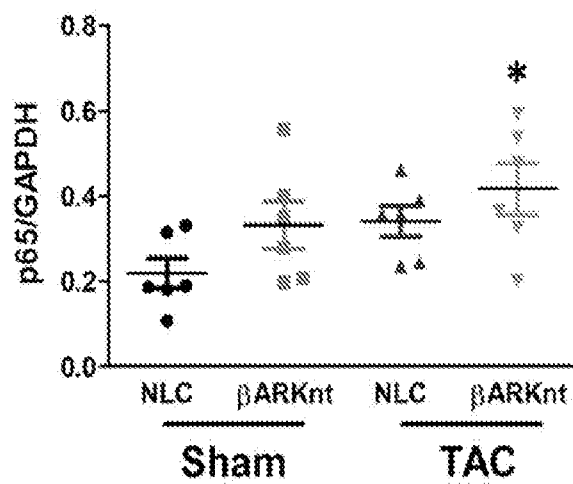
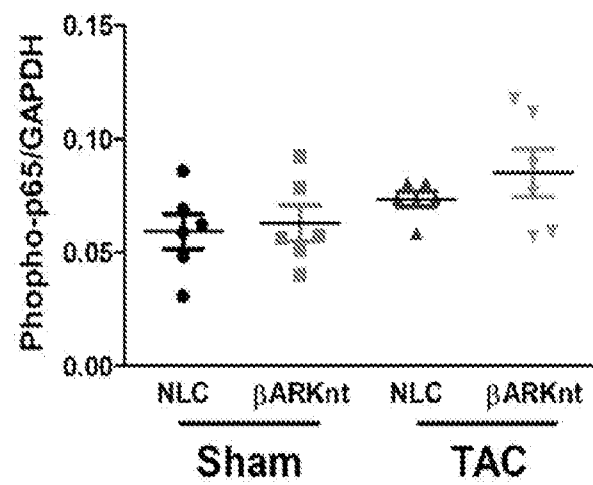


