Title

Urocortin 2 Gene Transfer Improves Heart Function in Aged Mice

Journal

Molecular Therapy, 28(1)

ISSN

1525-0016

Authors

Giamouridis, Dimosthenis
Gao, Mei Hua
Lai, N Chin
et al.

Publication Date

2020

DOI

10.1016/j.ymthe.2019.10.003

Peer reviewed

Molecular Therapy
Original Article

Urocortin 2 Gene Transfer Improves Heart Function in Aged Mice

Dimosthenis Giamouridis,[1,2,3,4] Mei Hua Gao,[1,2] N. Chin Lai,[1,2] Tracy Guo,[1,2] Atsushi Miyanohara,[2] W. Matthijs Blankesteijn,[3] Erik A.L. Biessen,[4,5] and H. Kirk Hammond[1,2]

[1]Department of Medicine, University of California, San Diego, Veterans Affairs San Diego Healthcare System, San Diego, CA 92161, USA; [2]Department of Medicine, University of California San Diego, San Diego, CA, USA; [3]Department of Pharmacology and Toxicology, Cardiovascular Research Institute Maastricht, Maastricht University, the Netherlands; [4]Department of Pathology, Cardiovascular Research Institute, Maastricht University, the Netherlands; [5]Institute for Molecular Cardiovascular Research, RWTH Aachen University, Aachen, Germany

Prevalence of left ventricular (LV) systolic and diastolic dysfunction increases with aging. We previously reported that *urocortin 2* (*Ucn2*) gene transfer increases heart function in mice with heart failure with reduced ejection fraction. Here, we test the hypotheses that (1) *Ucn2* gene transfer will increase LV function in aged mice and that (2) *Ucn2* gene transfer given in early life will prevent age-related LV dysfunction. Nineteen-month-old (treatment study) and 3-month-old (prevention study) mice received *Ucn2* gene transfer or saline. LV function was examined 3–4 months (treatment study) or 20 months (prevention study) after *Ucn2* gene transfer or saline injection. In both the treatment and prevention strategies, *Ucn2* gene transfer increased ejection fraction, reduced LV volume, increased LV peak −dP/dt and peak +dP/dt, and reduced global longitudinal strain. *Ucn2* gene transfer—in both treatment and prevention strategies—was associated with higher levels of LV SERCA2a protein, reduced phosphorylation of LV CaMKIIa, and reduced LV α-skeletal actin mRNA expression (reflecting reduced cardiac stress). In conclusion, *Ucn2* gene transfer restores normal cardiac function in mice with age-related LV dysfunction and prevents development of LV dysfunction.

INTRODUCTION

Heart failure (HF), a major cause of death, affects 6 million people in the United States, and its prevalence increases with age.[1] In recent decades, it has become apparent that patients with preserved left ventricular (LV) ejection fraction (EF) who have symptoms of HF (HFpEF), formerly referred to as diastolic HF, should be considered separately from those with HF and reduced EF (HFrEF).[2] Patients with HFpEF constitute up to 50% of the HF population.[3] They have abnormal ventricular filling, often due to hypertension and to associated hypertrophy and increased stiffness of the myocardium. HFpEF is associated with diabetes, myocardial ischemia, and advanced age.[3,4] In addition, there is usually concomitant impaired systolic function when assessment is done via less load-dependent indices of systolic function than EF, such as peak rate of LV pressure development (+dP/dt) and global longitudinal strain (LV strain, hereinafter).[5]

There are several interventions (pharmacological and devices) that reduce hospitalization rates and mortality in patients with HFrEF, although the outlook remains poor even with optimal therapy. Unfortunately, patients with HFpEF do not respond similarly to such interventions—pharmacological therapies can provide some symptom relief, but do not influence clinical outcomes such as hospitalizations for HF or mortality.[4] Effective therapies for HFpEF are needed. In the present study, we test the effectiveness of *urocortin 2* (*Ucn2*) gene transfer in mice as a treatment for and prevention of age-related LV dysfunction, which includes both diastolic and systolic dysfunction in the setting of EF > 50%.

