



(43) International Publication Date  
11 July 2013 (11.07.2013)

(10) International Publication Number  
**WO 2013/103896 A1**

(51) International Patent Classification:

**C12N 15/861** (2006.01) **C07K 14/58** (2006.01)  
**A61P 9/00** (2006.01)

(21) International Application Number:

PCT/US20 13/020392

(22) International Filing Date:

4 January 2013 (04.01.2013)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/584,006 6 January 2012 (06.01.2012) 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, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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

**Declarations under Rule 4.17:**

- as to applicant's entitlement to apply for and be granted a patent (Rule 4.1 7(H))
- as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.1 7(in))

**Published:**

- with international search report (Art. 21(3))

(54) Title: TREATING CARDIOVASCULAR OR RENAL DISEASES

(57) Abstract: This document provides methods and materials for treating cardiovascular and/or renal diseases. For example, AAV9 vectors designed to express natriuretic polypeptides, nucleic acid molecules encoding natriuretic polypeptides, methods for making AAV9 vectors, and methods for using such vectors or molecules to treat cardiovascular and/or renal diseases are provided.



**WO 2013/103896 A1**

## TREATING CARDIOVASCULAR OR RENAL DISEASES

### CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority to U.S. Provisional Application Serial No. 61/584,006, filed on January 6, 2012. The disclosure of the prior application is  
5 considered part of (and is incorporated by reference in) the disclosure of this application.

### STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

This invention was made with government support under grant number HL098502 awarded by the National Institute of Health. The government has certain rights in the  
10 invention.

#### *1. Technical Field*

This document relates to methods and materials involved in treating cardiovascular and/or renal diseases. For example, this document relates to adeno-  
15 associated virus serotype 9 (AAV9) vectors designed to express natriuretic polypeptides, nucleic acid molecules encoding natriuretic polypeptides, methods for making AAV9 vectors, and methods for using such vectors or molecules to treat cardiovascular and/or renal diseases.

#### *2. Background Information*

Hypertension is a highly common condition that, if not controlled, progresses toward more severe cardiovascular and renal morbidity. Its major clinical phenotype is hypertensive heart disease (HHD), which is characterized by diastolic dysfunction, cardiac remodeling, and fibrosis. Overtime, diastolic dysfunction evolves into systolic  
25 impairment, which leads to the worsening of overall cardiac function and to increased morbidity and mortality.

### SUMMARY

This document provides methods and materials for treating cardiovascular and/or  
30 renal diseases. For example, this document provides AAV9 vectors designed to express

natriuretic polypeptides, nucleic acid molecules encoding natriuretic polypeptides, methods for making AAV9 vectors, and methods for using such vectors or molecules to treat cardiovascular and/or renal diseases. AAV9 was isolated from human tissues and shown to have serological characteristics distinct from previously described serotypes  
5 (Gao *et al*, *J. Virol*, 78:6381-6388 (2004)).

As described herein, AAV9 vectors can be designed to have a nucleic acid sequence that encodes a natriuretic polypeptide such as an atrial natriuretic polypeptide (ANP), a B-type natriuretic polypeptide (BNP), a C-type natriuretic polypeptide (CNP), or a chimeric natriuretic polypeptide called CDNP. Such AAV9 vectors can be  
10 administered to a mammal (e.g., a human patient identified as suffering from a cardiovascular and/or renal disease) to treat that mammal's cardiovascular and/or renal disease. For example, an AAV9 vector provided herein can successfully deliver nucleic acid to cardiac cells for expression (e.g., sustained expression) of a natriuretic polypeptide without any short- or long-term toxicological effects and any signs of tolerance. In some  
15 cases, the sustained cardiac natriuretic polypeptide (e.g., BNP or CDNP) overexpression can reduce blood pressure (BP) and improve left ventricular (LV) function after a single administration (e.g., a single intravenous injection). In some cases, the AAV9 vectors provided herein can be used to reduce or prevent the development of hypertensive heart disease (HHD). Having the ability to deliver and express natriuretic polypeptide in  
20 cardiac cells as described herein can allow patients and clinicians to treat cardiovascular and/or renal diseases in an efficient and effective manner.

In general, one aspect of this document features an AAV9 vector comprising, or consisting essentially of, a nucleic acid sequence encoding a natriuretic polypeptide. The natriuretic polypeptide can be a human BNP polypeptide. The natriuretic polypeptide can  
25 be a CDNP polypeptide. The natriuretic polypeptide can be a B-CDNP polypeptide. The natriuretic polypeptide can be a C-CDNP polypeptide.

In another aspect, this document features a composition comprising, or consisting essentially of, an AAV9 vector in combination with a pharmaceutically acceptable delivery vehicle. The AAV9 vector comprises, or consists essentially of, a nucleic acid  
30 sequence encoding a natriuretic polypeptide. The natriuretic polypeptide can be a human BNP polypeptide. The natriuretic polypeptide can be a CDNP polypeptide. The

natriuretic polypeptide can be a B-CDNP polypeptide. The natriuretic polypeptide can be a C-CDNP polypeptide.

In another aspect, this document features a method for a cardiovascular or renal disease. The method comprises, or consists essentially of, administering a vector or a composition to a mammal. The vector can be an AAV9 vector comprising, or consisting essentially of, a nucleic acid sequence encoding a natriuretic polypeptide, and the composition can comprise, or consist essentially of, an AAV9 vector in combination with a pharmaceutically acceptable delivery vehicle. The natriuretic polypeptide can be a human BNP polypeptide. The natriuretic polypeptide can be a CDNP polypeptide. The natriuretic polypeptide can be a B-CDNP polypeptide. The natriuretic polypeptide can be a C-CDNP polypeptide. The mammal can be a human.

In another aspect, this document features a method for prolonging survival time for a mammal with hypertensive heart disease. The method comprises, or consists essentially of, administering a vector or a composition to the mammal. The vector can be an AAV9 vector comprising, or consisting essentially of, a nucleic acid sequence encoding a natriuretic polypeptide, and the composition can comprise, or consist essentially of, an AAV9 vector in combination with a pharmaceutically acceptable delivery vehicle. The natriuretic polypeptide can be a human BNP polypeptide. The natriuretic polypeptide can be a CDNP polypeptide. The natriuretic polypeptide can be a B-CDNP polypeptide. The natriuretic polypeptide can be a C-CDNP polypeptide. The mammal can be a human.

In another aspect, this document features a method for improving cardiac function in a mammal with hypertensive heart disease. The method comprises, or consists essentially of, administering a vector or a composition to the mammal under conditions wherein cardiac function is improved at least five months following the administration. For example, cardiac function can be improved for a period of time extending from about five months following the administration to about twelve months following the administration, from about five months following the administration to about ten months following the administration, or from about five months following the administration to about six months following the administration). The vector can be an AAV9 vector comprising, or consisting essentially of, a nucleic acid sequence encoding a natriuretic

polypeptide, and the composition can comprise, or consist essentially of, an AAV9 vector in combination with a pharmaceutically acceptable delivery vehicle. The natriuretic polypeptide can be a human BNP polypeptide. The natriuretic polypeptide can be a CDNP polypeptide. The natriuretic polypeptide can be a B-CDNP polypeptide. The natriuretic polypeptide can be a C-CDNP polypeptide. The mammal can be a human.

In another aspect, this document features a method for improving cardiac function in a mammal with polycystic kidney disease. The method comprises, or consists essentially of, administering a vector or a composition to the mammal under conditions wherein cardiac function is improved at least two months following the administration.

For example, cardiac function can be improved for a period of time extending from about two months following the administration to about twelve months following the administration, from about two months following the administration to about ten months following the administration, or from about two months following the administration to about five months following the administration). The vector can be an AAV9 vector comprising, or consisting essentially of, a nucleic acid sequence encoding a natriuretic polypeptide, and the composition can comprise, or consist essentially of, an AAV9 vector in combination with a pharmaceutically acceptable delivery vehicle. The natriuretic polypeptide can be a human BNP polypeptide. The natriuretic polypeptide can be a CDNP polypeptide. The natriuretic polypeptide can be a B-CDNP polypeptide. The natriuretic polypeptide can be a C-CDNP polypeptide. The mammal can be a human.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and

advantages of the invention will be apparent from the description and drawings, and from the claims.

### DESCRIPTION OF DRAWINGS

5           Figure 1. Generation of pre-proBNP-expressing AAV9 vector. (A) Schematic representation of the AAV9 vector encoding rat pre-proBNP. SD; splice donor, SA; splice acceptor, WPRE; woodchuck hepatitis virus post-transcriptional regulatory elements. CMV, Cytomegalovirus; SD, splice donor; SA, splice acceptor; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element. (B) Verification of proBNP  
10       expression in 293T cells transfected with pAAV-r-proBNP. Immunoreactive BNP in cell lysates and culture supernatants were detected by anti-rat BNP45 antibody. HMW, high molecular weight. (C) Immunostaining of rat BNP in pAAV-r-proBNP-transfected mouse cardiomyocytes (HL1 cells). When the plasmid encoding for the full length of pre-proBNP was introduced in HL1 cells, immunoreactive BNP signals were detected supra-  
15       nuclearly and in cytoplasmic secretory vesicles.

          Figure 2. AAV9 vector facilitates efficient cardiac gene delivery in spontaneously hypertensive rats (SHR). (A) Distribution of luciferase activities in firefly luciferase-expressing AAV9 vector-administered SHR organs was monitored by Xenogen IVIS Living Image. Strong luciferase expression in heart demonstrated efficient cardiac gene  
20       delivery by AAV9 in SHR (n=2) (left panel). Higher magnifications of Luciferase signals were found in both atria and ventricles (right panels). (B) Detection of luciferase by immunostaining. Luciferase in the sections of heart ventricles were detected by anti-firefly luciferase antibody, confirming the efficient cardiac luciferase gene expression upon AAV9 vector-mediated gene transfer. (C) No apparent toxicity observed in AAV9  
25       vector-administered SHR. Toxicological and pharmacological parameters in vector-injected SHR (n=3) were measured at 4 days and 3 weeks after vector administration. Averages of three rats were shown. \*P<0.05 vs respective untreated controls. WBC, white blood cells; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; PLT, platelets; ALB, albumin; ALP, alkaline phosphatase; ALT, alanine transferase; AMY, amylase; TBIL, total bilirubin; BUN, blood urea nitrogen; PHOS, phosphorus; CRE,  
30       creatinine. (D) Sustained BNP expression in the proBNP-expressing vector-administered

rats (n=3). The levels of plasma immunoreactive BNP were measured at 4 days and 3 weeks after vector administration by the rat BNP45 ELISA. Error bars indicate  $\pm$  SD. \*P<0.05 vs respective untreated controls.

Figure 3. Effects of long-term BNP overexpression in SHR. (A) No apparent toxicity observed in SHR at 4 months after proBNP-expressing AAV9 vector-administration. Toxicological and pharmacological parameters of the treated and untreated SHR were shown (n=4). No toxicity was observed in the rats, while plasma immunoreactive BNP45 was significantly elevated in the AAV9 vector-treated group. WBC, white blood cells; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; PLT, platelets; ALB, albumin; ALP, alkaline phosphatase; ALT, alanine transferase; AMY, amylase; TBIL, total bilirubin; BUN, blood urea nitrogen; PHOS, phosphorus; CRE, creatinine; GLU, glucose; TP, total protein. (B) BP measurements of proBNP-expressing AAV9 vector-administered SHR. BP in treated and untreated SHR were measured by tail-cuff method (n=8). \*P<0.05 vs respective untreated controls. (C) M-mode echocardiography of untreated and AAV9-proBNP treated SHR at four and nine months post AAV9 vector injection. AAV9 vector-treated SHR had significantly improved diastolic functions at four months and both diastolic and systolic functions at nine months as compared with untreated SHR. LVDs, left ventricular end-systolic dimension; LVDd, left ventricular end-diastolic dimension.

Figure 4. Effects of long-term BNP overexpression on cardiac remodeling. (A) Intra-arterial measurements of heart rate (HR), systolic blood pressure (SBP), and diastolic blood pressure (DBP) in anesthetized, treated and untreated, SHR are indicated  $\pm$  SD. (B) Plasma immunoreactive BNP45, plasma cGMP, and urinary cGMP are shown (n=4). Error bars indicate  $\pm$  SD. \*P<0.05 vs respective untreated controls. (C) Cross sections were stained by Mason's trichrome staining for muscle fibers (red staining) and collagen/fibrosis (blue staining). Representative images of whole section of treated and untreated SHR (upper panels) and higher magnifications of heart images of rats (n=4, lower panels) are shown. (D) Connective tissue deposition was evaluated as percent blue signals/red signals by KS400 Image Analysis software. The averages of four heart samples in treated and untreated SHR groups are shown.

Figure 5. Effects of non-cardiac BNP overexpression in SHR. (A) Distribution of luciferase activities in firefly luciferase-expressing AAV2 vector-administered SHR organs was monitored by Xenogen Living Image. Strong luciferase expression was evident in peritoneum by AAV2 in SHR (left panel), while no detectable luciferase expression was observed in heart (right panels). (B) BP measurements of proBNP-expressing AAV2 and AAV9 vector-administered SHR. BP in AAV2-treated (n=5), AAV9-treated (n=3), and untreated SHR (n=5) were measured by tail-cuff method. Error bars indicate  $\pm$  SD. \*P<0.05 vs respective untreated controls. (C) Plasma immunoreactive BNP45 and the heart weight/body weight ratios are shown. Error bars indicate  $\pm$  SD. \*P<0.05 vs respective untreated controls.