Figure 29C

Figure 29D

Figure 30B

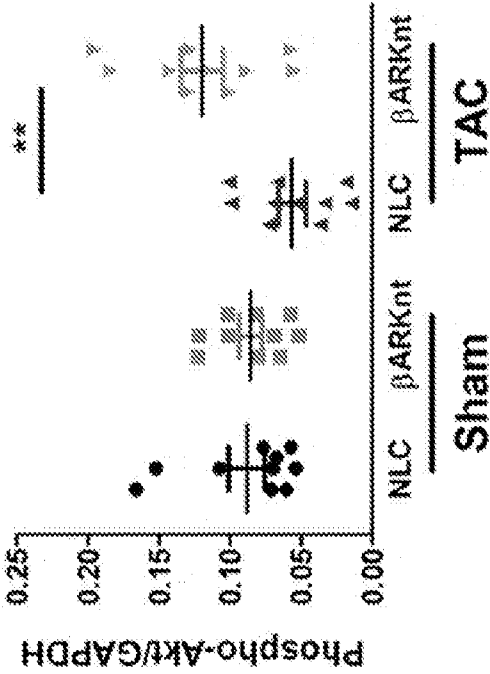


Figure 30A

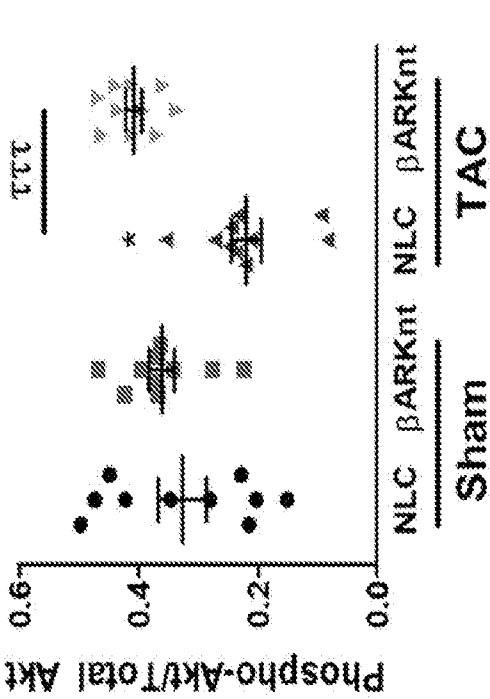


Figure 30D

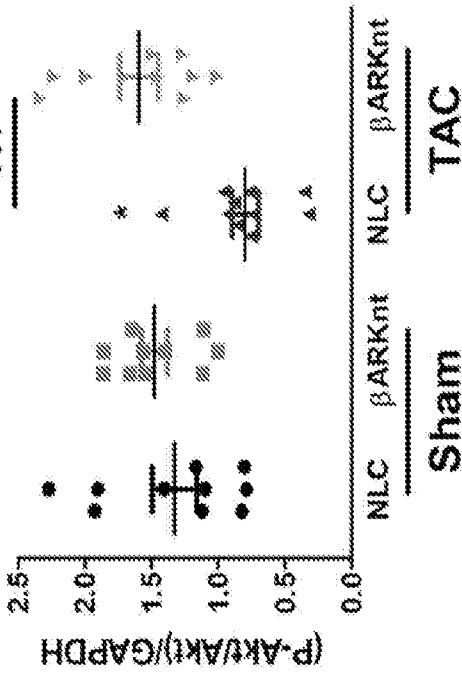


Figure 30C

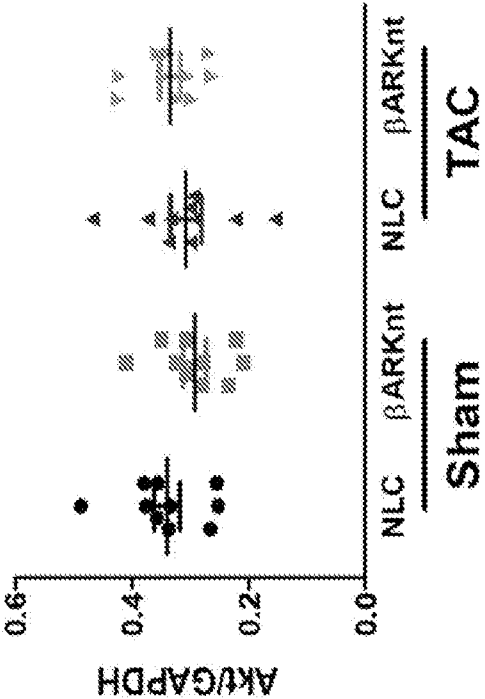


Figure 30

Figure 31B

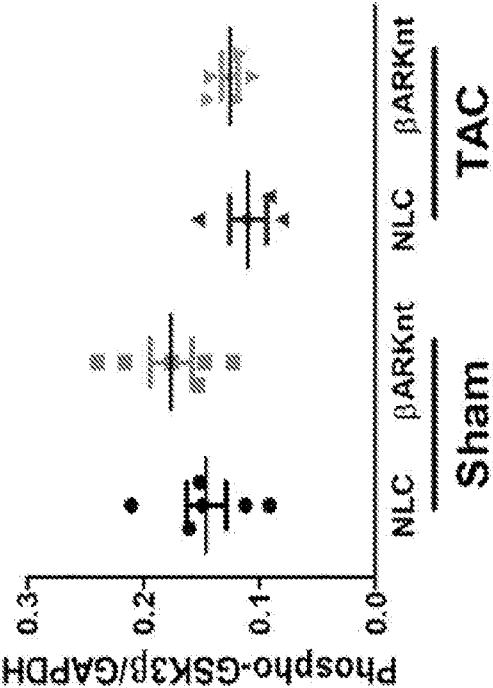


Figure 31A

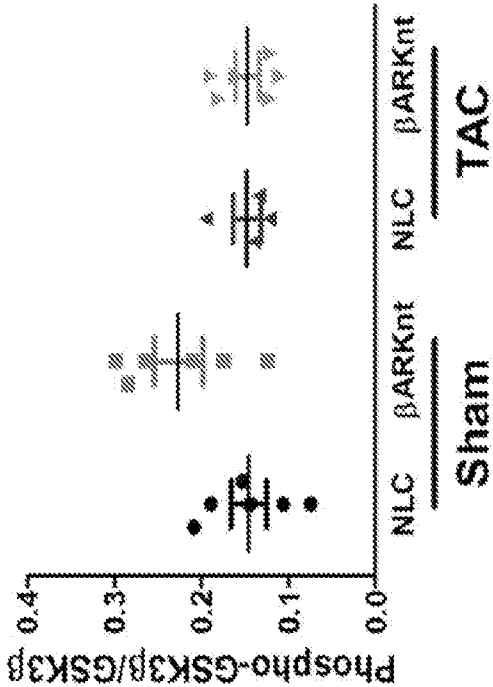


Figure 31D

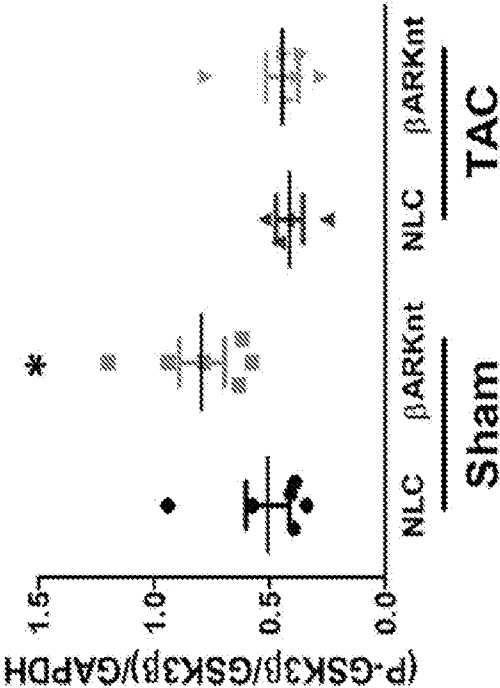


Figure 31C

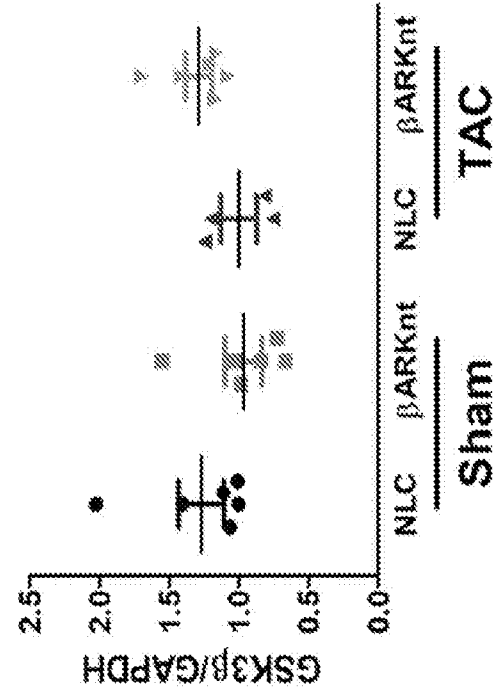


Figure 31

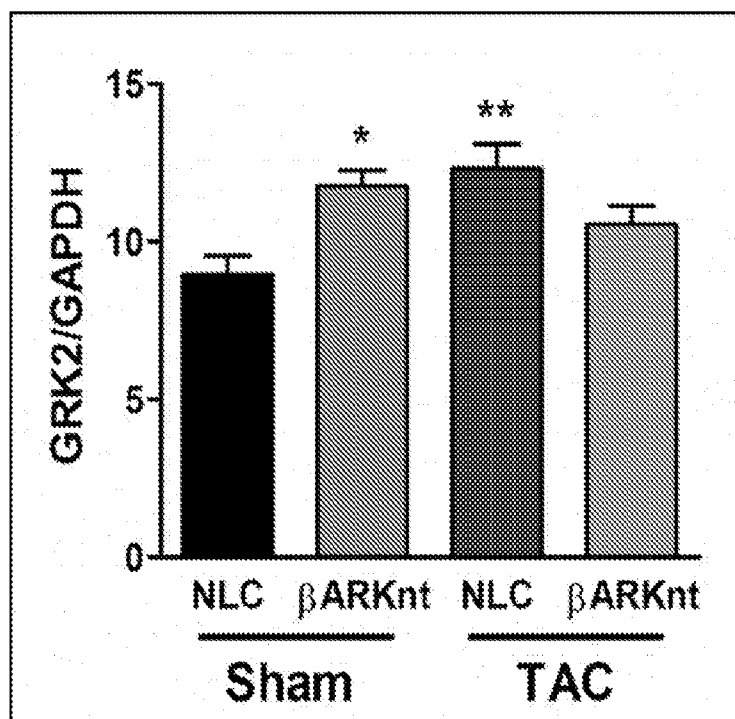
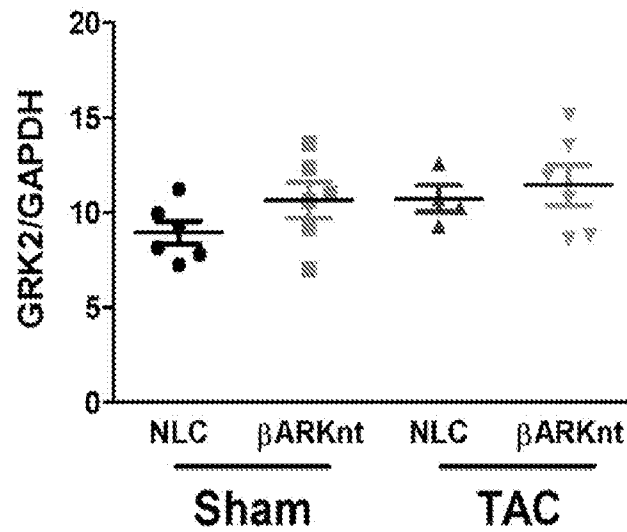


Figure 32
SUBSTITUTE SHEET (RULE 26)