Ucn2, a member of the corticotropin-releasing factor (CRF) family, is a 38-aa peptide that binds with high affinity to the corticotropin-releasing hormone receptor 2 (CRHR2). A preclinical study found that Ucn2 peptide infusion increased LV contractile function in mice with HFrEF and improved LV diastolic function.[6] Studies in large animals[7] and humans[8–10] have confirmed the safety and the beneficial effects of Ucn2 peptide infusion on measures of heart function in HFrEF. To our knowledge, no clinical studies have investigated the effects of Ucn2 peptide infusions in the setting of HFpEF.

Considering the short half-life of Ucn2 (15 min),[11] continuous intravenous infusion of Ucn2 peptide would be necessary in order to have sustained effects on the failing heart. This limitation precludes the use of Ucn2 peptide infusion as a long-term HF treatment. To overcome this impediment, we have used *Ucn2* gene transfer to provide a sustained increase in the plasma levels of Ucn2. We have previously demonstrated in preclinical studies that such an approach is feasible. For example, intravenous delivery of an adeno-associated virus vector encoding *Ucn2* (AAV8.*mUcn2*) raised plasma Ucn2 levels and increased LV

Received 11 July 2019; accepted 4 October 2019;
https://doi.org/10.1016/j.ymthe.2019.10.003.

Correspondence: H. Kirk Hammond, Department of Medicine, University of California, San Diego, Veterans Affairs San Diego Healthcare System, 3350 La Jolla Village Drive, San Diego, CA 92161, USA.
E-mail: khammond@ucsd.edu

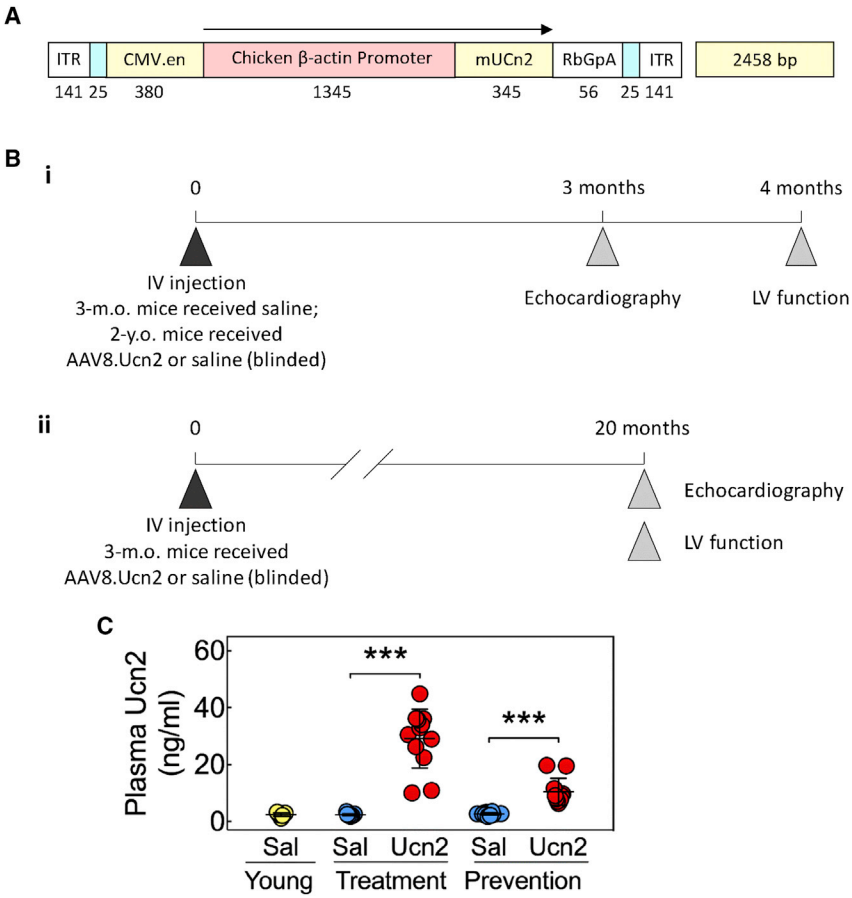

Figure 1. Urocortin 2 Vector Construct and Plasma Levels

(A) AAV8.CBA.mUcn2 vector map. ITR, inverted terminal repeat; CMV.en, cytomegalovirus enhancer; CBA, chicken β-actin promoter; Ucn2, murine urocortin 2; βGpA, β-globin polyadenylation signal. (B) Study design. (i) Aged mice study; (ii) prevention study. m.o., months old; y.o., years old. (C) Ucn2 plasma levels in 3-month-old (Young) and 2-year-old (Treatment) mice 4 months after intravenous delivery of AAV8.*mUcn2* (Ucn2, 1.9×10^{13} gc/kg) or saline (Sal). Ucn2 plasma levels in 3-month-old mice were studied 20 months (Prevention) after saline (Sal) or AAV8.*mUcn2* (Ucn2; 1.9×10^{13} gc/kg) administration. Individual data are indicated (mean ± SE). The p value is from Student's t test (unpaired, two-tailed); ***$p < 0.001$.