Figure 6. Effects of AAV9 vector-mediated long-term BNP expression in normal Wistar rats. (A) Efficient cardiac transgene expression upon systemic AAV9 vector-administration. Normal rats were injected by GFP-carrying AAV9 vector. Four weeks after injection, heart sections were analyzed for GFP expression. (B) Plasma immunoreactive BNP and the heart weight/body weight ratios are shown. Error bars indicate  $\pm$  SD. \*P<0.05 vs respective untreated controls. (C) Intra-arterial measurements of heart rate (HR), systolic blood pressure (SBP), and diastolic blood pressure (DBP) in anesthetized, treated and untreated, SHR are indicated  $\pm$  SD.

Figure 7 contains graphs plotting heart, lung, or kidney weights as a percentage of body weight for untreated SHR or SHR treated with a AAV9 viral vector designed to express proBNP or proCDNP.

Figure 8 contains graphs plotting the levels of CDNP in heart and circulation, which were measured by an ELISA kit detecting NT-proCNP.

Figure 9 contains photographs of tissues from spontaneously hypertensive rats 18 months after being exposed to an AAV9 vector designed to express luciferase. The tissues were assessed for luciferase activity. In both cases, the heart tissue exhibited high levels of luciferase activity.

Figure 10 is graph plotting percent survival of 9 month-old SHR rats with established hypertensive heart disease that were treated with an AAV9 vector designed to express BNP. Controls were comparable rats not treated with the AAV9 vector.

Figure 11 is a table demonstrating that sustained cardiac proBNP over-expression by the AAV9 vector improved cardiac function and structure in mammals with established hypertensive heart disease.

Figure 12A contains schematics of various AAV vectors that can be designed to express the indicated natriuretic polypeptides. Figure 12B is a photograph of a Western blot analysis demonstrating the expression of CNP from an AAV9-CNP vector and the expression of CDNP from an AAV9-C-CDNP vector.

Figure 13 is a table demonstrating that sustained cardiac proBNP over-expression by the AAV9 vector improved cardiac function and structure in mammals with polycystic kidney disease.

Figure 14 contains amino acid sequences for ANP, pre-pro-ANP, mANP, and pre-pro-mANP as well as a codon optimized nucleic acid sequence that encodes pre-pro-mANP.

## DETAILED DESCRIPTION

This document provides methods and materials for treating cardiovascular and/or renal diseases. For example, this document provides AAV9 vectors designed to express natriuretic polypeptides, nucleic acid molecules encoding natriuretic polypeptides, methods for making AAV9 vectors, and methods for using such vectors or molecules to treat cardiovascular and/or renal diseases. In some cases, an AAV8 vector can be used in place of an AAV9 vector described herein to obtain an AAV8 vector designed to express one or more natriuretic polypeptides. Such AAV8 vectors can be used as described herein with respect to AAV9 vectors.

As described herein, an AAV9 vector can be configured to include a nucleic acid sequence that encodes a natriuretic polypeptide. Examples of natriuretic polypeptides that can be expressed using an AAV9 vector as described herein include, without limitation, ANP (e.g., human ANP), BNP (e.g., human BNP), CNP (e.g., human CNP), CDNP, DNP, mANP, and ASBNP. The core amino acid sequence of CDNP can be as follows: GLSKGCFGLKLDRIQMSGLGCP SLRDPRPNAP STSA (SEQ ID NO:6). The amino acid sequence for ANP, pre-pro-ANP, mANP, and pre-pro-mANP can be as set forth in Figure 14. In some cases, the nucleic acid sequence encoding a natriuretic

polypeptide can be codon optimized. For example, a codon optimized nucleic acid sequence designed to encode pre-pro-mANP as set forth in Figure 14 can be used to make an AAV9 or AAV8 vector provided herein.

In some cases, an AAV9 vector can be configured to include two or more different nucleic acid sequences that encode natriuretic polypeptides. For example, an AAV9 vector can be configured to include a nucleic acid sequence that encodes human BNP and a nucleic acid sequence that encodes CDNP.

In some cases, the one or more natriuretic polypeptides to be expressed using an AAV9 vector can include the N-terminal region of a natural natriuretic polypeptide that includes non-active components of an active natriuretic polypeptide such as a signal peptide sequence and other sequences that can be involved in polypeptide processing, folding, and stabilization. Examples of such N-terminal regions include, without limitation, those set forth in SEQ ID NO: 1, 4, or 5. In some cases, one or more of the following sequences can be used as an N-terminal region of a natriuretic polypeptide to be expressed using an AAV9 vector: BNP signal peptide + NT-proBNP, CNP signal peptide + NT-proCNP, and ANP signal peptide + NT-proANP. Examples of amino acid sequences encoding a natriuretic polypeptide that includes such an N-terminal region include, without limitation, those amino acid sequences set forth in SEQ ID NO: 3, 7, 8, or 13.

A nucleic acid sequence (e.g., a nucleic acid sequence optimized for human codon usage) encoding a natriuretic polypeptide described herein can be inserted into any appropriate AAV9 viral vector. For example, a nucleic acid sequence encoding a human CDNP can be inserted into an AAV9 vector having a nucleic acid sequence as set forth in GenBank® Accession No. AY530557 (GINo. 46487760), JA400113.1 (GI No. 346220229), JA232063 (GI No. 330731135), JA231827 (GINo. 330729561), or JA062576 (GI No. 328343515). In some cases, an AAV9 vector can have the sequence as described elsewhere. See, e.g., WO2003/052052, U.S. Patent Application Publication No. 20110236353, EP2345731, EP2292780, EP2292779, or EP2298926. In some cases, an AAV vector (e.g., AAV9 or AAV8 vectors) can be designed to express BNP, CNP, or CDNP as set forth in Figure 12A.

In some cases, a promoter sequence can be operably linked to a nucleic acid sequence that encodes a natriuretic polypeptide (e.g., BNP, pre-proBNP, CDNP, B-CDNP, or C-CDNP) to drive expression of the natriuretic polypeptide. Examples of such promoter sequences include, without limitation, CMV, EF1 alpha, BNP, CNP, ANP, MYH6, and MYH7 promoters. In some cases, a promoter sequence that is active under conditions of elevated blood pressure with minimal, or no, activity under conditions of normal or low blood pressure can be operably linked to a nucleic acid sequence that encodes a natriuretic polypeptide (e.g., BNP, pre-proBNP, CDNP, B-CDNP, or C-CDNP) to drive expression of the natriuretic polypeptide under conditions of elevated blood pressure. Examples of such blood pressure sensitive promoter sequences include, without limitation, BNP and ANP promoters.

In one aspect, this document provides AAV9 vectors containing a nucleic acid sequence that encodes a natriuretic polypeptide. Such AAV9 vectors can infect cardiac cells and direct the expression of the natriuretic polypeptide by the infected cells.

Any appropriate method can be used to insert nucleic acid (e.g., nucleic acid encoding a natriuretic polypeptide) into the genome of an AAV9 vector. For example, standard molecule biology techniques such as restriction enzyme cutting, ligations, and homologous recombination can be used to insert nucleic acid into the genome of an AAV9 vector. Any appropriate method can be used to identify AAV9 vectors containing a nucleic acid molecule that encodes a natriuretic polypeptide. Such methods include, without limitation, PCR and nucleic acid hybridization techniques such as Northern and Southern analysis. In some cases, immunohistochemistry and biochemical techniques can be used to determine if an AAV9 vector contains a particular nucleic acid molecule by detecting the expression of a polypeptide encoded by that particular nucleic acid molecule.

In another aspect, this document provides nucleic acid molecules that encode a natriuretic polypeptide. For example, a nucleic acid molecule provided herein can be a single nucleic acid molecule that encodes the N-terminal region of a natural natriuretic polypeptide that includes one or more non-active components of an active natriuretic polypeptide such as a signal peptide sequence and other sequences that can be involved in polypeptide processing, folding, and stabilization upstream of an active component of a

natriuretic polypeptide. In some cases, such a nucleic acid molecule can have a nucleic acid sequence that encodes a natriuretic polypeptide set forth in SEQ ID NO: 3, 7, 8, or 13.

In some cases, a nucleic acid molecule provided herein can include a promoter  
5 sequence operably linked to the nucleic acid sequence encoding a natriuretic polypeptide. For example, a nucleic acid molecule provided herein can include a promoter sequence that is active under conditions of elevated blood pressure operably linked to a nucleic acid sequence encoding a natriuretic polypeptide (e.g., CDNP).

The term "nucleic acid" as used herein encompasses both RNA and DNA,  
10 including cDNA, genomic DNA, and synthetic (e.g., chemically synthesized) DNA. A nucleic acid can be double-stranded or single-stranded. A single-stranded nucleic acid can be the sense strand or the antisense strand. In addition, a nucleic acid can be circular or linear.

This document also provides methods for treating cardiovascular and/or renal  
15 diseases (e.g., to reduce blood pressure, cardiomyocyte hypertrophy, cardiac fibrosis, or renal fibrosis, or to improve systolic and diastolic dysfunctions). For example, an AAV9 vector provided herein can be administered to a mammal having a cardiovascular and/or renal disease to reduce blood pressure within the mammal. An AAV9 vector provided herein can be produced in human cell lines, such as 293T cells, or other types of cells  
20 such as insect cells, which can be concentrated typically by at least 100-fold, or even by as much as 5,000- to 10,000-fold, through ultracentrifugation. A viral titer typically is assayed by measuring the viral vector copy numbers in concentrated/purified vector preparations.

An AAV9 vector provided herein can be administered to a patient (e.g., human  
25 patient) by, for example, direct injection into a group of cardiac cells or intravenous delivery to cardiac cells. An AAV9 vector provided herein can be administered to a patient in a biologically compatible solution or a pharmaceutically acceptable delivery vehicle such as saline, by administration either directly into a group of cardiac cells or systemically (e.g., intravenously). Suitable pharmaceutical formulations depend in part  
30 upon the use and the route of entry. Such forms should not prevent the composition or formulation from reaching a target cell (i.e., a cell to which the virus is desired to be

delivered to) or from exerting its effect. For example, pharmacological compositions injected into the blood stream should be soluble.

While dosages administered will vary from patient to patient, an effective dose can be determined by setting as a lower limit the concentration of virus proven to be safe and escalating to higher doses of up to  $10^{13}$  vector genome copies (vg)/kg, while  
5 monitoring for a response (e.g., a reduction in blood pressure) along with the presence of any deleterious side effects. Escalating dose studies can be used to obtain a desired effect for a given viral treatment.

An AAV9 vector provided herein can be delivered in a dose ranging from, for  
10 example, about  $10^3$  vg/kg to about  $10^{13}$  vg/kg. A therapeutically effective dose can be provided in repeated doses. Repeat dosing is appropriate in cases in which observations of clinical symptoms or monitoring assays indicate that the degree of viral activity (e.g., natriuretic polypeptide expression) is declining. Repeat doses can be administered by the same route as initially used or by another route. A therapeutically effective dose can be  
15 delivered in several discrete doses (e.g., days, weeks, months, or years apart).

An AAV9 vector provided herein can be directly administered to cardiac cells. For example, a virus can be injected directly into heart tissue. In some cases, ultrasound guidance can be used in such a method. In some cases, an AAV9 vector provided herein can be delivered systemically. For example, systemic delivery can be achieved  
20 intravenously via injection. The course of therapy with an AAV9 vector provided herein can be monitored by evaluating changes in clinical symptoms.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

## 25 **EXAMPLES**

### Example 1- Long-Term Cardiac proBNP Gene Delivery Prevents the Development of Hypertensive Heart Disease in Spontaneously Hypertensive Rats

#### *SHR and Wistar rats*

Four week-old SHR and five week-old Wistar rats were purchased from Charles  
30 River. SHR served as a model of progressive HHD. Strains of rats, number of animals,

treatment, and duration of treatment in each experiment was summarized in Table 1. All animal studies were approved by the Institutional Animal Care and Use Committee.

Table 1. Summary of the rats used.

| Study                            | Strain | Treatment | Number | Duration (weeks) |
|----------------------------------|--------|-----------|--------|------------------|
| <b>Cardiac delivery</b>          | SHR    | AAV9-Luc  | 2      | 3                |
| <b>Toxicology</b>                | SHR    | Untreated | 3      | 3                |
|                                  | SHR    | AAV9-Luc  | 3      | 3                |
|                                  | SHR    | AAV9-BNP  | 3      | 3                |
| <b>Pharmacokinetics/dynamics</b> | SHR    | Untreated | 8      | 40               |
|                                  | SHR    | AAV9-BNP  | 8      | 40               |
| <b>Non-cardiac delivery</b>      | SHR    | Untreated | 5      | 8                |
|                                  | SHR    | AAV2-BNP  | 5      | 8                |
|                                  | SHR    | AAV9-BNP  | 3      | 8                |
| <b>Normotensive rat study</b>    | Wistar | AAV9-GFP  | 6      | 4                |
|                                  | Wistar | AAV9-BNP  | 6      | 4                |

5 SHR, spontaneously hypertensive rats; Luc, luciferase; GFP, green fluorescent protein.

#### *Cell culture*

HEK 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 50 U/mL penicillin, and 50 µg/mL streptomycin. A  
 10 murine atrial cardiomyocyte cell line, HL-11, was obtained from Dr. William C. Claycomb (Louisiana State University Medical Center, New Orleans) and cultured in Claycomb's medium with 10% FBS, 100 µM norepinephrine, and 4 mM L-glutamine on 0.02% gelatin/fibronectin-coated flasks or plates.