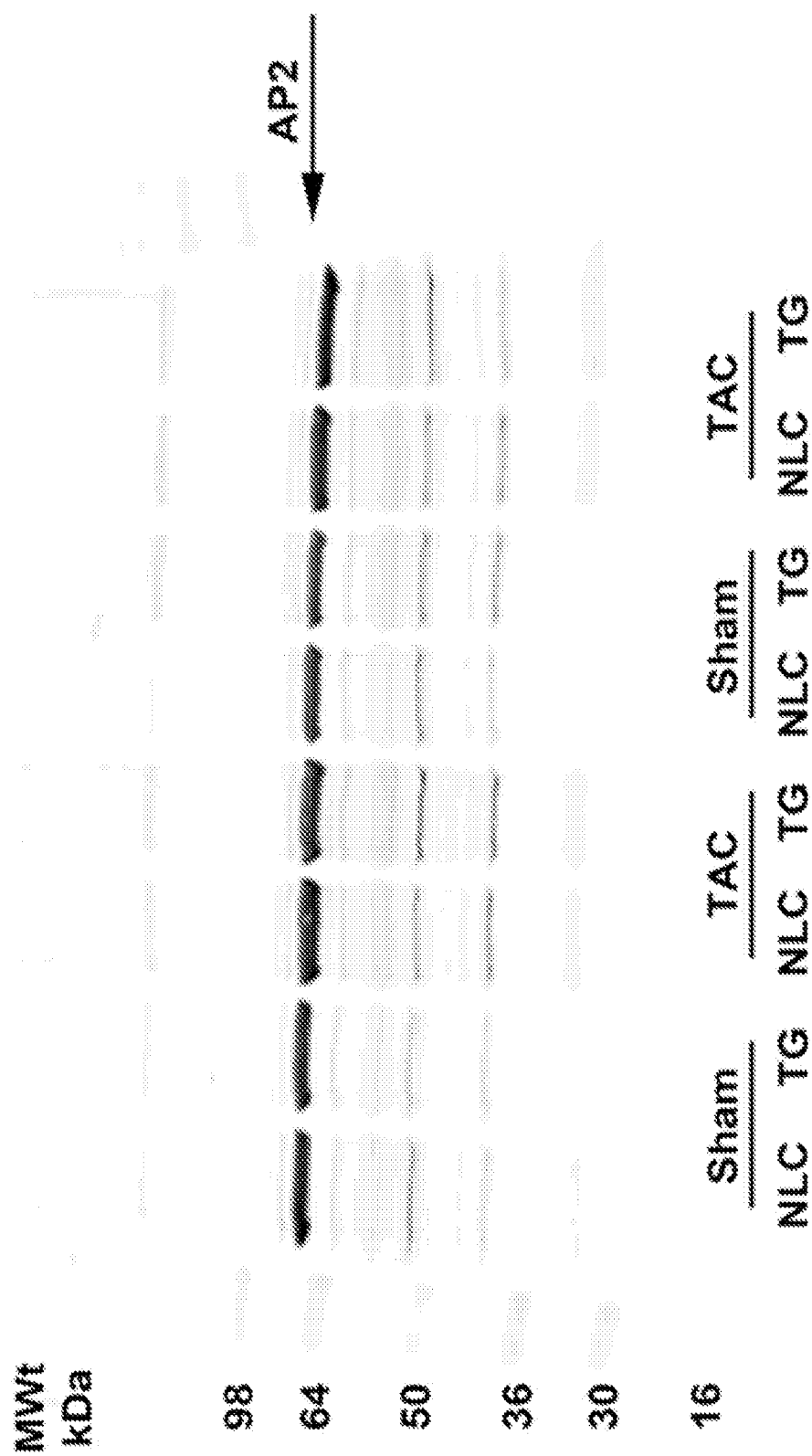


Figure 33A

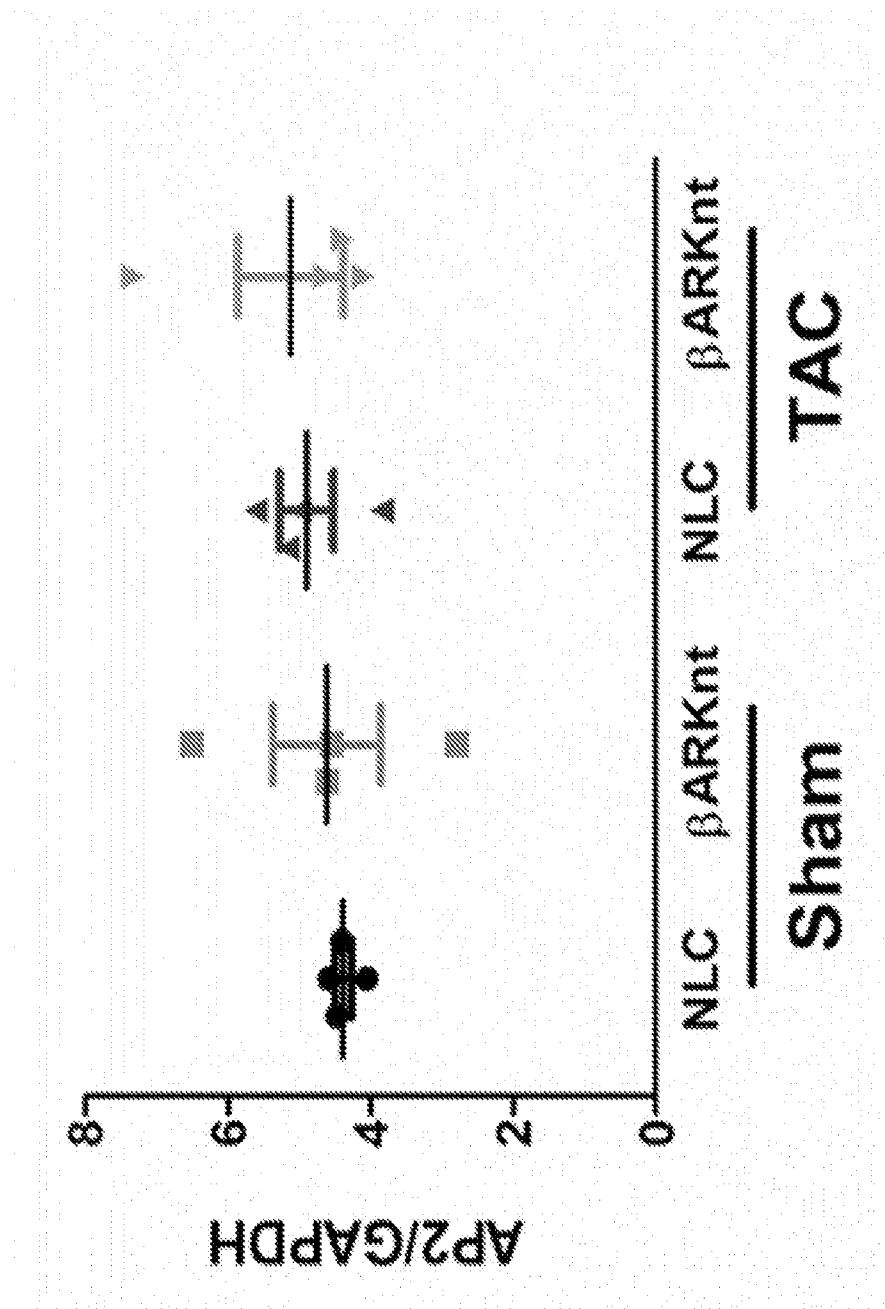


Figure 33B

Figure 34B

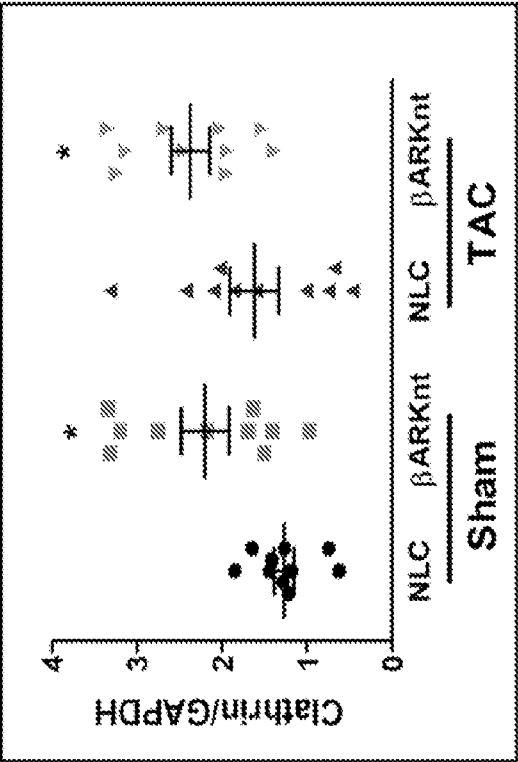


Figure 34A

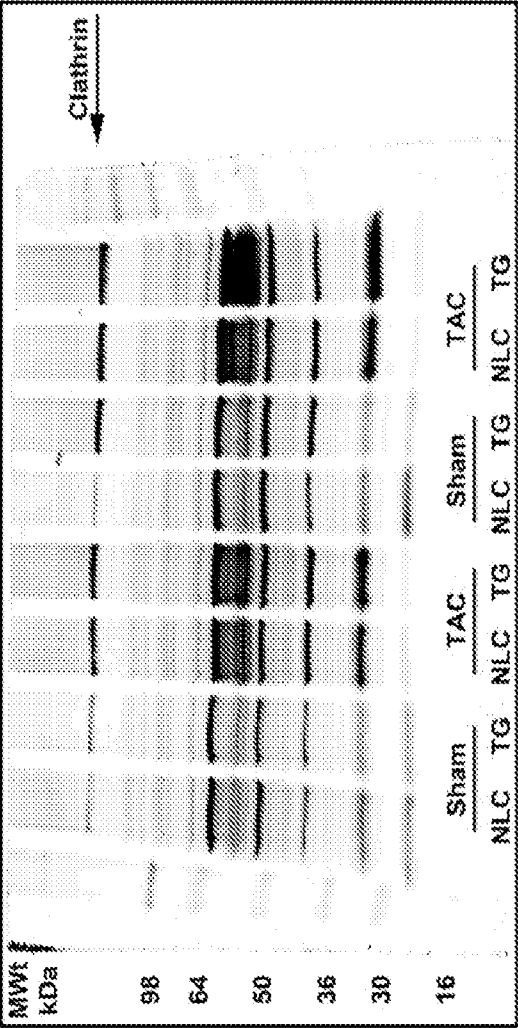


Figure 34

Figure 35B

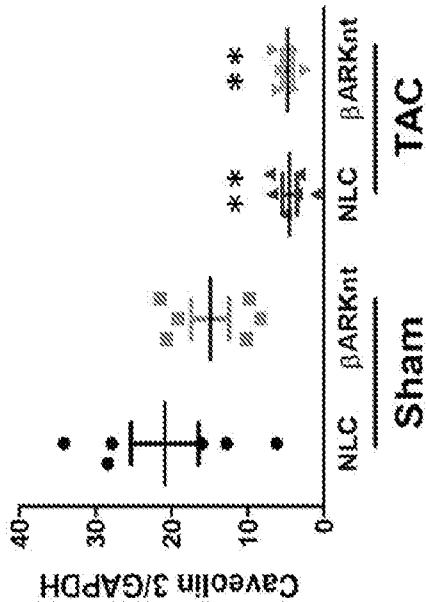


Figure 35A

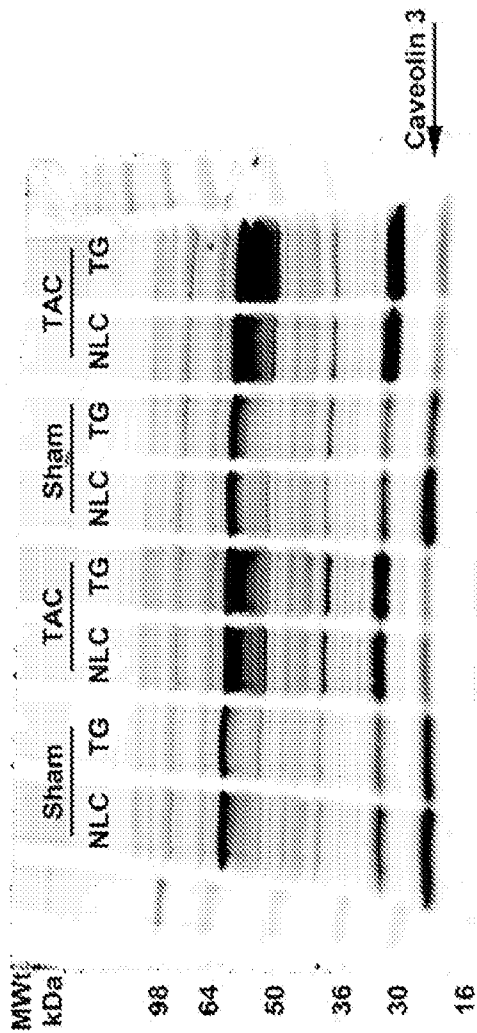


Figure 35

Figure 36A

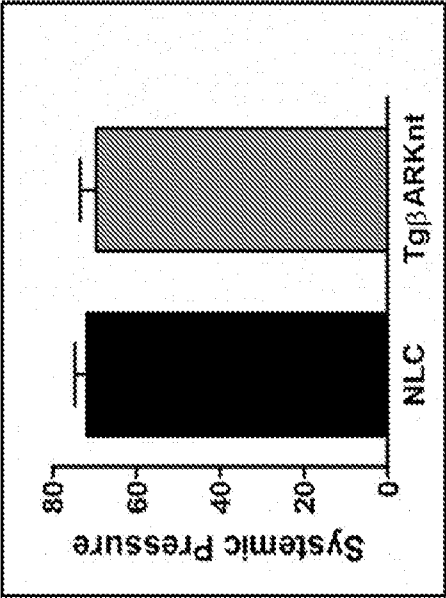


Figure 36B

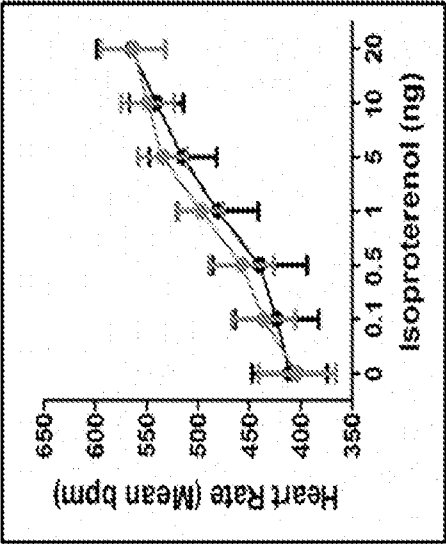


Figure 36C

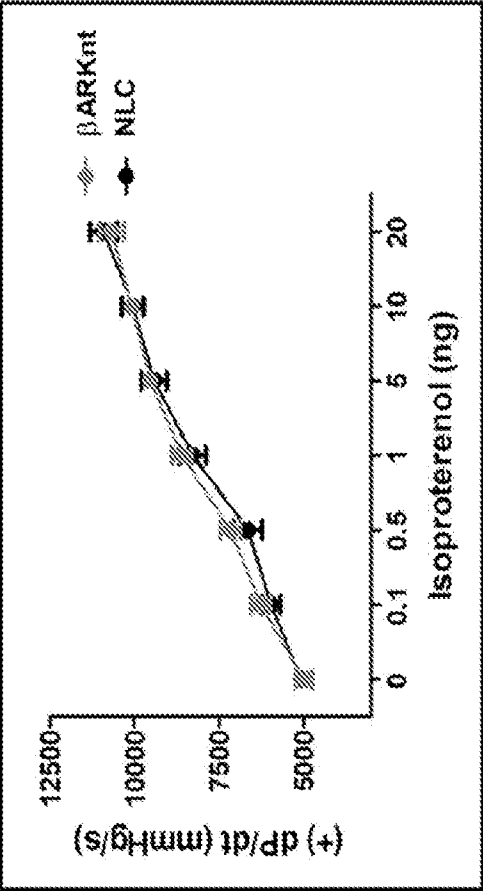


Figure 36D

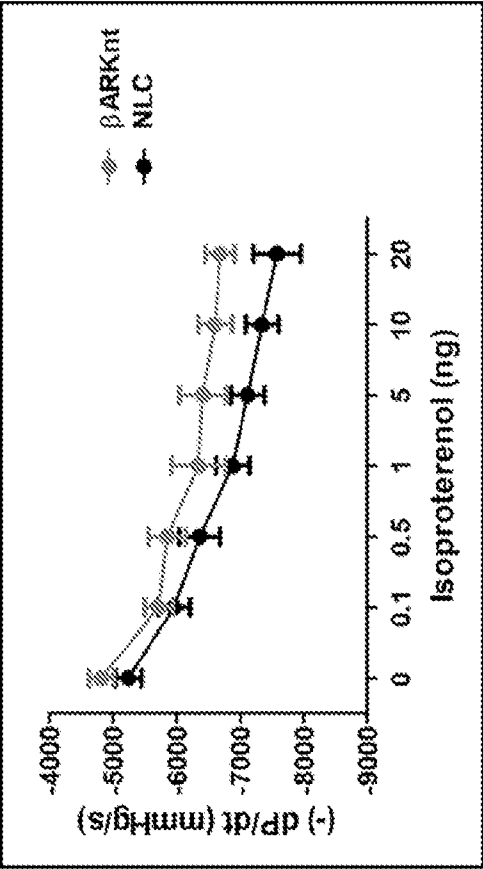


Figure 36

Figure 37E

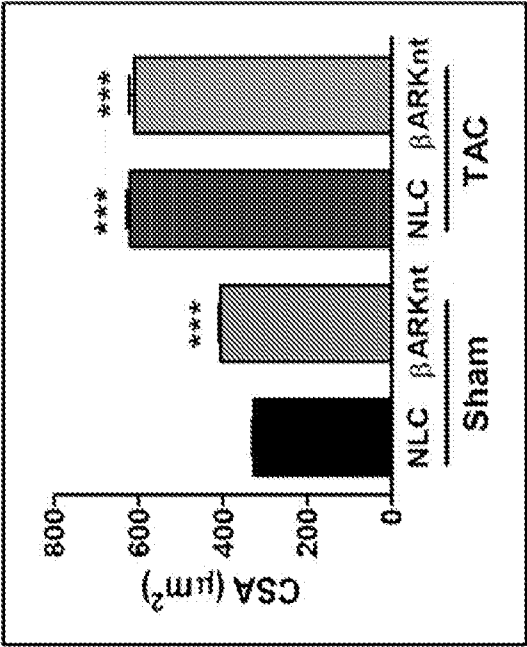


Figure 37B

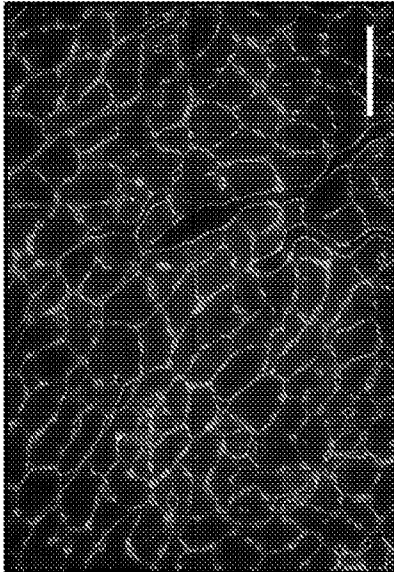


Figure 37A

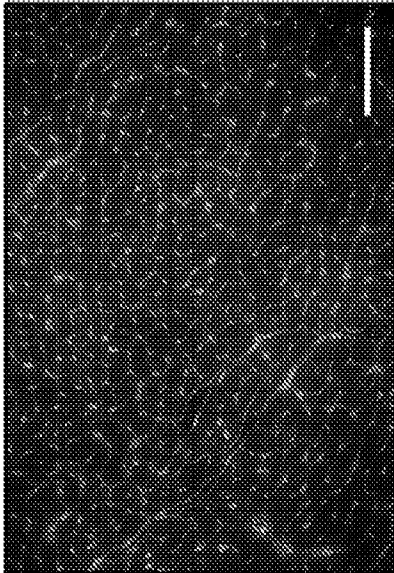


Figure 37D

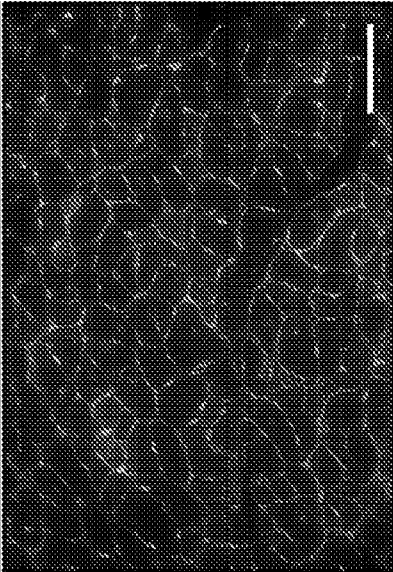


Figure 37C

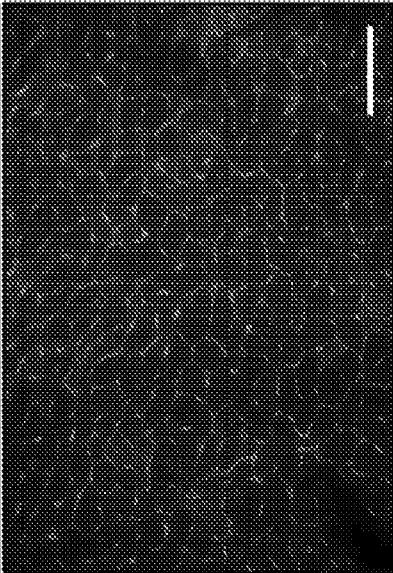


Figure 37

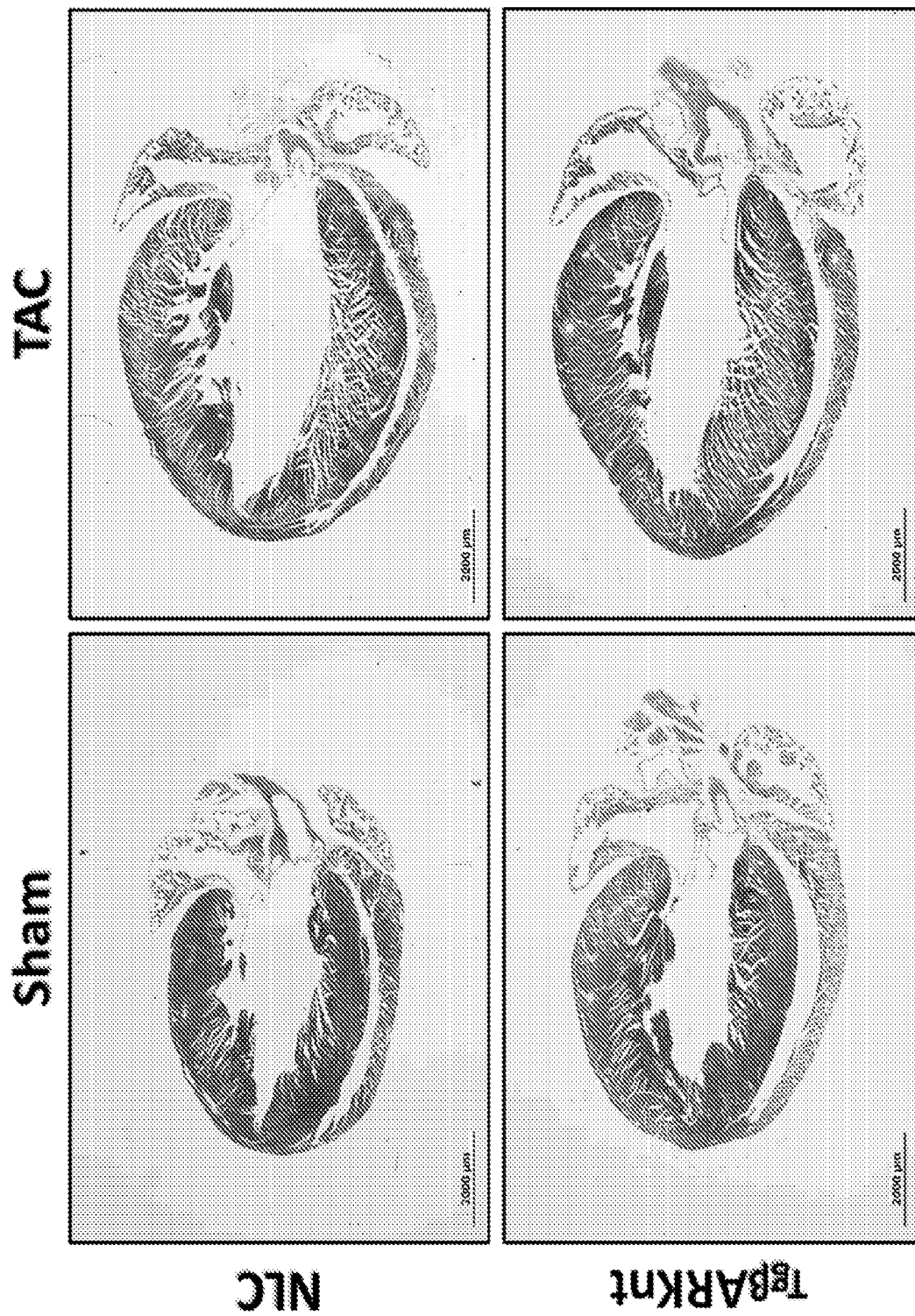