systolic and diastolic function in normal mice and in mice with HFrEF.[12–14] However, *Ucn2* gene transfer has not previously been tested in mice with impaired diastolic function and preserved EF. A second goal of the present study was to determine whether *Ucn2* gene transfer that was performed in early life (at 3 months old) could thwart age-related HFpEF in late life. Since many HF patients are of advanced age, this is particularly relevant, because treatment of middle-aged patients with early HF might be advantageous in later life. Therefore, in the present study, we test two hypotheses: (1) *Ucn2* gene transfer will increase LV function in aged mice, and (2) *Ucn2* gene transfer in early life will prevent the development of LV dysfunction later in life. In both instances, EF prior to randomization was >50%.

RESULTS

Plasma Ucn2

Plasma Ucn2 was measured 4 months after the delivery of saline or AAV8.*mUcn2* in the treatment study, and 20 months after the delivery of saline or AAV8.*mUcn2* in the prevention study. In addition, plasma Ucn2 was measured in 7-month-old mice that received saline at 3 months of age. Plasma Ucn2 levels were similar in the 7-month-old mice, aged mice that received saline, and in aged mice 20 months after saline injection (Figure 1).

Treatment Study

Only male mice were used in the treatment study (aged female mice were unavailable from the vendor). Plasma Ucn2 levels were increased 4 months after delivery (saline: 2.3 ± 0.1 ng/mL, n = 11; Ucn2: 29.1 ± 3.0 ng/mL, n = 12; $p < 0.0001$; Figure 1).

Prevention Study

Plasma Ucn2 levels were increased 20 months after delivery in the prevention study (saline: 2.7 ± 0.1 ng/mL, n = 12; Ucn2: 10.4 ± 1.4 ng/mL, n = 11; $p < 0.0001$; Figure 1). Females had lower plasma Ucn2 than males (females: 7.5 ± 0.4 ng/mL, n = 6; males: 13.9 ± 2.4 ng/mL, n = 5; $p = 0.017$).

Echocardiography

Treatment Study

Three months after gene transfer or saline treatment, echocardiography was performed to assess LV dimensions and EF. Comparing saline-treated aged mice with saline-treated young mice, the LV posterior wall was thicker ($p = 0.0004$) and LV strain was higher ($p = 0.02$) in aged mice. However, EF was not reduced in aged compared to young mice (Table 1). Compared to saline-treated aged mice, aged mice that received *Ucn2* gene transfer showed higher values for EF ($p < 0.0002$) and velocity of circumferential fiber shortening (VCFc; $p < 0.0002$) and showed lower LV end-diastolic diameter (EDD; $p = 0.004$), LV end-systolic diameter (ESD; $p < 0.0002$), heart rates (HRs), and LV strain ($p < 0.0002$).

Prevention Study

Three-month-old animals were treated with either saline or with *Ucn2* gene transfer (Table 2). When they were compared 20 months later, *Ucn2* gene transfer was associated with higher EF ($p = 0.0002$) and VCFc ($p = 0.0003$) and with lower LV strain ($p = 0.002$) and ESD ($p = 0.002$).

Table 1. Treatment Study, Echocardiography

		Aged Mice		
	Young Mice (n = 10)	+Saline (n = 11)	+*Ucn2* (n = 12)	p Value
HR (bpm)	550 ± 7	545 ± 7	510 ± 8	.008
EDD (mm)	3.9 ± 0.1	4.1 ± 0.1	3.6 ± 0.1	.004
ESD (mm)	2.6 ± 0.1	2.9 ± 0.1	1.9 ± 0.1	<0.0002
IVSd (mm)	.8 ± 0.01	.8 ± 0.02	.9 ± 0.03	.7
PWd (mm)	.7 ± 0.03	.9 ± 0.03*	.8 ± 0.03	.2
EF (%)	64 ± 3	58 ± 3	80 ± 2	<0.0002
VCFc (circ/s)	23 ± 2	21 ± 2	33 ± 2	<0.0002
LV Strain (%)	−12.5 ± 0.6	−9.7 ± 0.6**	−15.3 ± 0.9	<0.0002