#### 15 *Transfection, Immunoblotting and Immunostaining*

Eugene6 (Roche) was used for transfection. For immunoblotting, immuno-reactive rat BNPs were detected using rabbit anti-rat BNP 1-45 antibody (AssayPro) and HRP-conjugated anti-rabbit IgG antibody. Immunostaining of immuno-reactive rat BNP

was performed using the same anti-rat BNP1-45 antibody and FITC-conjugated anti-rabbit IgG antibody.

#### *WIS imaging*

5           The cardiac luciferase expression was monitored by Xenogen IVIS biophotonic imaging machine. Upon luciferin administration through IP, anesthetized rats were euthanized, and the organs were harvested immediately. Harvested tissues were placed on the 10-cm plates on the imaging chamber, and a background photo of the tissues and a color overlay of the emitted photon data were obtained.

10

#### *Toxicological and pharmacological tests*

For toxicological and pharmacological tests, hematological parameters (VetScan HM2 Hematology System; 50  $\mu$ L blood in EDTA for WBC counts, WBC histogram, Hb, Hct, MCV, MCH, MCHC, RDW, graphic RBC histogram, PLT count, MPV, PCT, PDW and Graphic platelet histogram) and chemistry (VetScan Classic; 100  $\mu$ L blood in lithium heparin; ALB, ALP, ALT, AMY, BUN, CA<sup>++</sup>, CRE, GLOB, GLU, K<sup>+</sup>, Na<sup>+</sup>, PHOS, TBIL, TP) were measured.

15

#### *Plasmids*

20           The codon-optimized rat pre-proBNP was synthesized by GenScript, and cloned into a lentiviral vector, pSIN-CSGWdlNotI. The BamHI-XhoI short fragment, which contained rat pre-proBNP and WPRE post-transcriptional regulatory element, was then cloned into the mammalian expression plasmid, pAAV-MCS (Stratagene), resulting in pAAV-rat-pre-proBNP .

25

#### *AAV9 and AAV2 vectors*

The AAV9 vector stocks were produced in human 293T cells using the helper-free transfection method according to the manufacturer's protocol (Stratagene). For AAV9 vector production, AAV9 capsid-expressing plasmid pRep2Cap9 (obtained from Dr. Hiroyuki Nakai) was used, while AAV2 vector was made with the AAV2 capsid-expressing plasmid, pAAV-RC (Stratagene). Firefly luciferase-, humanized recombinant

30

green fluorescent protein (GFP)-, or rat proBNP-encoding AAV genome constructs were packaged. Three days after transfection, AAV9 vector-producing 293T cells were harvested for vector purification. The cells were lysed by freeze and thaw cycling, followed by ultracentrifuge concentration (62,500 rpm for 2 hours) through Optiprep  
5 Density Gradient Medium (Sigma). The resulting AAV9 vectors were desalted and further concentrated using Amicon Ultra- 15 100k filtration (Amicon). The titers (genomic copy numbers/mL) of concentrated AAV9 vector stocks were determined by quantitative PCR using plasmid DNA standards, AAV genomic sequence-specific primers, and a fluorescent probe.

10

#### *Non-invasive tail blood pressure measurement*

The blood pressure (BP) of conscious rats was measured by the CODA High-Throughput Non-Invasive Tail BP System (Kent Scientific).

#### 15 *Echocardiography (ECHO) for non-invasive assessment of ventricular function and structure*

To evaluate cardiac function and structure, both standard ECHO and Two-Dimensional Speckle-Derived Strain ECHO (2DSE) examinations were performed at four and nine months post injections in the BNP-treated and the untreated SHR. Standard  
20 ECHO and 2DSE also were performed in normal Wistar rats at 4 weeks after AAV9 injections. All ECHO examinations were performed by a skilled sonographer blinded to the treatment.

Standard ECHO was performed as follows. After removing chest hair, ultrasonic scans was performed in all rats in supine position using a Vivid 7 system (GE Healthcare,  
25 Milwaukee, WI) equipped with a 10S ultrasound probe (11.5 MHz) with ECG monitoring. M-mode images and gray scale 2D images (300-350 frames/sec) of the parasternal long-axis and mid-LV was recorded for off-line analysis. LV end-diastolic (LVDd) and end-systolic (LVDs) dimensions and septal diastolic (SWTd) and posterior wall diastolic (PWTd) and systolic (PWTs) thicknesses were measured from M-mode  
30 images. LV mass was calculated according to uncorrected cube assumptions as LV mass = 1.055 x [(LVDd+SWTd+PWTd)<sup>3</sup>-(LVDd)<sup>3</sup>], where 1.055 is the specific gravity of

myocardium. LV mass was corrected for body weight (LVMI) for analysis. End-systolic (ESV), end-diastolic and stroke volumes (SV), and ejection fraction (EF) was calculated using the Teichholz formula:  $LV\ volume = 7 \times [(LVDd)^3 / (2.4 + LVDd)]$ . Relative wall thickness (RWT) was calculated as  $RWT = (SWTd + PWTd) / LVDd$ . All parameters

5 represented the average of three beats.

2DSE was performed as follows. Using EchoPAC software (EchoPAC PC - 2D strain, BTO 6.0.0, GE Healthcare, Milwaukee, WI), which included a high resolution speckle tracking analysis library for off-line analysis, endocardial border was carefully manually traced at end-systole in LV short-axis views at the middle level (i.e., at the level

10 of papillary muscles). Ideal width of circular region of interest was chosen in order to include the entire myocardial wall. Speckle tracking was performed by the software and global strain, and circumferential strain rate parameters were measured computing the mean of the six middle LV segments. The analysis included peak circumferential systolic strains (sS) and strain rates (sSR) for evaluation of myocardial systolic function and peak

15 early circumferential strain rates (dSR-E) for evaluation of myocardial diastolic function. All parameters represented the average of three beats. Using standard ECHO and 2DSE, significant improvement were detected in both systolic and diastolic function in a rat model of cardiac dysfunction when compared to the untreated SHR.

#### 20 *Acute experiment procedure*

Rats for the acute protocol were anesthetized with isoflurane (1.5% in oxygen). Placement of PE-50 tubing into the carotid artery for BP monitoring and blood sampling were performed. A portion of the neck skin was removed, and the carotid artery were isolated and cleared. A cut was made with micro-scissors, and a PE-50 tubing was

25 introduced into the vessel for direct BP monitoring. Blood was drawn to evaluate toxicological reactions in AAV9-BNP transduced rats and to measure BNP and cGMP. At the end of the experiments, rat organs were harvested for further analysis.

#### *Masson 's Trichrome staining*

The sections of frozen cardiac samples were assessed by Trichrome staining for collagen contents. Percent blue signals were analyzed by KS400 Image Analysis Software (version 3.0, Zeiss).

5 *Sample size and statistical analysis*

Groups were compared by unpaired t-test, and changes within-groups were assessed by paired t-test. Comparisons of BP values between groups were performed by two-way ANOVA for repeated measurements. Data were expressed as mean  $\pm$  SD. Significance was accepted for  $p < 0.05$ .

10

*Results*

*In vitro expression and localization of proBNP*

After successfully engineering AAV9 encoding for the rat pre-proBNP, which included the signal peptide (SP), NT-proBNP, and BNP1-45 domains (Figure 1A), the protein expression of BNP was verified in 293T cells. Non-glycosylated proBNP (10 kDa) and high molecular weight (HMW, 12-24 kDa) glycosylated proBNP were detected in the cell lysates by Western blotting analysis. Of note, HMW forms of BNP were predominantly secreted (Figure 1B). When the pre-proBNP was expressed in mouse cardiomyocytes (HL-1 cells obtained from Dr. William C. Claycomb) and analyzed by immunostaining with an anti-rat BNP 1-45 antibody, clear supranuclear localization of immunoreactive BNP (red staining) as well as discrete cytoplasmic body signals (red staining) were detected (Figure 1C).

25 *In vivo cardiac specific tropism of AAV9 vector-mediated gene transfer*

The rat pre-proBNP- or firefly luciferase-expressing vectors were packaged in AAV9 capsid, and the influence of AAV9 vector-mediated gene delivery was examined in SHR. Four week-old SHR (n=2) were used. Three weeks after tail intravenous injection of AAV9 carrying luciferase ( $10^{12}$  genome copy/animal), the tissue specificity of the AAV9 vector was determined by luciferase expression in the SHR, which demonstrated high levels of luciferase expression in myocardium (Figure 2A-B). To confirm luciferase expression in the heart, the heart section was stained with anti-

luciferase antibody, and the signals were detected predominately in the cardiomyocytes (Figure 2B). When AAV9-luciferase (n=3) and AAV9-pre-proBNP (n=3) were injected and the acute (4 days) and chronic (3 weeks) toxicological responses were compared to those of the untreated SHR (n=3), no notable toxicity was observed among these three groups of rats (Figure 2B). However, plasma BNP, by rat BNP1-45 ELISA, was significantly higher in the AAV9-pre-proBNP -treated SHR compared with untreated SHR both at four days and three weeks after injections (Figure 2D), thus confirming the sustained BNP expression upon AAV9 vector-mediated gene delivery.

10 *Effects of sustained proBNP expression in SHR*

The effects of sustained proBNP expression in SHR through cardiac proBNP delivery by AAV9 vector were monitored. Four months after AAV9-pre-proBNP injections in SHR, there was no toxicological reaction compared to untreated SHR. Importantly, plasma immune reactive BNP was significantly higher in the AAV9-pre-proBNP -treated group compared with the untreated SHR (Figure 3A). Tail cuff BP measurements indicated significant reduction in SBP, DBP, and MAP in the AAV9-pre-proBNP -treated SHR as compared with untreated SHR. Indeed, in the AAV9-pre-proBNP, SBP was significantly reduced one month after injection and followed by a reduction in both DBP and MAP at two months post-injection as compared with the untreated SHR. These reductions in SBP, DBP, and MAP in conscious rats remained throughout the nine month study (Figure 3B).

Echocardiographic parameters in untreated SHR and in AAV9 pre-proBNP treated SHR were summarized in Table 2. While no difference was detected in HR between AAV9 pre-proBNP treated and untreated SHR both at four and nine months post injection, echo analysis indicated a significant improvement of diastolic function at four and nine months as well as systolic function at nine months post injection in AAV9 pre-proBNP treated SHR as compared with untreated SHR (Figure 3C). Of note, EF, PWTd, LVDD, and dSR-E circumferential were improved, and LVMi was lower at nine months in the AAV9 pre-proBNP treated SHR even when compared to four months untreated SHR (Table 2).

Table 2. Echocardiographic parameters in untreated (n=8) and BNP treated SHR (n=8).

|                              | 4 Month p.i. |             | 9 Months p.i. |             |
|------------------------------|--------------|-------------|---------------|-------------|
|                              | Untreated    | BNP Treated | Untreated     | BNP Treated |
| <b>HR</b>                    | 403±27.3     | 392±22.3    | 381±25.2      | 393±13      |
| <b>SWTd</b>                  | 2.09±0.1     | 1.86±0.1*   | 2.71±0.1†     | 2.17±0.2*†  |
| <b>PWTd</b>                  | 2.03±0.1     | 1.86±0.2*   | 2.16±0.3      | 1.87±0.1*‡  |
| <b>LVDd</b>                  | 6.77±0.2     | 6.71±0.1    | 7.56±0.6†     | 7.57±0.4†‡  |
| <b>LVDs</b>                  | 3.83±0.4     | 3.47±0.1*   | 4.66±0.6†     | 3.96±0.3*†  |
| <b>Ejection Fraction</b>     | 80±4.1       | 85±1.8*     | 74±4.5†       | 83±2.1*†‡   |
| <b>LV Mass Index</b>         | 0.44±0.01    | 0.4±0.02*   | 0.49±0.01     | 0.4±0.01*†‡ |
| <b>sSR Circumferential</b>   | -4.6±0.7     | -4.75±0.6   | -3.74±0.4†    | -5.04±0.4*  |
| <b>dSR-E Circumferential</b> | 2.41±0.8     | 4.07±1.5*   | 2.09±0.8      | 3.25±0.9*‡  |
| <b>sSR-Radial</b>            | 7.17±1.1     | 6.77±0.8    | 6.34±1.6      | 8.13±1.9*†  |
| <b>dSR-E Radial</b>          | -3.29±1.2    | -5.72±2.5*  | -2.41±1.3     | -4.57±2.1*† |

\*P<0.05 vs respective Untreated; †P<0.05 vs four months within group; ‡P<0.05 between nine months BNP-treated and four months Untreated. HR, heart rate; SWTd, septal wall thickness at end diastole; PWTd, posterior wall thickness at end diastole; LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; sSR, systolic strain rate; dSR, diastolic strain rate.

At nine months post-injection, four rats per group were sacrificed for acute experiments. Direct intra-carotid systolic and diastolic blood pressure was reduced in the AAV9 pre-proBNP treated anesthetized SHR (Figure 4A), while no significant differences were found in weights and heart rates between treated and untreated rats. The heart weight corrected for the body weight was significantly reduced in the BNP-treated as compared with the control SHR (0.37±0.01 vs 0.43±0.02, respectively, p<0.05). Plasma BNP was higher in the BNP-treated as compared with the control SHR (Figure 4B). Although plasma cGMP was not different, urinary cGMP was greater in the BNP-

treated as compared with the control SHR (Figure 4B). Connective tissue (assessed by Mason's trichrome staining) tended to increase in heart sections of untreated SHR as compared with AAV9-preproBNP treated SHR (Figure 4C-D).