Figure 38

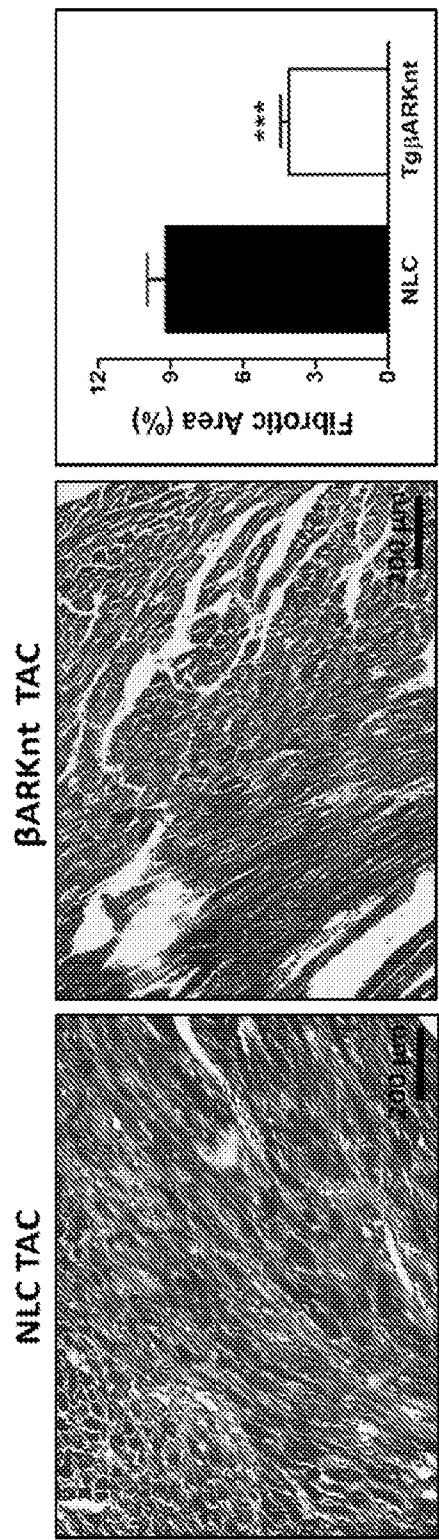


Figure 39

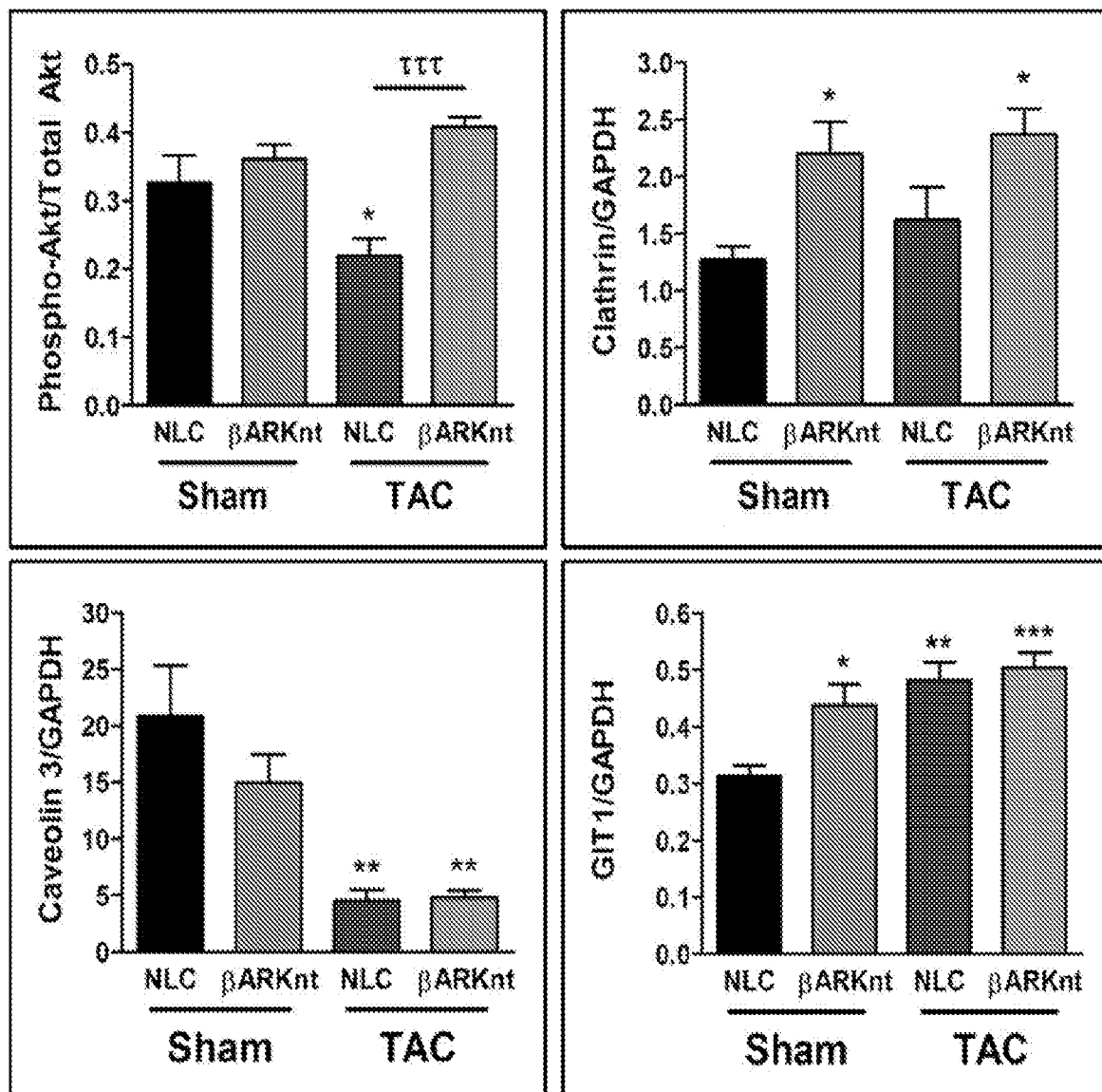


Figure 40
SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/35897

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 48/00; A61K 38/00; C12N 15/00; C07H 21/04 (2017.01)

CPC - A61K 48/00; A61K 38/1709; C12N 15/8645; A61K 38/00; C07H 21/00; C07K 14/47

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	MURGA et al., High Affinity Binding of Beta-Adrenergic Receptor Kinase to Microsomal Membranes. J Biol Chem. 1996, Vol. 271(2), p. 985-94. Abstract; pg 985, col 2, lower para; pg 986, col 2, para 3; and pg 991, col 1, and col 2, lower para, and Fig 9	1 ----- 2-3
Y	UniProtKB_P21146, RecName: Full=Beta-adrenergic receptor kinase 1; Short=Beta-ARK-1; AltName: Full=G-protein-coupled receptor kinase 2. UniProtKB/Swiss-Prot, ACCESSION Number: P21146. 