Echocardiographic measurements were taken 3 months after saline (Young, +Saline) or AAV8.*mUcn2* (+*Ucn2*; 1.9×10^{13} gc/kg) administration. Young mice, 3-month-old saline-injected normal mice; Aged mice, 2-year-old mice; HR, heart rate; bpm, beats per minute; EDD, LV end-diastolic diameter; ESD, LV end-systolic diameter; IVSd, intraventricular septum thickness at end diastole; PWd, posterior wall thickness at end diastole; EF, ejection fraction; VCFc, velocity of circumferential fiber shortening (corrected for heart rate); circ, circumference; LV Strain, global longitudinal strain. The p values are from Student's t test (unpaired, two-tailed) with Bonferroni correction. Young versus Aged +Saline: *p = 0.0004; **p = 0.02.

Table 2. Prevention Study, Echocardiography

	Saline (n = 20)	*Ucn2* (n = 16)	p Value
HR (bpm)	538 ± 9	537 ± 8	.94
EDD (mm)	3.9 ± 0.1	3.6 ± 0.1	.12
ESD (mm)	2.7 ± 0.1	2.1 ± 0.1	.002
IVSd (mm)	.8 ± 0.03	.8 ± 0.03	.38
PWd (mm)	.9 ± 0.04	.9 ± 0.05	.73
EF (%)	58 ± 3	72 ± 2	.0002
VCFc (circ/s)	21 ± 1	29 ± 1	.0003
LV Strain (%)	−14.8 ± 1.3	−20.7 ± 1.2	.002

Echocardiographic measurements were taken 20 months after saline (saline) or AAV8.*mUcn2* (*Ucn2*, 1.9×10^{13} gc/kg) administration. HR, heart rate; bpm, beats per minute; EDD, LV end-diastolic diameter; ESD, LV end-systolic diameter; EF, ejection fraction; VCFc, velocity of circumferential fiber shortening (corrected for heart rate); circ, circumference; LV Strain, global longitudinal strain. The p values are from Student's t test (unpaired, two-tailed) with Bonferroni correction.

LV Function

Treatment Study

Aged mice were treated with *Ucn2* gene transfer or saline. Young mice were treated with saline. Four months later, peak rates of LV pressure development (LV +dP/dt) were measured using LV pressure catheters. Compared to young mice, aged mice that had received saline showed a 37% mean reduction in LV peak +dP/dt (p = 0.0004; Figure 2A) and a 39% reduction in LV peak −dP/dt (p = 0.001) (Figure 2B). There were also age-related reductions in LV developed pressure (p < 0.0002; Figure 2C) and heart rate (p = 0.05; Figure 2D). These data indicate age-related decrements in LV systolic function and diastolic function. However, EF was preserved (Table 1), an important component in the definition of clinical HFpEF. Compared to aged mice that received saline, aged mice that received *Ucn2* gene transfer had a 2-fold more rapid LV peak +dP/dt (p < 0.0002; Figure 2A), a 1.7-fold more rapid LV peak −dP/dt (p = 0.004; Figure 2B), and a higher heart rate (p = 0.0006; Figure 2D).

Prevention Study

Mice that received *Ucn2* gene transfer when they were young showed a 1.7-fold higher LV peak +dP/dt (p = 0.0005; Figure 3A) and a 1.4-fold higher LV peak −dP/dt (p = 0.03; Figure 3B) when studied 20 months after randomization. LV-developed pressure was also higher in mice that had received *Ucn2* gene transfer (p = 0.03; Figure 3C). No group differences in heart rate were observed.

Necropsy

Treatment Study

Comparing saline-treated aged mice with saline-treated young mice (Table 3), the body weight and liver:body-weight ratio showed no group differences. However, there were increases in LV weight (p = 0.03), LV:body-weight ratio (p = 0.04), and lung:body-weight ratio (p = 0.001) in the older control mice. There were no saline versus *Ucn2* gene transfer group differences in aged mice.

Prevention Study

There were no group differences in body or organ weights 20 months after saline or AAV8.*mUcn2* administration (Table 4).