#### 5 *Non-cardiac BNP-transduction by AAV2 vector*

The effects of non-cardiac proBNP gene delivery on BP, plasma BNP levels, and heart weight in SHR were assessed. For non-cardiac gene delivery, conventional AAV2 vectors were administered through intra-peritoneal injection. One month after administration of AAV2 vector carrying luciferase, high levels of luciferase expression  
 10 were found in peritoneum, but not in heart (Figure 5A), confirming efficient, but non-cardiac, gene delivery by AAV2 vector. To assess the influence of non-cardiac proBNP gene delivery, SHR were injected with pre-proBNP-carrying AAV2 vector and compared with untreated (n=5) and AAV9-pre-proBNP vector-administered rats (n=3). Tail cuff BP measurements indicated that AAV2-pre-proBNP administration had no significant  
 15 effects on SBP and DBP. In contrast, SBP was significantly reduced five and eight weeks after injection of the AAV9-pre-proBNP vector (Figure 5B). The pre-proBNP gene delivery by AAV9, but not AAV2, showed significantly higher plasma level of BNP (Figure 5C) and significant reduction in the heart weight/body weight ratio (Figure 5C), suggesting the requirement of cardiac pre-proBNP delivery for efficient BNP release and  
 20 the anti-hypertrophic effects of BNP in SHR.

#### *Effects of sustained proBNP expression in normotensive rats*

To investigate whether the beneficial effects on both cardiac structure and function observed in the BNP-treated SHR were mainly due to the sustained BP effects,  
 25 AAV9 encoding for GFP or pre-proBNP was injected in normal Wistar rats (n=12). Six rats per group underwent echocardiographic examination 4 weeks after injections and were sacrificed for acute experiments thereafter. Normal rats treated with AAV9 carrying GFP (n=6) showed wide spread GFP expression in cardiomyocytes 4 weeks after injection, further confirming the cardiac transduction of the AAV9 vector (Figure 6A).  
 30 Echocardiographic examination by strain analysis demonstrated that at 4 weeks post injection, AAV9-pre-proBNP treated normal rats (n=6) had a significantly improved

systolic function compared with AAV9-GFP as indicated by a thinner SWTd, and higher sSR circumferential, while LVMi was only slightly reduced ( $p=0.07$ , NS) (Table 3). AAV9-pre-proBNP treated normal rats had significantly higher plasma level of BNP compared to the GFP-control rats (Figure 6B). Although direct intra-carotid BP measurement found similar SBP, DBP, and MAP between the two groups, the heart weight corrected for the body weight was significantly reduced in the BNP-treated as compared with the GFP-control rats (Figures 6B and 6C).

Table 3. Echocardiographic parameters in control (n=6) and BNP treated (n=6) normal rats.

|                              | <b>4 Weeks p.i.</b> |                    |
|------------------------------|---------------------|--------------------|
|                              | <b>Controls</b>     | <b>BNP Treated</b> |
| <b>HR</b>                    | 407±27.2            | 408±24.5           |
| <b>SWTd</b>                  | 2.03±0.15           | 1.80±0.11*         |
| <b>PWTd</b>                  | 1.66±0.09           | 1.77±0.19          |
| <b>LVDd</b>                  | 7.0±0.67            | 7.1±0.53           |
| <b>LVDs</b>                  | 3.84±0.67           | 3.94±0.37          |
| <b>Ejection Fraction</b>     | 83±1.72             | 83±4.17            |
| <b>LV Mass Index</b>         | 0.35±0.01           | 0.33±0.01          |
| <b>sSR Circumferential</b>   | -5.37±0.5           | -6.54±0.8*         |
| <b>dSR-E Circumferential</b> | 5.09±0.7            | 5.98±1.0           |
| <b>sSR-Radial</b>            | 7.63±1.5            | 9.08±1.7           |
| <b>dSR-E Radial</b>          | -5.59±1.3           | -7.84±2.7          |

\* $P<0.05$  vs respective Controls. HR, heart rate; SWTd, septal wall thickness at end diastole; PWTd, posterior wall thickness at end diastole; LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; sSR, systolic strain rate; dSR, diastolic strain rate.

These results demonstrate successful *in vivo* cardiomyocyte transduction via a AAV9 vector that facilitated sustained cardiac proBNP overexpression. Long-term proBNP delivery led to reduced BP and improved LV function and structure in an HHD rat model without any short- or long-term toxicological adverse effects or development of tolerance. Although long-term proBNP delivery improved both systolic and diastolic function, the effect on diastolic performance was more remarkable and preceded the improvement in systolic function in this HHD model. Importantly, the effects on cardiac structure and function occurred independently of BP lowering effects in normal Wistar rats.

These results also demonstrated that rat BNP is released from pre-proBNP-expressing 293T cells as a HMW form.

In addition, rat proBNP overexpression was associated with significant and sustained BP reduction in SHR. Indeed, SBP, DBP and MAP were lower in the BNP-treated as compared with the control SHR from two months up to nine months post-AAV9 injection. This reduction of BP was rather modest and occurred without changes in HR. Of note, BP was reduced at the vector dose used in the current study, while it did not completely normalized BP, which remained elevated throughout the period of observation. It is possible that a higher vector dose would result in a more profound BP reduction. Although the use of telemetry would have helped in better assessing BP changes, a significant BP lowering effect of BNP was confirmed in unconscious BNP-treated SHR compared to the untreated SHR via direct intra-arterial BP measurements at the time of the acute experiments (9 months).

Plasma immunoreactive rat BNP45 was elevated in the BNP-treated as compared with the control SHR at four days, three weeks, and four and nine months post-injection, confirming a sustained overexpression of BNP in the heart. At nine months post-injection, plasma cGMP was not different between the BNP-treated and the control SHR. In contrast, urinary cGMP was increased in the BNP-treated as compared with the control SHR. Thus, the lack of elevation of plasma cGMP may be explained by the increased urinary cGMP excretion.

Chronic overexpression of proBNP prevented the development of HHD, which began at four weeks of age in the SHR. Indeed, AAV9 induced proBNP production

resulted in a sustained and significant reduction (up to nine months) of SBP and DBP. Thorough echo analysis demonstrated a significant improvement of diastolic function at four months post transfection in the BNP-treated group as compared with the untreated SHR. Importantly, at nine months, untreated SHR also developed signs of impaired  
5 systolic function which was prevented in the BNP-treated SHR. Of note, global cardiac function and remodeling were not only improved in the BNP-treated SHR compared to untreated SHR of the same age but also, BNP-treated SHR at nine months showed improved diastolic function and reduced cardiac hypertrophy even when compared with the untreated SHR at four months of age. This finding further supports the beneficial role  
10 of BNP in preventing cardiac dysfunction and remodeling. Of note, all these favorable actions occurred without signs of any short- or long-term toxicological side effects and BNP maintained its biological actions up to nine months post injection without developing tolerance. It should be noted that, although sustained, the BP reduction was minimal, thus further studies are required to address the pathogenic role of BNP in  
15 hypertension.

In SHR transduced with non-cardiac AAV2 vector, no increase in plasma immunoreactive BNP was observed. This could be due to a less efficient intracellular processing and/or release of rat BNP in non-cardiac cells. Furthermore, in these AAV2-transduced SHR, changes in heart weight compared to untreated controls were not  
20 observed. The work described herein used a comparable vector dose for both the AAV2 and AAV9 studies.

The work was extended to normal rats to investigate the anti-hypertrophic actions of BNP overexpression in the absence of hypertension. In this model, age induced systolic impairment was significantly ameliorated in the BNP-treated rats by echo strain  
25 analysis at 4 weeks. Also, cardiac mass was reduced in the BNP-treated rats as compared with the controls and heart weight/body weight was significantly lower in the BNP-treated rats as compare to the controls. Importantly, the improved cardiac function and the reduced cardiac mass were observed after 4 weeks post injections of the AAV9 vector and occurred despite any difference in BP (measured directly intra-carotid) between the  
30 BNP-treated and the control group.

Taken together, the results provided herein demonstrate that the use of chronic supplementation of the cardiorenal protective hormone BNP can be employed in hypertension to prevent the progression toward more severe stages of HHD and the onset of heart and renal failure. Instead of oral delivery, a gene transfection strategy was used to facilitate a nine month delivery of bioactive BNP with single intravenous injection of the AAV9 vector. As indicated herein, chronic overexpression of BNP in SHR reduced BP, decreased LVH, tended to reduce fibrosis, and improved systolic and diastolic function.

In summary, the results provided herein demonstrate the successful cardiac delivery of the AAV9 vector, which mediated sustained cardiac proBNP overexpression without any short- or long-term toxicological effects and any signs of tolerance. Importantly, sustained cardiac BNP overexpression reduced BP and improved LV function in a model of progressive HHD after a single intravenous injection. Although long-term proBNP delivery improved both systolic and diastolic function, the effect on diastolic performance was more remarkable and appeared earlier during the development of HHD. Ultimately, sustained overexpression of BNP in SHR prevented the development of HHD as nine month old BNP-treated SHR had a significantly improved cardiac function and structure even when compared with four month old untreated SHR. Non-cardiac BNP-overexpression was not associated with increase in plasma BNP, changes in BP, and reduced heart weight. The direct cardiac effects of overexpressed BNP seem to be, at least in part, independent of BP lowering action as indicated by the improved systolic function and reduced heart weight in the normotensives rats despite no changes in BP.

In addition, an AAV9 vector designed to express luciferase achieved long-term cardiac luciferase expression in SHR rats at least for 18 months (Figure 9). Generation of AAV9 vectors expressing CNP and CDNP as set forth in Figure 12A was verified by Western blotting (Figure 12B) using an anti-CNP antibody.

#### Example 2 - Use of natriuretic polypeptides to reduce heart volume

To deliver rat BNP-45 to rat cardiac cells, a cardio-tropic AAV9 vector was designed to express a rat proBNP polypeptide having a rat N-terminus proBNP amino

acid sequence (called an NT-proBNP region) upstream of a rat BNP-45 amino acid sequence. The amino acid sequence for the rat NT-proBNP region with its signal peptide of the rat proBNP polypeptide was MDLQKVLPQMILLLLFLNLSPLGGHSHPLG-SPSQSPEQSTMQKLELIREKSEEMAQRQLSKDQGPTKELLKRVLR (SEQ ID NO: 1). The amino acid sequence for the rat BNP-45 of the rat proBNP polypeptide was SQDSAFRIQERLRNSKMAHSSSCFGQKIDRIGAVSRLGCDGLRLF (SEQ ID NO:2). The amino acid sequence for the rat pre-proBNP polypeptide was MDLQKVLPQMILLLLFLNLSPLGGHSHPLGSPSQSPEQSTMQKLELIREKSEEMAQRQLSKDQGPTKELLKRVLR SQDSAFRIQERLRNSKMAHSSSCFGQKIDRIGAVSRLGCDGLRLF (SEQ ID NO:3).

To deliver CDNP to rat cardiac cells, two cardio-tropic AAV9 vectors were designed. One AAV9 vector was designed to express a polypeptide designated B-CDNP, and the other AAV9 vector was designed to express a polypeptide designated C-CDNP. The B-CDNP polypeptide included a human N-terminus proBNP amino acid sequence (called an NT-proBNP region) upstream of the CDNP amino acid sequence, while the C-CDNP polypeptide included a human N-terminus proCNP amino acid sequence (called an NT-proCNP region) with its signal peptide upstream of the CDNP amino acid sequence. The amino acid sequence for human NT-proBNP region with its signal peptide of the B-CDNP polypeptide was with its signal peptide MDPQTAPSRALLLLLFL-HLAFLGGRSHPLGSPGSASDLETSGLQEQRNHLQGKLSLQVEQTSLEPLQESPRPTGVWKSREVATEGIRGHRKMVLYTLRAPR (SEQ ID NO:4), and the amino acid sequence for the rat NT-proCNP region with its signal peptide of the C-CDNP polypeptide was MHLSQLLACALLLTLLSLRPSEAKPGAPPKVPRTPPAEELAEPQ-AAGGGGKKGDKAPGGGGANLKGDRSRLRLRVDTKSRAAWARLLGEHPNAR KYKGANKK (SEQ ID NO:5). The amino acid sequence for CDNP of the B-CDNP and C-CDNP polypeptides was GLSKGCFGLKLDRI GMSGLGCPSLRDP RP NAPSTSA (SEQ ID NO:6). The amino acid sequence for B-CDNP polypeptide was MDPQTAPSRALLLLLFLHLAFLGGRSHPLGSPGSASDLETSGLQEQRNHLQGKLSLQVEQTSLEPLQESPRPTGVWKSREVATEGIRGHRKMVLYTLRAPRGLSKGCFGLKLDRI GMSGLGCPSLRDP RP NAPSTSA (SEQ ID NO:7), and the amino acid sequence for C-CDNP polypeptide was MHLSQLLACALLLTLLSLRPSEAKPGAPPKVPRTPPAE-

ELAEPQAAGGGQKKGDKAPGGGGANLKGDRSRLRLDLRVDTKSRAAWARLLQ  
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGCPSLRDPRPNAPSTSA (SEQ  
ID NO:8).