26 November 2014. [online]. [Retrieved on 2017.09.19]. Retrieved from the Internet: <URL: https://www.ncbi.nlm.nih.gov/protein/114151?sat=21&satkey=3407977> LOCUS; Definition; and ORIGIN, the region between amino acid residues 50-145	2-3
A	KEYS et al., Cardiac hypertrophy and altered Beta-adrenergic signaling in transgenic mice that express the amino terminus of Beta-ARK1. Am J Physiol Heart Circ Physiol. 2003, Vol. 285(5), p. H2201-11. Entire documentation, especially Abstract; and pg H2201, col 1, and col 2, middle para	1-3
A	ROCKMAN et al., Expression of a b-adrenergic receptor kinase 1 inhibitor prevents the development of myocardial failure in gene-targeted mice. Proc Natl Acad Sci U S A. 1998, Vol. 95(12), p. 7000-5. Entire documentation, especially Abstract	1-3
A	TACHIBANA et al., Level of Beta-Adrenergic Receptor Kinase 1 Inhibition Determines Degree of Cardiac Dysfunction After Chronic Pressure Overload-Induced Heart Failure. Circulation. 2005, Vol. 111(5), p. 591-7. Entire documentation, especially Abstract	1-3
A	US 2009/0082410 A1 (OAKLEY et al.) 26 March 2009 (26.03.2009), Abstract, para [0003], [0005], [0013], [0061], and SEQ ID NO: 1, the region between amino acid residues 50-145	1-3

☐ 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

"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

01 August 2017

Date of mailing of the international search report

30 OCT 2017

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/35897

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
- a. ☒ forming part of the international application as filed:
☐ in the form of an Annex C/ST.25 text file.
☒ on paper or in the form of an image file.
- b. ☒ furnished together with the international application under PCT Rule 13*ter*. 1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*. 1(a)).
☐ on paper or in the form of an image file (Rule 13*ter*. 1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/35897

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. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+, Claims 1-8, directed to a composition comprising an amino-terminal domain of Beta.adrenergic receptor kinase-1 (Beta.ARKnt) peptide; or a nucleic acid encoding Beta.ARKnt. The composition will be searched to the extent that the composition encompasses Beta.ARKnt peptide, a variant thereof, or a fragment thereof. It is believed that claims 1(in part), 2-3 encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass the composition encompasses Beta.ARKnt peptide, a variant thereof, or a fragment thereof. Additional composition will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected composition. Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group.