LV SERCA2a and CaMKII Protein Expression

Treatment Study

LV sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA2a) protein levels were similar in young mice and aged mice that received saline. However, in aged mice, *Ucn2* gene transfer was associated with a 2.9-fold increase in LV SERCA2a (p = 0.0004; Figure 4A). LV calcium/calmodulin-dependent protein kinase IIa (CaMKIIa) phosphorylation was not different between young and aged saline-treated animals. However, in aged mice, *Ucn2* gene transfer was associated with a 46% lower level of LV p-CaMKII (p = 0.01; Figure 4B).

Prevention Study

In aged mice that had received *Ucn2* gene transfer 20 months previously, LV SERCA2a protein levels were 1.8-fold higher versus those in mice that received saline (p = 0.01; Figure 4C), and they showed a 39% reduction of LV p-CaMKII (p = 0.05; Figure 4D).

LV mRNA Expression of Markers of Hypertrophy and Fibrosis and Duration of Liver Expression

The mRNA expression of several relevant proteins was determined by RT-PCR.

Treatment Study

Ucn2 gene transfer in aged mice resulted in lower LV mRNA levels of *AT1aR* (p = 0.005), *B cell lymphoma 2* (*Bcl-2*; p = 0.008), *brain natriuretic peptide* (*BNP*; p = 0.04), and *α-skeletal actin* (p = 0.0008) (Tables S1 and S2). As expected, mRNA expression of murine *Ucn2* was increased (p = 0.0004) in aged mice that received *Ucn2* gene

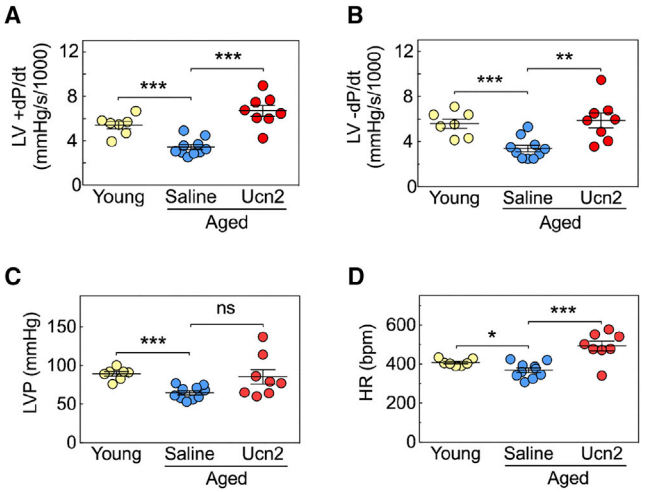

Figure 2. Treatment Study: *In Vivo* Assessment of LV Function

Young versus aged mice, 4 months after intravenous delivery of AAV8.*mUcn2* (1.9×10^{13} gc/kg) or saline, underwent physiological studies to assess LV function. (A and B) Peak rate of LV pressure development (A; +dP/dt) and peak rate of LV pressure decay (B; −dP/dt). (C) LV-developed pressure (LVP) was higher in mice that received *Ucn2* gene transfer. (D) Heart rate (HR) in anesthetized animals was lower in young and aged mice that received saline compared to the aged mice that received *Ucn2* gene transfer. LV, left ventricle; Young, 3-month-old saline-injected mice; Aged, 2-year-old mice. Individual data are indicated (mean ± SE). The p values are from Student's t test (unpaired, two-tailed) with Bonferroni correction for multiple comparisons; *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant.

transfer. Expression levels of *four-and-a-half LIM domain protein* 1 (FHL-1) were higher in mice that received *Ucn2* gene transfer (p = 0.01).

Prevention Study

LV protein expression was assessed 20 months after saline or AAV8.*mUcn2* administration (Table S3). Mice that received *Ucn2* gene transfer had lower mRNA levels of *α-skeletal actin* (p = 0.05) and increased levels of *FHL-1* (p = 0.008). Murine *Ucn2* expression in the LV was elevated 20 months after gene transfer (p = 0.008).