The nucleic acid sequences encoding B-CDNP and C-CDNP were codon-  
5 optimized for expression in human cells. The nucleic acid sequence encoding B-CDNP  
was as follows 5'-ATGGACCCACAGACAGCTCCCAGTAGGGCTTTGCTTCTT-  
TTGCTTTTCCTGCACCTGGCTTTTCTGGGCGGACGATCCCATCCACTGGGTAG  
CCCTGGCTCCGCCTCAGATCTGGAGACTAGTGGACTGCAGGAGCAGCGCAAT  
CACTTGCAGGGCAAACGTCCGAGCTGCAGGTGGAACAAACGAGCCTCGAGC  
10 CCCTGCAGGAGAGCCCTAGACCTACCGGGGTGTGGAAGTCTCGAGAGGTAGC  
GACAGAAGGCATTAGAGGGCACAGGAAGATGGTACTGTATACTCTGAGGGCC  
CCAAGGGGACTGAGCAAGGGCTGTTTTGGCCTGAAGCTGGATCGGATTGGCA  
GCATGTCCGGCCTGGGCTGCCCTTCCCTGCGGGACCCACGGCCAAATGCCCCC  
TCCACCAGCGCCTAA-3' (SEQ ID NO:9), while the nucleic acid sequence encoding  
15 C-CDNP was as follows 5'-ATGCATCTGTCCCAACTGCTGGCTTGTGCTCTCC-  
TGCTGACTCTGCTGAGCCTCCGGCCTAGCGAGGCCAAGCCTGGAGCACCACC  
TAAGGTCCCCAGGACTCCTCCAGCCGAAGAACTGGCTGAGCCTCAGGCTGCC  
GGGGGCGGGCAGAAGAAAGGAGACAAAGCCCCTGGAGGGGGCGGGGCTAAT  
CTCAAGGGCGATAGGTCCAGACTGCTGAGGGATCTGAGAGTGGACACAAAGT  
20 CCAGGGCCGCCTGGGCACGGCTCCTGCAAGAGCACCCTAACGCTCGGAAGTA  
CAAAGGGGCCAATAAGAAGGGCCTCAGCAAAGGCTGCTTTGGCCTGAACTG  
GACAGAATTGGCTCCATGTCCGGCCTCGGCTGCCCTTCCCTGCGGGACCCTCG  
GCCCAATGCCCTTCCACTAGCGCTTAA-3' (SEQ ID NO: 10).

Spontaneously hypertensive rats (SHR) were divided into groups of six rats each.  
25 One group included rats that were untreated, while the other groups were treated with a  
single intravenous injection of AAV9 vectors designed to express the rat proBNP  
polypeptide, the B-CDNP polypeptide, or the C-CDNP polypeptide. After five weeks,  
the rats were examined to determine the weights of their hearts, lungs, and kidneys. No  
significant difference was detected for the lungs and kidneys between the treated and  
30 untreated animals (Figure 7). There, however, was a statistically significant difference in  
the weight of the hearts for the treated animals as compared to the weight of the hearts for

the untreated animals (Figure 7). Administration of AAV9-C-CDNP facilitated long-term cardiac CDNP expression for five weeks in six treated rats as compared to six untreated rats (Figure 8).

These results demonstrate that a single intravenous administration of a natriuretic polypeptide-carrying AAV9 vector can protect the heart from excessive fibrosis and hypertrophy in mammals with hypertension and/or with renal dysfunction. These results also demonstrate that the methods and materials provided herein can be used for long-term natriuretic polypeptide treatment (e.g., long-term CNDP treatment) of patients with cardiovascular and/or renal diseases (e.g., those patients with severe cardiac hypertrophy and dysfunction).

In another set of experiments, an AAV9 vector designed to express BNP was used to mediate cardiac BNP expression and extend the survival of rats with established hypertensive heart disease (Figure 10). Briefly, 9 month-old SHR rats with established hypertensive heart disease were treated with the AAV9 vector expressing rat proBNP. Treated and untreated rats were monitored for survival. AAV9 vector-mediated cardiac BNP expression extended the survival of rats with established hypertensive heart disease (Figure 10).

Sustained cardiac proBNP over-expression by the AAV9 vector improved cardiac function and structure in established hypertensive heart disease (Figure 11). Aged SHR rats (9 months old) with impaired cardiac functions were randomly assigned to two groups, and 6 rats were intravenously injected with proBNP-expressing AAV9 vector ( $1 \times 10^{12}$  genome particles/rat). The effects of sustained proBNP expression on cardiac functions/remodeling were analyzed at 5 months post injection. When compared to controls, septum wall thickness and LV mass were significantly reduced, while ejection fraction was significantly higher in BNP-treated rats (Figure 11).

Expression of proBNP using an AAV9 vector designed to express proBNP resulted in improved cardiac functions in a rat model of polycystic kidney disease (Figure 13). Short-term (2 months) AAV9-proBNP treatment significantly improved the cardiac function in a rat model of polycystic kidney disease (PKD). Briefly, four week old PCK rats (a rat model of PKD) were treated with the proBNP-encoding AAV9 vector. Two

months after systemic vector administration, statistically significant improvements in cardiac structure and functions (output) were observed (Figure 13).

Example 3 - Design of BNP and CDNP viral vectors for human use to treat  
cardiovascular and/or renal diseases

To deliver human BNP to human cardiac cells, a cardio-tropic AAV9 vector was designed to express a human proBNP polypeptide having a human N-terminus proBNP amino acid sequence (called an NT-proBNP region) upstream of a human BNP-32 amino acid sequence. The amino acid sequence for the signal peptide and NT-proBNP region of the human proBNP polypeptide was MDPQTAPSRALLLLFLHLAFLGGRSHPLG-  
 10 SPGSASDLETSGLQEQRNHLQGKLSLQVEQTSLEPLQESPRPTGVWKSREVATE  
 GIRGHRKMOVLYTLRAPR (SEQ ID NO:4). The amino acid sequence for the human BNP-32 of the human proBNP polypeptide was SPKMOVQSGCFGRKMDRI-  
 SSSSGLGCKVLRH (SEQ ID NO: 11). The amino acid sequence for the human  
 15 proBNP polypeptide was MDPQTAPSRALLLLFLHLAFLGGRSHPLGSPG-  
 SASDLETSGLQEQRNHLQGKLSLQVEQTSLEPLQESPRPTGVWKSREVATEGIR  
 GHRKMOVLYTLRAPRSPKMOVQSGCFGRKMDRISSSSGLGCKVLRH (SEQ ID  
 NO: 12).

To deliver CDNP to human cardiac cells, two cardio-tropic AAV9 vectors were  
 20 designed. One AAV9 vector was designed to express a polypeptide designated human B-  
 CDNP, and the other AAV9 vector was designed to express a polypeptide designated  
 human C-CDNP. The human B-CDNP polypeptide was designed to include a human N-  
 terminus proBNP amino acid sequence (called an NT-proBNP region) upstream of the  
 CDNP amino acid sequence, while the C-CDNP polypeptide was designed to include a  
 25 human N-terminus proCNP amino acid sequence (called an NT-proCNP region) upstream  
 of the CDNP amino acid sequence. The amino acid sequence for the human NT-proBNP  
 region with signal peptide of the human B-CDNP polypeptide was MDPQTAPSR-  
 ALLLLFLHLAFLGGRSHPLGSPGSASDLETSGLQEQRNHLQGKLSLQVEQTSLEP  
 LQESPRPTGVWKSREVATEGIRGHRKMOVLYTLRAPR (SEQ ID NO:4), and the  
 30 amino acid sequence for the human NT-proCNP region of the human C-CDNP  
 polypeptide was MHLSQLLACALLLTLLSLRPSEAKPGAPPKVPRTPPAE-

ELAEPQAAGGGGKKGDKAPGGGGANLKGDRSRLRLRDLRVDTKSRAAWARLLG  
 EHPNARKYKGANKK (SEQ ID NO:5). The amino acid sequence for CDNP of the  
 human B-CDNP and human C-CDNP polypeptides was GLSKGCFGLKLDRI-  
 GSMSGLGCPSLRDPRPNAPSTSA (SEQ ID NO:6). The amino acid sequence for  
 5 human B-CDNP polypeptide was MDPQTAPSRALLLLFLHLAFLGGRSHPLGS-  
 PGSASDLETSLQEQRNHLQGKLSELQVEQTSLEPLQESPRPTGVWKSREVATEG  
 IRGHRKMVLYTLRAPRGLSKGCFGLKLDRIGSMSGLGCPSLRDPRPNAPSTSA  
 (SEQ ID NO: 7), and the amino acid sequence for human C-CDNP polypeptide was  
 MHLSQLLACALLLTLLSLRPSEAKPGAPPKVPRTPPAEELAEPQAAGGGQKKGDK  
 10 APGGGGANLKGDRSRLRLRDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLS  
 KGCFGLKLDRIGSMSGLGCPSLRDPRPNAPSTSA (SEQ ID NO:8).

The nucleic acid sequences encoding human B-CDNP and human C-CDNP were  
 codon-optimized for expression in human cells. The nucleic acid sequence encoding  
 human B-CDNP was as follows 5'-ATGGACCCACAGACAGCTCCCAGTAGGG-  
 15 CTTTGCTTCTTTTGCTTTTCCTGCACCTGGCTTTTCTGGGCGGACGATCCCATC  
 CACTGGGTAGCCCTGGCTCCGCCTCAGATCTGGAGACTAGTGGACTGCAGGA  
 GCAGCGCAATCACTTGCAGGGCAAACCTGTCCGAGCTGCAGGTGGAACAAACG  
 AGCCTCGAGCCCCTGCAGGAGAGCCCTAGACCTACCGGGGTGTGGAAGTCTC  
 GAGAGGTAGCGACAGAAGGCATTAGAGGGCACAGGAAGATGGTACTGTATA  
 20 CTCTGAGGGCCCCAAGGGGACTGAGCAAGGGCTGTTTTGGCCTGAAGCTGGA  
 TCGGATTGGCAGCATGTCCGGCCTGGGCTGCCCTTCCCTGCGGGACCCACGGC  
 CAAATGCCCCCTCCACCAGCGCCTAA-3' (SEQ ID NO:9), while the nucleic acid  
 sequence encoding human C-CDNP was as follows 5'-ATGCATCTGTCCCAA-  
 CTGCTGGCTTGTGCTCTCCTGCTGACTCTGCTGAGCCTCCGGCCTAGCGAGGC  
 25 CAAGCCTGGAGCACCACCTAAGGTCCCCAGGACTCCTCCAGCCGAAGAACTG  
 GCTGAGCCTCAGGCTGCCGGGGGCGGGCAGAAGAAAGGAGACAAAGCCCCT  
 GGAGGGGGCGGGGCTAATCTCAAGGGCGATAGGTCCAGACTGCTGAGGGATC  
 TGAGAGTGGACACAAAGTCCAGGGCCGCCTGGGCACGGCTCCTGCAAGAGCA  
 CCCTAACGCTCGGAAGTACAAAGGGGCCAATAAGAAGGGCCTCAGCAAAGG  
 30 CTGCTTTGGCCTGAAACTGGACAGAATTGGCTCCATGTCCGGCCTCGGCTGCC

Attorney Docket No.: 07039-1 107WO1

CTTCCCTGCGGGACCCTCGGCCCAATGCCCCTTCCACTAGCGCTTAA-3 ' (SEQ  
ID NO: 10).

### OTHER EMBODIMENTS

5           It is to be understood that while the invention has been described in conjunction  
with the detailed description thereof, the foregoing description is intended to illustrate and  
not limit the scope of the invention, which is defined by the scope of the appended claims.  
Other aspects, advantages, and modifications are within the scope of the following  
claims.

10

**WHAT IS CLAIMED IS:**

1. An AAV9 vector comprising a nucleic acid sequence encoding a natriuretic polypeptide.  
5
2. The vector of claim 1, wherein said natriuretic polypeptide is a human BNP polypeptide.
3. The vector of claim 1, wherein said natriuretic polypeptide is a CDNP  
10 polypeptide.
4. The vector of claim 1, wherein said natriuretic polypeptide is a B-CDNP polypeptide.
- 15 5. The vector of claim 1, wherein said natriuretic polypeptide is a C-CDNP polypeptide.
6. A composition comprising an AAV9 vector of claim 1 in combination with a pharmaceutically acceptable delivery vehicle.  
20
7. The composition of claim 6, wherein said natriuretic polypeptide is a human BNP polypeptide.
8. The composition of claim 6, wherein said natriuretic polypeptide is a CDNP  
25 polypeptide.
9. The composition of claim 6, wherein said natriuretic polypeptide is a B-CDNP polypeptide.
- 30 10. The composition of claim 6, wherein said natriuretic polypeptide is a C-CDNP polypeptide.

11. A method for treating a cardiovascular or renal disease, wherein said method comprises administering a vector of any one of claims 1-5 or a composition of any one of claims 6-10 to a mammal.

5

12. The method of claim 11, wherein said mammal is a human.

13. A method for prolonging survival time for a mammal with hypertensive heart disease, wherein said method comprises administering a vector of any one of claims 1-5 or a composition of any one of claims 6-10 to said mammal.

10

14. The method of claim 13, wherein said mammal is a human.

15. A method for improving cardiac function in a mammal with hypertensive heart disease, wherein said method comprises administering a vector of any one of claims 1-5 or a composition of any one of claims 6-10 to said mammal under conditions wherein cardiac function is improved at least five months following said administration.