*****Continued in the extra sheet*****

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(in part), 2-3, limited to Beta.ARKnt peptide

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

International application No.

PCT/US 17/35897

Continuation of:
Box No III (unity of invention is lacking)

(Continuation of Groups I+) Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be the composition comprising a nucleic acid encoding Beta.ARKnt, a variant thereof or a fragment thereof [claims 1(in part), 4-8].

Group II, claims 9-19, directed to a method for treating or preventing heart failure a subject; or a method for altering Beta.-adrenergic receptor (Beta.AR) density in a subject.

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

Special Technical Feature

Groups I+ and II are related as a product (Groups I+), and methods of potentially using the product (Group II).

Group II includes the special technical feature of treating or preventing heart failure or altering Beta.-adrenergic receptor (Beta.AR) density in a subject, not required by Groups I+.

Among Groups I+, a peptide is structurally different from a nucleic acid, each requires a different carrier for delivery.

Common Technical Features

The inventions of Groups I+ and II share the technical feature of a composition comprising an amino-terminal domain of Beta.adrenergic receptor kinase-1 (Beta.ARKnt) peptide, a variant thereof, or a fragment thereof; or a nucleic acid encoding Beta.ARKnt, a variant thereof or a fragment thereof (claim 1).

However, these shared technical features do not represent a contribution over prior art as being anticipated by an article entitled 'High Affinity Binding of Beta-Adrenergic Receptor Kinase to Microsomal Membranes' by Murga et al., (hereinafter 'Murga'; J Biol Chem. 1996, Vol. 271(2), p. 985-94) as follows:

Murga discloses a composition comprising an amino-terminal domain of Beta.adrenergic receptor kinase-1 (Beta.ARKnt) peptide (pg 986, col 2, para 3 - 'GST-bARK Fusion Proteins - Fusion proteins containing amino acids 50-145 (FP1)', wherein 'bARK...amino acids 50-145 (FP1)' is 'an amino-terminal domain of Beta.adrenergic receptor kinase-1 (Beta.ARKnt) peptide', and wherein 'GST-bARK Fusion Proteins... containing amino acids 50-145 (FP1)', is 'a composition comprising an amino-terminal domain of Beta.adrenergic receptor kinase-1 (Beta.ARKnt) peptide'; Abstract - 'b-adrenergic receptor kinase (bARK) modulates b-adrenergic and other G protein-coupled receptors'; pg 991, Fig 9, Legend - 'amino-terminal GST-bARK fusion proteins'; pg 991, col 2, lower para - 'GST-bARK fusion proteins comprising amino acids ... 50-145 (FP1), which is 'a composition comprising an amino-terminal domain of Beta.ARKnt peptide'; Please see Specification: pg 51, ln 9 - 'GRK2 residues 50-145 (Beta.ARKnt)'; pg 1, ln 16-19 - 'G protein-coupled receptors (GPCRs) ...GPCR kinases (GRKs) and one in particular, GRK2 (originally known as Beta.ARK1)'; or -- a nucleic acid encoding Beta.ARKnt (pg 991, col 1 - 'bovine bARK1 and an additional construct containing amino acids 50-145 of the kinase (FP1)', wherein 'amino acids 50-145 of the kinase (FP1)' is 'an amino-terminal domain of Beta.ARKnt peptide', as discussed above, and wherein 'bARK1 ...construct containing amino acids 50-145 of the kinase (FP1)' is 'a composition comprising a nucleic acid encoding Beta.ARKnt'; pg 991, col 2, lower para - 'recombinant bARK in the presence of different GSTbARK constructs (Fig. 9A); pg 991, Fig 9A, wherein 'GST-bARK contract' - 'GST_50-145' is 'a composition comprising a nucleic acid encoding Beta.ARKnt'; pg 986, col 2, para 3 - 'Fusion proteins containing amino acids 50-145 (FP1) ... bovine bARK1 were prepared... amplify the desired cDNA fragments. The amplified fragments were ligated ...glutathione S-transferase (GST) gene fusion vector...GST-bARK1 constructs').

Without a shared special technical feature, the inventions lack unity with one another.

Groups I+ and II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Note:

I) Claim 1 is objected as lacking a definition for the first appeared abbreviation "Beta.ARKnt" limitation in claims. For the purposes of this ISR, the first "a Beta.ARKnt" in claim 1 is rewritten as "an amino-terminal domain of Beta.adrenergic receptor kinase-1 (Beta.ARKnt)", based on the Specification and the prior art (Specification: pg 1, ln 16-19 - 'G protein-coupled receptors (GPCRs) ...GPCR kinases (GRKs) and one in particular, GRK2 (originally known as Beta.ARK1)'; pg 2, ln 23-24 - 'Beta.adrenergic receptor (Beta.AR)'; pg 49, ln 24-27 - 'GRK2 contains a putative amino-terminal Regulator of G protein Signaling (RGS) domain (Beta.ARK-RGS) ...a fragment of this RGS domain (Beta.ARKnt)'; pg 51, ln 9 - 'GRK2 residues 50-145 (Beta.ARKnt)'; Please also see an article entitled 'Cardiac hypertrophy and altered Beta-adrenergic signaling in transgenic mice that express the amino terminus of Beta-ARK1' by Keys et al. (Am J Physiol Heart Circ Physiol. 2003, Vol. 285(5), p. H2201-11: pg H2201, col 1 - 'Beta-ADRENERGIC RECEPTOR KINASE-1 (Beta-ARK1)'; pg H2201, col 2, middle para - 'Beta-ARK1 has a three-domain... amino terminal (NT) and CT domains... NT domain (Beta-ARKnt)...Beta.ARKnt (amino acids 50-145)').

II) Claim 5 is objected as lacking a definition for the first appeared abbreviation "AAV" limitation in claims. For the purposes of this ISR, "AAV" in claim 5 is rewritten as "adeno-associated virus (AAV)", based on the Specification (Specification: pg 31, ln 26 - 'adeno-associated virus (AAV)').

III) Claim 7 is objected as lacking a definition for the first appeared abbreviation "EF1alpha" limitation in claims. For the purposes of this ISR, "a EF1alpha" in claim 7 is rewritten as "an Elongation Growth Factor-1alpha (EF1alpha)", based on the Specification (Specification: pg 32, ln 30 - 'promoter is Elongation Growth Factor-1a (EF-1a)').