Liver *Ucn2* expression was evaluated 4 months and 20 months after *Ucn2* gene transfer in similarly aged male mice (Table S4), which indicated a 73% reduction in mRNA levels at 20 months versus 4 months after gene transfer (p = 0.09). Because we saw sex differences in plasma levels of Ucn2 (male > female), we examined male versus female liver *Ucn2* mRNA expression in mice in the prevention study, finding no differences: females: $0.5 \times 10^6 \pm 0.1 \times 10^6$ copies per microgram of cDNA, n = 6; males: $1.5 \times 10^6 \pm 0.8 \times 10^6$ copies per microgram of cDNA, n = 5; p = 0.21.

Histological Evaluation of LV and Liver

Treatment Study

Qualitative evaluation of LV and liver tissues showed no differences in inflammatory infiltrates or fibrosis among the three groups (Figure S1).

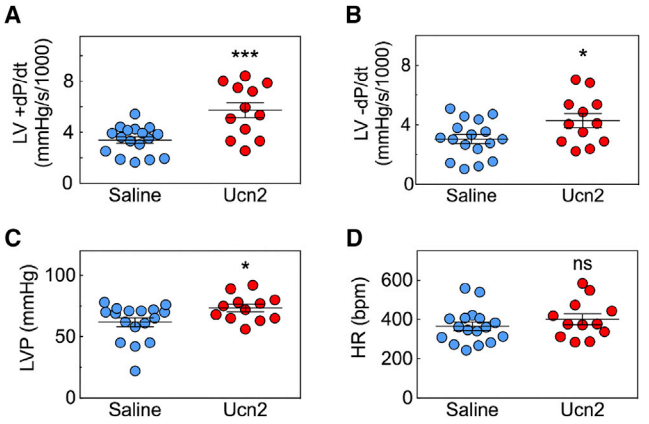

Figure 3. Prevention Study: *In Vivo* Assessment of LV Function 20 Months after Saline or AAV8.*mUcn2* (1.9×10^{13} gc/kg) Administration

(A and B) Peak rate of LV pressure development (A; +dP/dt) and peak rate of LV pressure decay (B; −dP/dt). These data indicate that AAV8.*mUcn2* (Ucn2) gene transfer increased both peak +dP/dt and peak −dP/dt. (C) LV developed pressure (LVP) was increased by *Ucn2* gene transfer. (D) Heart rate (HR) was not altered by *Ucn2* gene transfer. LV, left ventricle. Individual mouse data are shown (mean ± SE are indicated); p values are from Student's t test; *p < 0.05; ***p < 0.001; ns, not significant.

Prevention Study

No inflammatory infiltrates or differences in the degree of fibrosis were found in LV and liver 20 months after saline or AAV8.*mUcn2* injection (Figure S2).

DISCUSSION

The two most important findings in the present study are that urocortin 2 gene transfer (1) increases LV function in aged mice and (2) prevents the development of age-related impairment of LV diastolic function when delivered to young mice. Preserved EF and impaired diastolic function, in combination with symptoms of HF, are characteristics of clinical HFpEF, which are seen with increased prevalence in older patients.[1–4]

In this study of mice, we could not evaluate symptoms, but we found age-related impairment of LV diastolic function in the context of EF > 50%, LV hypertrophy, and pulmonary congestion, findings analogous to what is seen in clinical settings. A recent paper[15] highlighted the complexity of selecting suitable models of clinical HFpEF, and our model complies with the majority of the suggested requirements. Our data indicate that *Ucn2* gene transfer has beneficial effects in an animal model that shares features of clinical age-related LV dysfunction.

It is noteworthy that aging in mice is associated with similar declines in both LV peak +dP/dt (despite normal EF) and LV peak −dP/dt and that *Ucn2* gene transfer provides a remedy for both impairments. Importantly, aged control mice had higher LV strain than young control mice, while their EFs were similar (Table 1), which is analogous to what is seen in clinical age-related HFpEF.[16,17] In general, we found,

Table 3. Treatment Study, Necropsy

		Aged Mice		
	Young Mice (n = 10)	Saline (n = 11)	*Ucn2* (n = 12)	p Value
BW (g)	31.0 ± 0.5	31.1 ± 0.6	32.8 ± 0.6	.1
LV (mg)	104 ± 4	121 ± 5*	113 ± 5	.6
LV/BW (mg/g)	3.3 ± 0.1	3.9 ± 0.2**	3.5 ± 0.1	.1
Lung/BW (mg/g)	4.8 ± 0.1	5.9 ± 0.2***	6 ± 0.3	1
Liver/BW (mg/g)	45 ± 2	49 ± 2	50 ± 3	1

Necropsy data are from 4 months after saline (Young, +saline) or AAV8.*mUcn2* (+*Ucn2*, 1.9×10^{13} gc/kg) administration. BW, body weight; LV, left ventricle; young mice, 3-month-old saline-injected normal mice; aged mice, 2-year-old mice. The p values are from Student's t test (unpaired, two-tailed) with Bonferroni correction. *p = 0.03; **p = 0.04; ***p = 0.001.