15

16. The method of claim 15, wherein said mammal is a human.

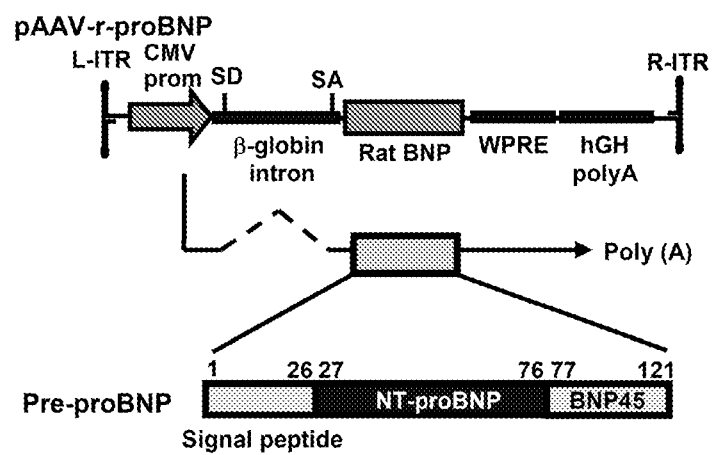
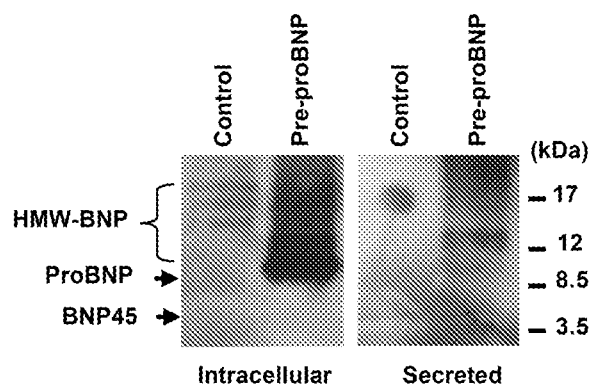
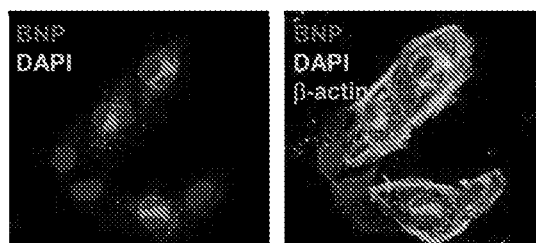
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17. A method for improving cardiac function in a mammal with polycystic kidney disease, wherein said method comprises administering a vector of any one of claims 1-5 or a composition of any one of claims 6-10 to said mammal under conditions wherein cardiac function is improved at least two months following said administration.

25

18. The method of claim 17, wherein said mammal is a human.

Fig. 1

**A****B****C**

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Fig. 2

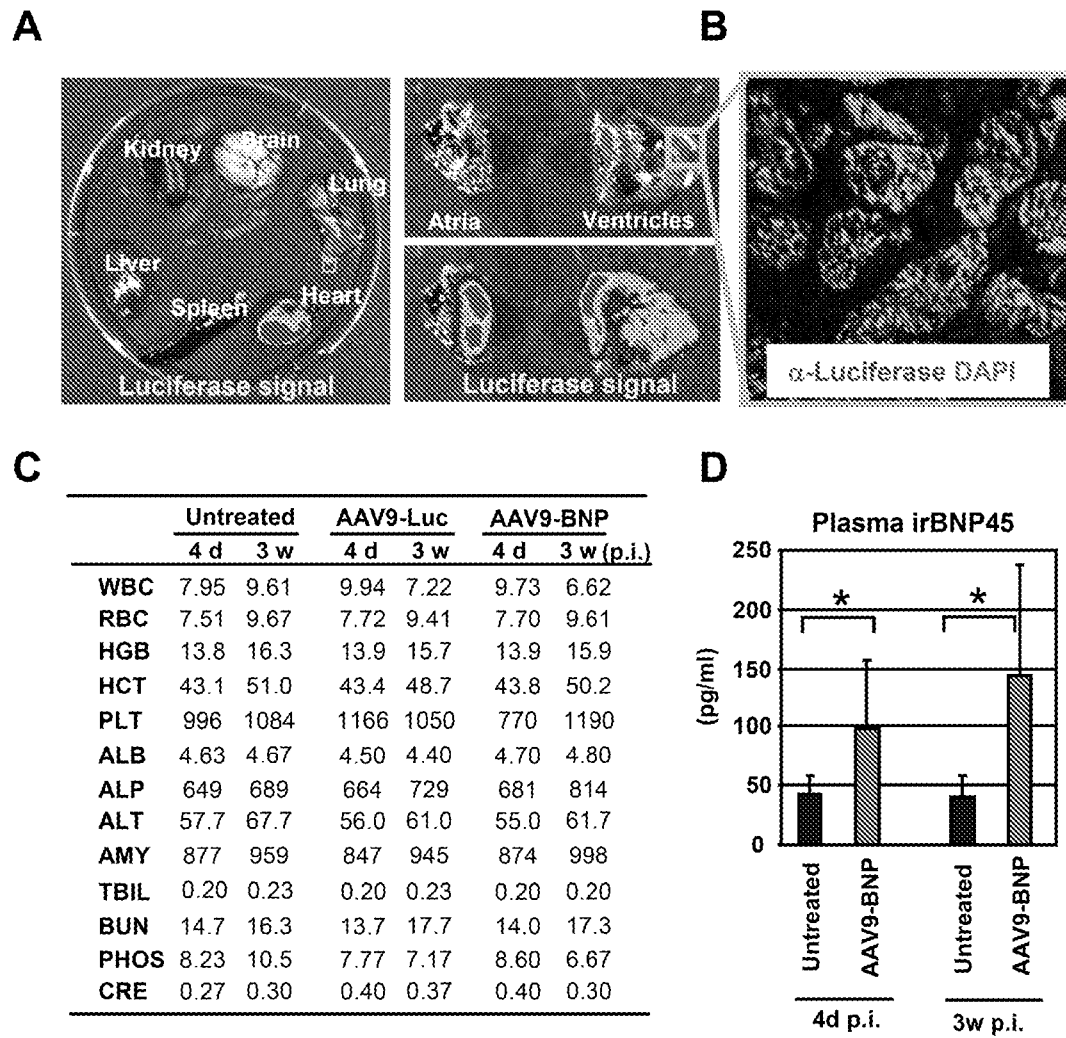
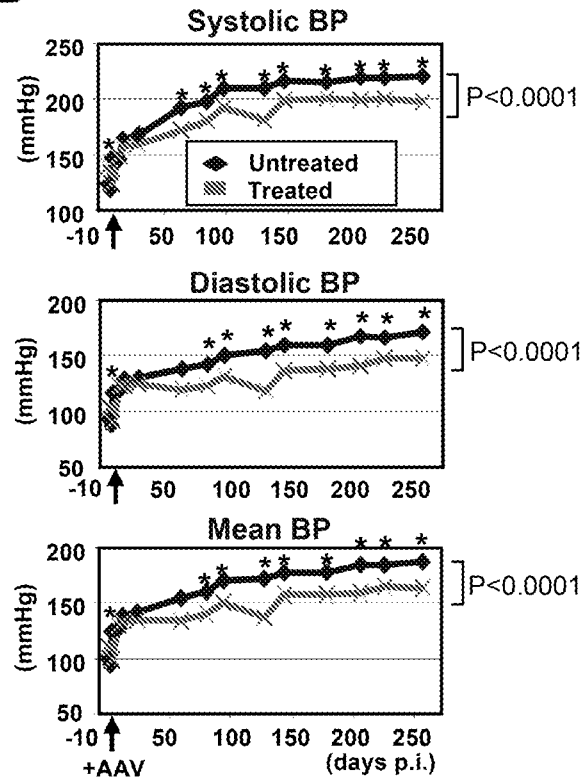
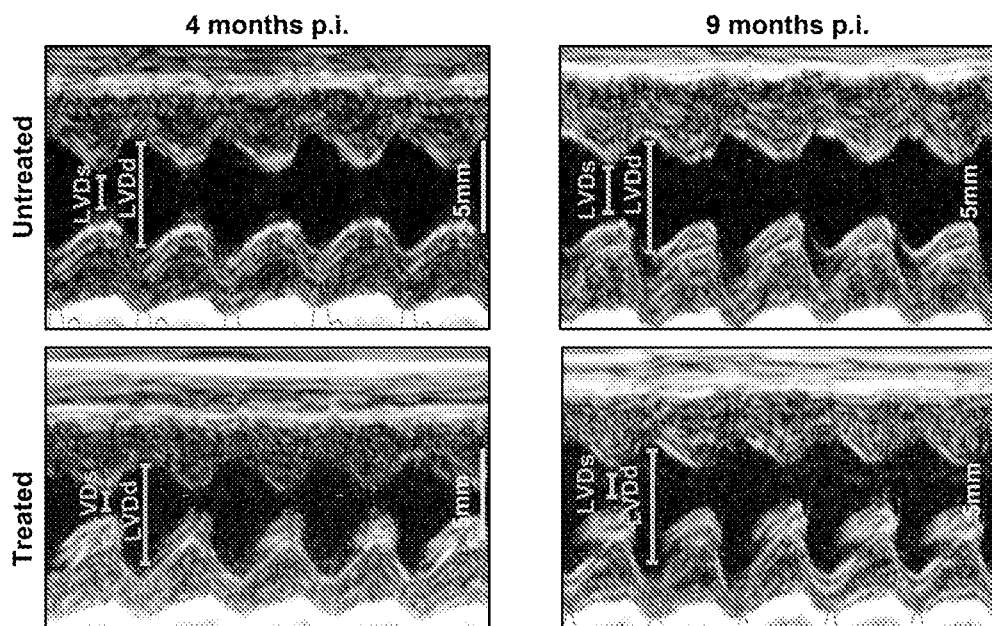


Fig. 3

**A**

|         | Untreated | Treated     |
|---------|-----------|-------------|
| irBNP45 | 60.6±15.1 | 433.3±17.0* |
| WBC     | 7.22±1.17 | 8.58±2.37   |
| RBC     | 10.5±0.31 | 10.9±0.22   |
| HGB     | 17.4±0.80 | 17.9±0.33   |
| HCT     | 48.4±1.32 | 49.8±1.94   |
| PLT     | 898±42.7  | 933±65.9    |
| ALB     | 5.13±0.29 | 5.28±0.38   |
| ALP     | 503±28.5  | 546±39.0    |
| ALT     | 68.0±1.83 | 69.8±2.87   |
| AMY     | 889±59.8  | 881±64.5    |
| TBIL    | 0.20±0    | 0.23±0.05   |
| BUN     | 18.0±1.41 | 18.3±0.5    |
| PHOS    | 5.3±0.29  | 5.95±1.29   |
| CRE     | 0.45±0.06 | 0.45±0.1    |
| GLU     | 186±16.5  | 208±12.8    |
| TP      | 6.90±0.22 | 7.15±0.24   |

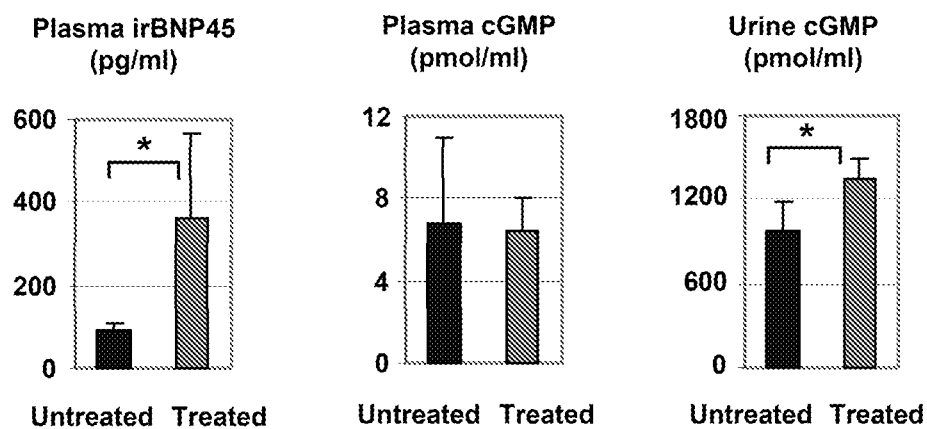
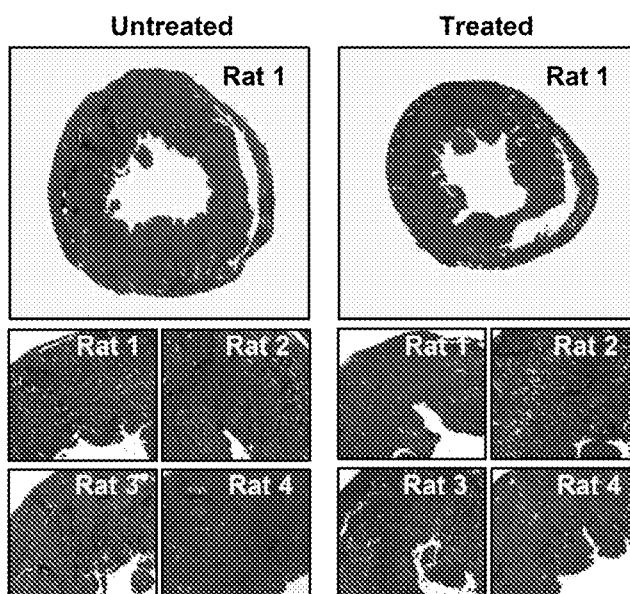
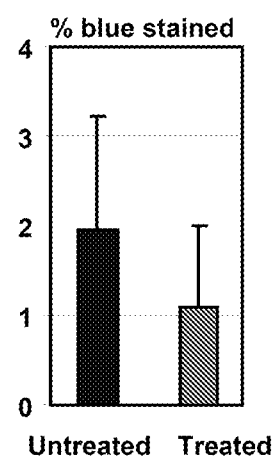
**B****C**

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Fig. 4

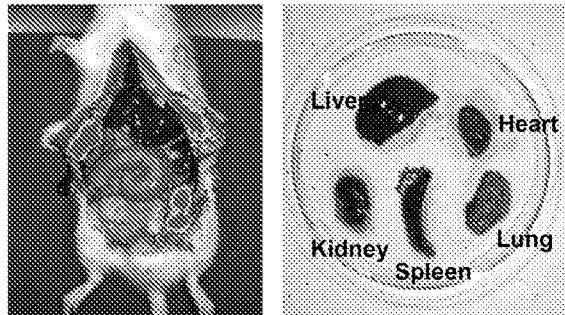
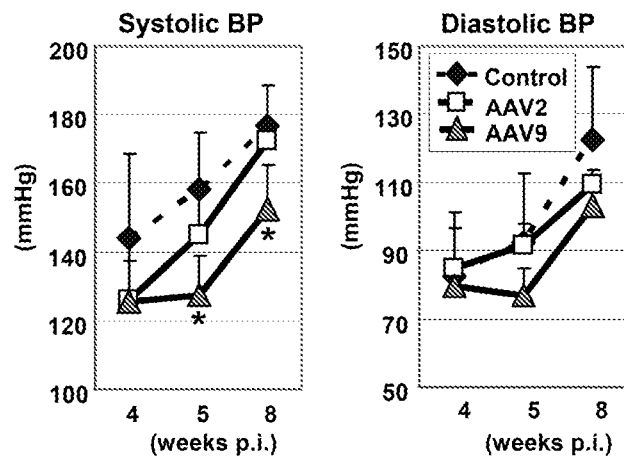
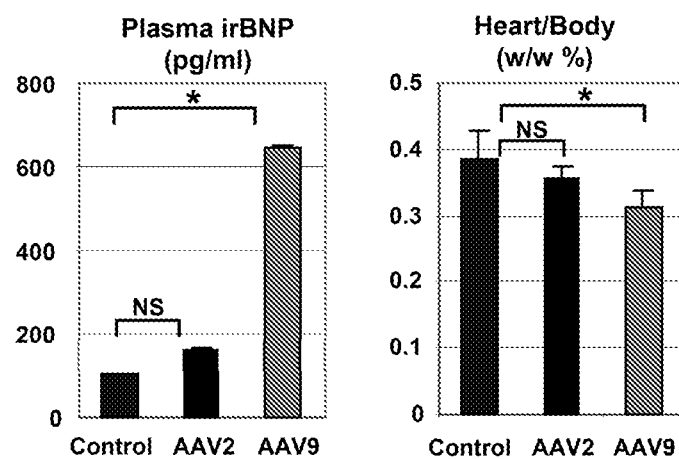
| 9 months p.i.  |           |           |
|----------------|-----------|-----------|
|                | Untreated | Treated   |
| HR (arterial)  | 318±33.3  | 320±45.7  |
| SBP (arterial) | 184±13.7  | 121±11.7* |
| DBP (arterial) | 148±24.2  | 103±5.6*  |

\*P&lt;0.05 vs respective untreated rats (n=4)

**B****C****D**

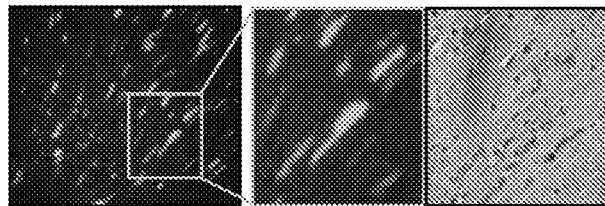
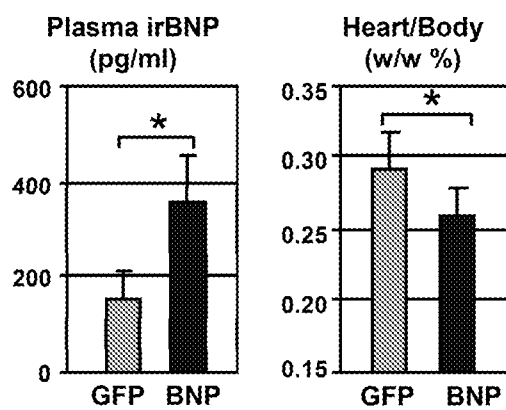
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Fig. 5

**A****B****C**

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Fig. 6

**A****B****C**

| 4 weeks p.i.   |           |             |
|----------------|-----------|-------------|
|                | Controls  | BNP Treated |
| HR (arterial)  | 311±30.8  | 317±29.7    |
| SBP (arterial) | 100±11.1  | 99.8±7.7    |
| DBP (arterial) | 85.8±10.8 | 80.0±8.6    |

No significant difference was observed between BNP vector treated and GFP vector-treated rats (n=6).

Fig. 7

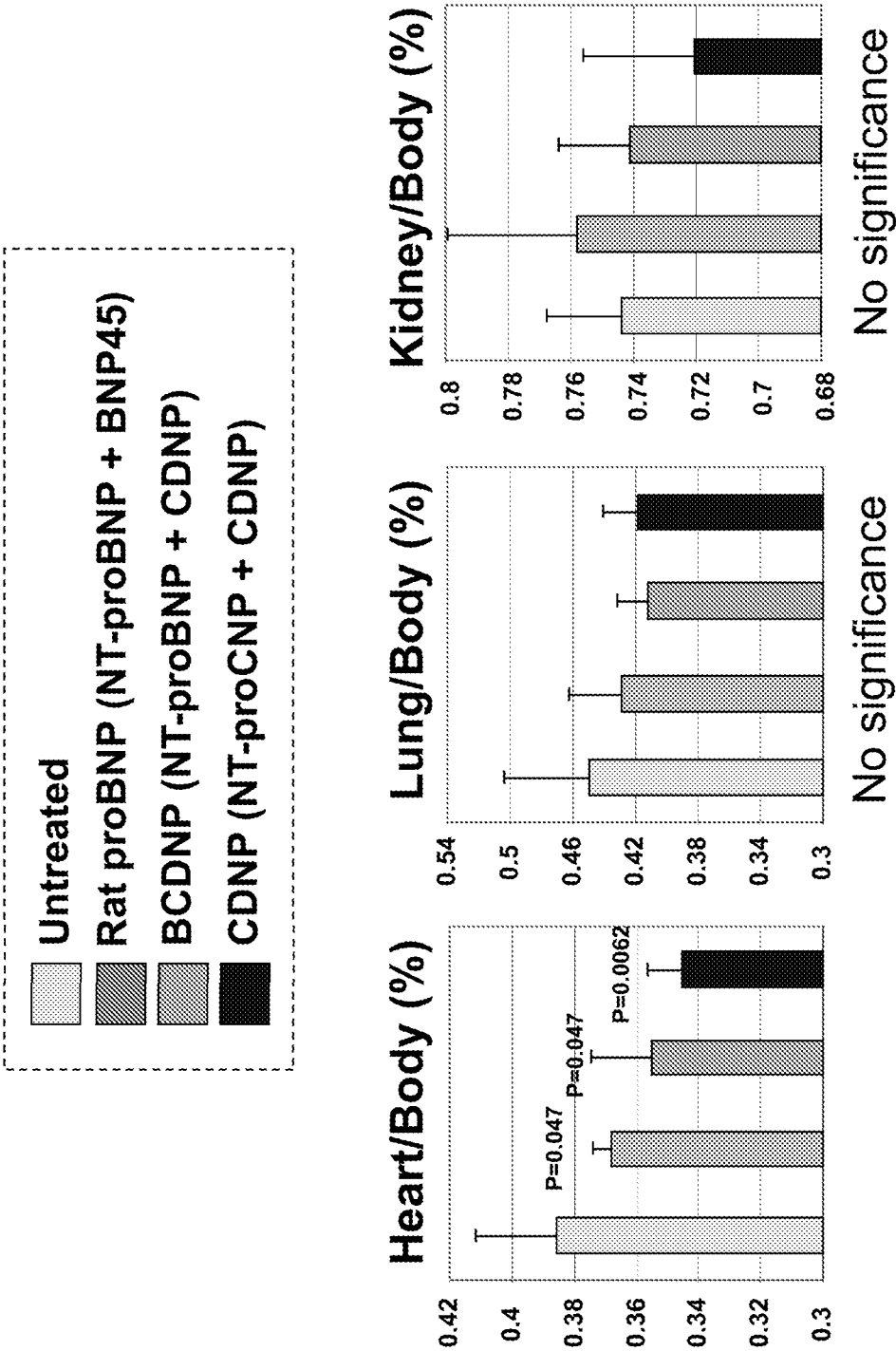


Fig. 8

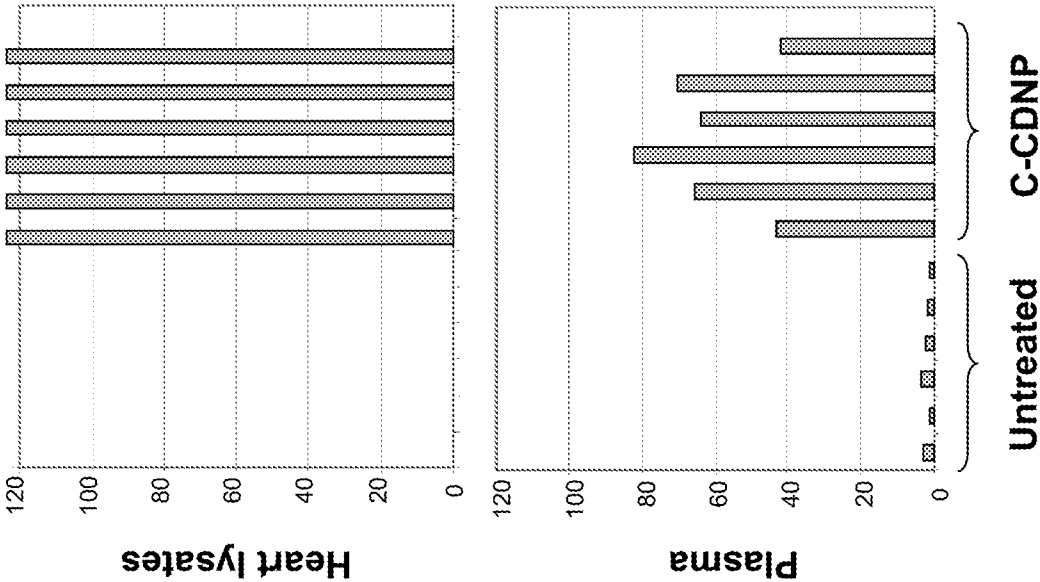
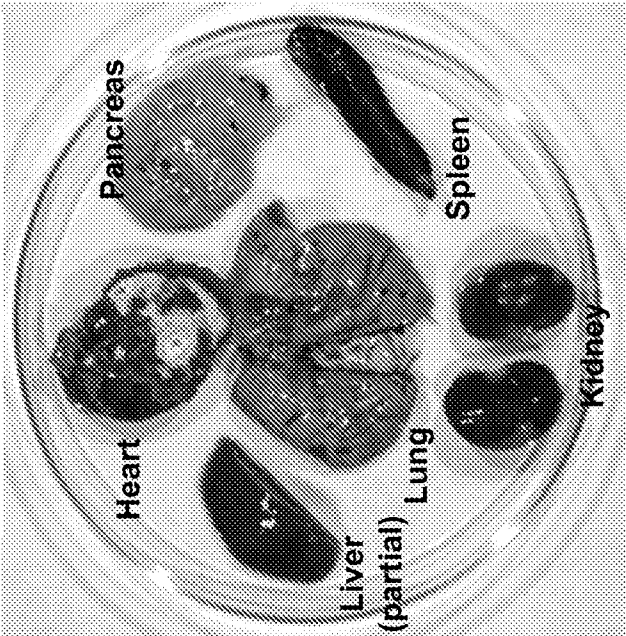


Figure 9

SHR #1



SHR #2

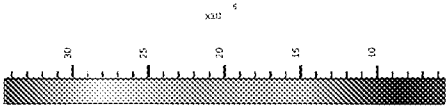
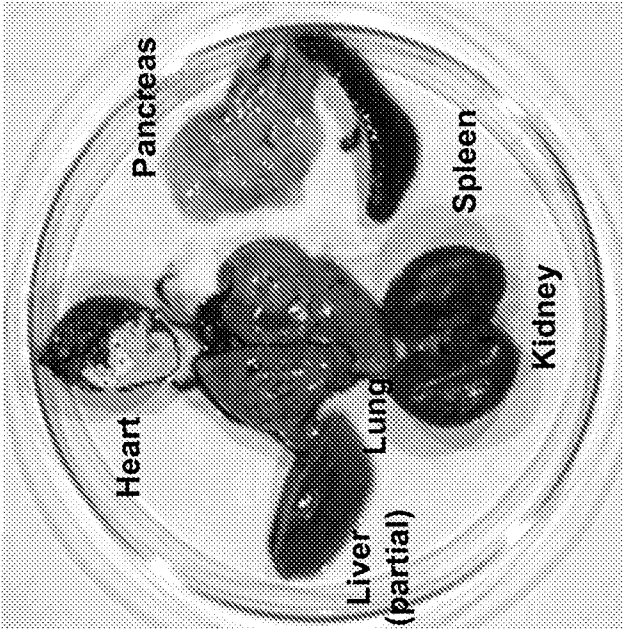


Figure 10

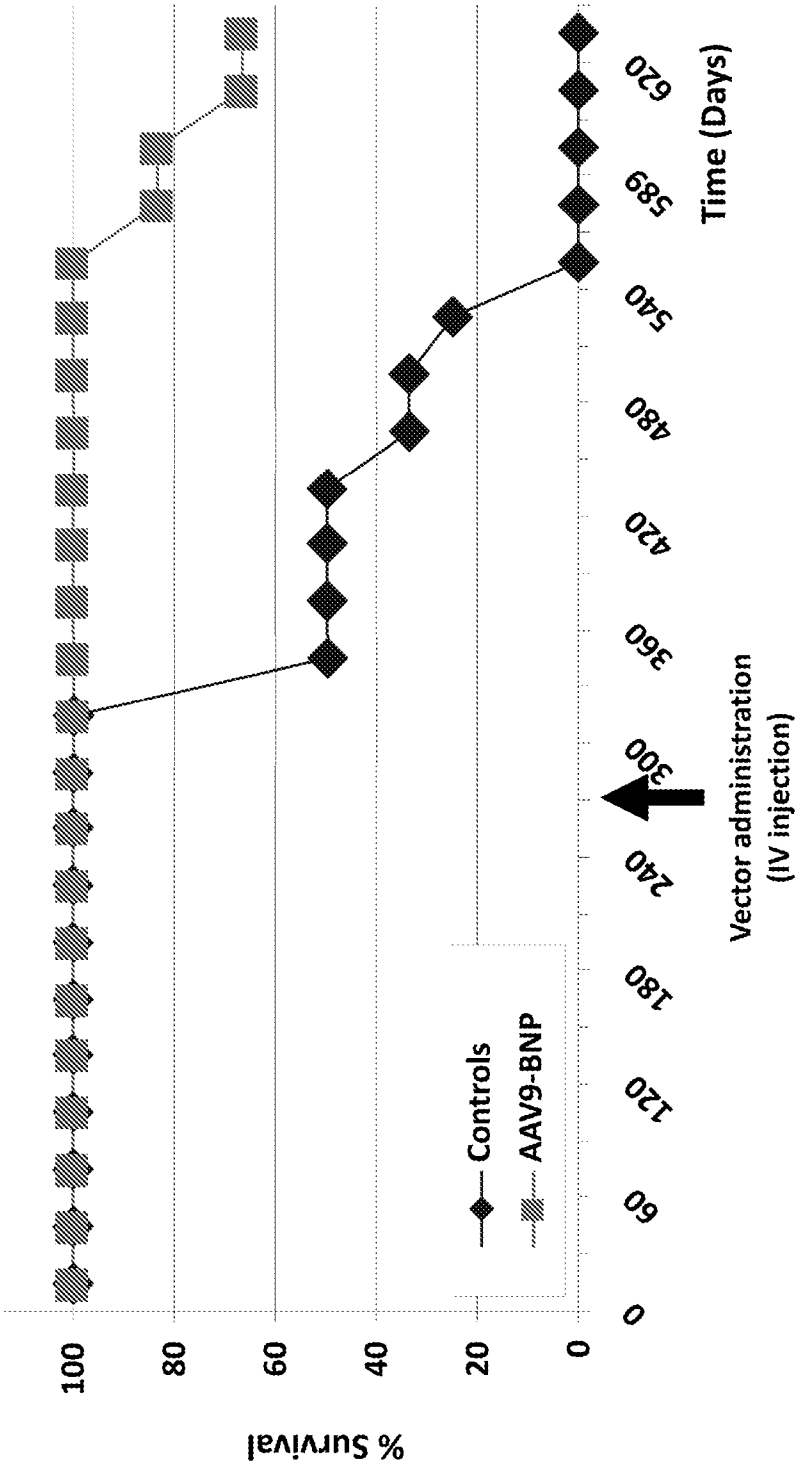


Figure 11

|                  | Control<br>(n=7) | proBNP<br>(n=6) |
|------------------|------------------|-----------------|
| HR               | 359±24.2         | 385±18.9        |
| SWTd             | 2.63±0.28        | 2.28±0.20*      |
| PWTd             | 2.28±0.13        | 2.26±0.23       |
| LVDd             | 8.67±0.88        | 7.81±0.76       |
| SWTs             | 3.84±0.38        | 3.35±0.19*      |
| PWTs             | 2.93±0.35        | 3.43±0.33*      |
| LVDs             | 6.00±1.35        | 4.69±0.56       |
| LV Mass diastole | 2.16±0.36        | 1.77±0.14*      |
| EF (Cube)        | 66.4±12.6        | 78.2±3.3*       |
| EF (Teich)       | 63.7±12.8        | 76.2±3.2*       |
| %FS              | 31.4±8.5         | 40±3.0*         |

Figure 12

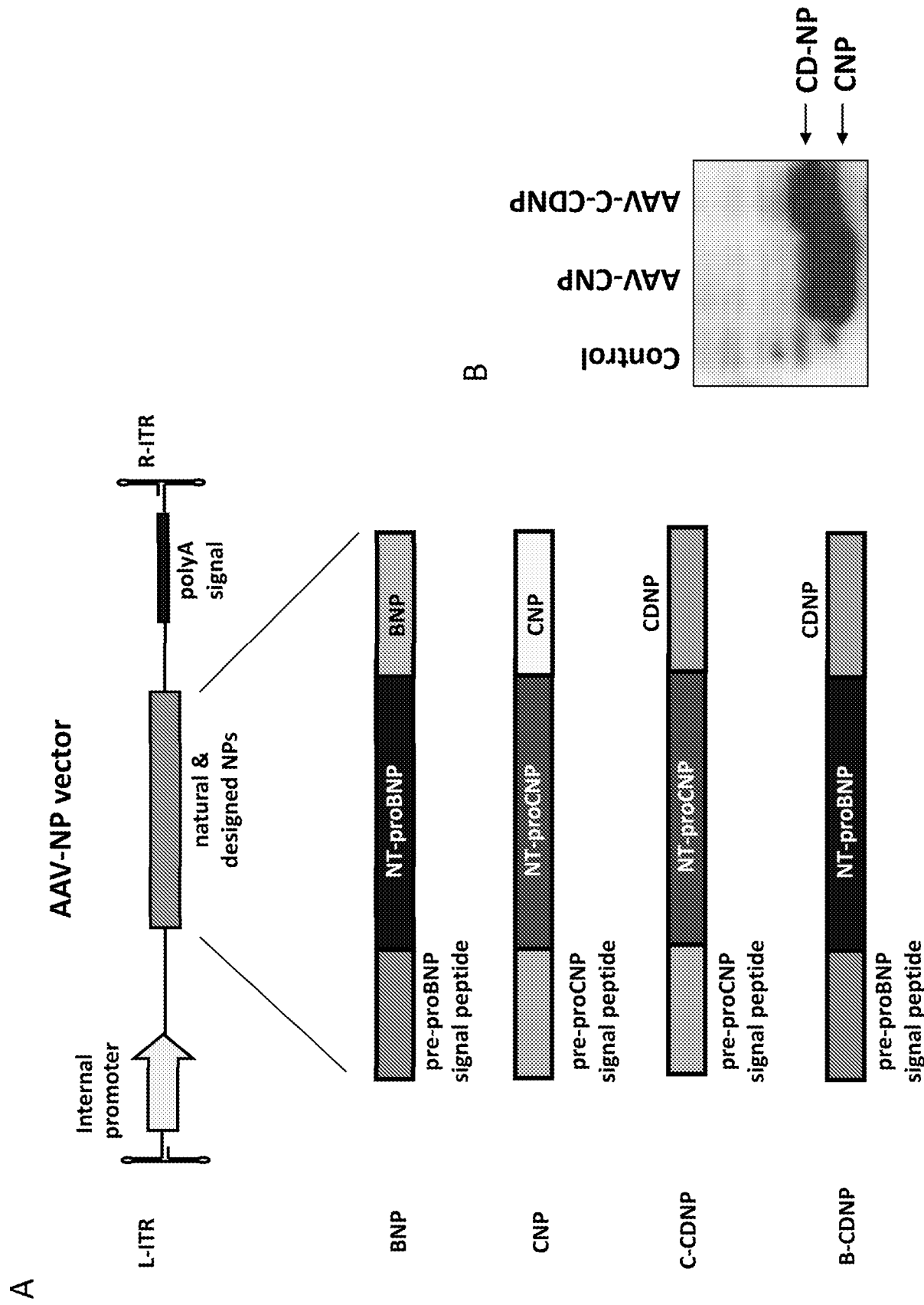


Figure 13

| Parameters for<br>cardiac functions | Control | AAV9<br>proBNP |
|-------------------------------------|---------|----------------|
| IVSd                                | 2.22    | 1.66*          |
| LVIDd                               | 7.25    | 7.85*          |
| LVPWd                               | 1.84    | 1.69           |
| IVSs                                | 3.19    | 2.83           |
| LVIDs                               | 4.45    | 4.88           |
| LVPWs                               | 2.57    | 2.46           |
| EFcube                              | 75.6    | 75.7           |
| EFteich                             | 73.4    | 73.3           |
| %FS                                 | 38.5    | 38.0           |
| LVd Mass                            | 1.49    | 1.37           |
| VLs Mass                            | 1.41    | 1.38           |
| HR                                  | 352.8   | 378.7          |
| Cardiac Output                      | 0.22    | 0.29*          |

Figure 14

**pre-pro-ANP amino acid sequence**

(NCBI Reference Nucleotide Sequence: NM\_006172.3)

MSSFSTTVSFLLLAFQLLGQTRANPMYNAVSNADLMDFKNLLDHLEEKM  
 PLEDEVPPQVLSEPNEEAALSPLEVPPTGEVSPAQRDGGALGRGPW  
 DSSDRSALLKSKLRALLTAPRSLRRSSCFGGRMDRIGAQSGLCNSFRY

ANP

**pre-pro-mANP (frame-shifted mutant ANP) amino acid sequence**

MSSFSTTVSFLLLAFQLLGQTRANPMYNAVSNADLMDFKNLLDHLEEKM  
 PLEDEVPPQVLSEPNEEAALSPLEVPPTGEVSPAQRDGGALGRGPW  
 DSSDRSALLKSKLRALLTAPRSLRRSSCFGGRMDRIGAQSGLCNSFRYRI

TAREDKQGW

mANP (= fsANP)

**Synthesized mANP-encoding nucleotide sequence (codon usage-optimized)**

ATGAGCTCCTTTCCACTACTGTGTCCTTCTGCTGCTGGCCTCCAGCTGTGGCCAAACCCGGCTAACCC  
 AATGTACAACGCCGTCAGCAATGCTGATCTGATGGATTTAAGAATCTGCTGGATCACCTGGAGGAAAGATGCCTCTGG  
 AAGACGAAGTGGTCCCTCCTCAAGTCCTGTCCGAACCTAACGAGGAAGCCGGCTGCCCTGAGCCCTCTCCCTGAAGT  
 CCCACCTGGACAGGGGAGGTCAGCCCCGCACAGAGGGACGGGGCACTGGGAAGAGGCCCATGGGATAGCTCC  
 GACCGGAGCGCTCTGCTGAAAAGCAAACTGAGGGCTCTGCTGACCGCCCTAGAAAGCCTCAGGAGAAGCTCCTGCTTC  
 GCGGGAAGGATGGACCGGATTGGGGCACAAAGCGGCTGGGATGTAACTCCTCCGGTATAGGATTACAGCTAGAGAA  
 GATAACAGGGCTGGCTTAA

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2013/020392

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                                                                                                                                                                                                                                                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                       |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| A. CLASSIFICATION OF SUBJECT MATTER                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |                                                                                                                                                                                                                                                             | <b>C12N 15/861 (2006.01)</b><br><b>A61P 9/00 (2006.01)</b><br><b>C07K 14/58 (2006.01)</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |                       |
| According to International Patent Classification (IPC) or to both national classification and IPC                                                                                                                                                                                                                                                                                                                                                                                                                                                             |                                                                                                                                                                                                                                                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                       |
| B. <u>FIELDS SEARCHED</u>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                                                                                                                                                                                                                                                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                       |
| Minimum documentation searched (classification system followed by classification symbols)                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                                                                                                                                                                                                                                                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                       |
| C12N 15/86 1, A61P 9/00, C07K 14/58                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |                                                                                                                                                                                                                                                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                       |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched                                                                                                                                                                                                                                                                                                                                                                                                                                 |                                                                                                                                                                                                                                                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                       |
| Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                                                                                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                       |
| PAJ, Esp@cent, PCTonline, USPTODB, WIPO, RUPTO, EAPATIS, PatSearch                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                                                                                                                                                                                                                                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                       |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                                                                                                                                                                                                                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                       |
| Category*                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                          |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | Relevant to claim No. |
| X<br>Y                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | CATALIOTTI A. et al. Long-term cardiac proBNP gene delivery prevents the development of hypertensive heart disease in spontaneously hypertensive rats, Circulation, 2011, Vol. 123, no. 12, pp. 1297-1305. doi: 10.1161/CIRCULATIONAHA.110.981720, pp. 1-19 |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | 1-2, 11-18<br>3-10    |
| Y                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | DICKY D.M. et al. Novel bifunctional natriuretic peptides as potential therapeutics, J Biol Chem, 2008, Vol. 283, no. 50, pp. 35003-35009, especially, p 35003                                                                                              |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | 3-7, 7-10             |
| Y                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | US 2011/0236353 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 29.09.2011, claim 4                                                                                                                                                                     |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | 6-10                  |
| <input type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.                                                                                                                                                                                                                                                                                                                                                                                                              |                                                                                                                                                                                                                                                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                       |
| * Special categories of cited documents:                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |                                                                                                                                                                                                                                                             | "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<br>"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<br>"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<br>"&" document member of the same patent family |                       |
| "A" document defining the general state of the art which is not considered to be of particular relevance<br>"E" earlier document but published on or after the international filing date<br>"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)<br>"O" document referring to an oral disclosure, use, exhibition or other means<br>"P" document published prior to the international filing date but later than the priority date claimed |                                                                                                                                                                                                                                                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                       |
| Date of the actual completion of the international search                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                                                                                                                                                                                                                                                             | Date of mailing of the international search report                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                       |
| 25 March 2013 (25.03.2013)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                                                                                                                                                                                                                                                             | 18 April 2013 (18.04.2013)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                       |
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