Table 4. Prevention Study, Necropsy

	Saline (n = 21)	*Ucn2* (n = 15)	p Value
BW (g)	27.8 ± 1	28 ± 1.2	.94
LV (mg)	118 ± 6	115 ± 8	.77
LV/BW (mg/g)	4.3 ± 0.3	4.3 ± 0.4	.9
Lung/BW (mg/g)	6.9 ± 0.4	6.3 ± 0.2	.24
Liver/BW (mg/g)	51.9 ± 2.3	56 ± 3.1	.29

Necropsy data are from 20 months after saline or AAV8.*mUcn2* (*Ucn2*, 1.9×10^{13} gc/kg) administration. BW, body weight; LV, left ventricle. The p values are from Student's t test.

in both the treatment and prevention strategies, that *Ucn2* gene transfer increased LV diastolic and systolic function, reduced LV dilation, and reduced LV strain.

We found group differences in direct measures of LV contraction and relaxation, using micromanometer catheters in the LV cavity. Such data provide less load-dependent measures of LV function than does EF. LV peak +dP/dt and peak −dP/dt were higher in aged mice that had received *Ucn2* gene transfer 4 months previously, indicating increased LV systolic and diastolic function. Increased heart rate (HR) can contribute to increased +dP/dt, and we saw an increase in heart rate in aged mice that received *Ucn2* gene transfer (versus saline). However, the force-frequency effect would account for a smaller change than the 2-fold increase we found.[18] Additionally, aged mice that received *Ucn2* gene transfer (versus saline) had lower heart rates during echocardiography (light anesthesia) but higher heart rates during deeper anesthesia and mechanical ventilation, which probably reflects variability in response to anesthetic.

In the prevention study, we asked whether *Ucn2* gene transfer performed early in life might prevent age-related abnormalities in LV function. These studies showed that 20 months after *Ucn2* gene transfer (versus saline), age-related LV dysfunction was averted. Indeed, EF, LV strain, EDD, and ESD were similar whether the mice received gene transfer when young or as a treatment when old. Finally, we have shown that a single intravenous delivery of AAV8.*mUcn2* results in sustained high plasma Ucn2 levels 20 months after delivery.

We recently showed that intravenous delivery of AAV8.*mUcn2* (2×10^{13} genome copies [gc]/kg) in male mice provided significant expression 8 weeks later only in liver ($p < 0.0001$) and, to a lesser extent, in left ventricle ($p = 0.018$).[19] In the present study, which included females, plasma Ucn2 levels were lower in females than in males, despite having received the same dose of AAV8.*mUcn2*. We have seen lower plasma Ucn2 in normal young female versus male mice after AAV8.*mUcn2* injections of the same dose (unpublished data), suggesting a sex difference in *Ucn2* expression or fate. A plausible explanation for this observation is that androgens can significantly affect hepatocyte gene transfer.[20] In the present study, no sex differences were found in liver *Ucn2* mRNA after *Ucn2* gene transfer.

The beneficial effects on LV strain and LV −dP/dt were somewhat less in females than males, perhaps due to reduced plasma Ucn2 levels in females. Data analysis included both sexes in the prevention study. Plasma Ucn2 levels were lower in males that received gene transfer 20 versus 4 months previously, despite having received a similar dose of AAV8.*mUcn2*. Liver *Ucn2* mRNA was somewhat lower 20 months versus 4 months after *Ucn2* gene transfer ($p = 0.09$). A decline in plasma transgene levels was seen also after AAV8-mediated factor IX gene transfer in macaques, which was attributed to reductions in liver expression.[21] Reduced plasma Ucn2 over time may reflect hepatocyte turnover, which leads to reduction of the vector DNA over time, since only one daughter cell will carry the transgene's DNA.[22,23] Other possibilities, including liver inflammation or necrosis, were excluded by the absence of histological abnormalities of liver (Figure S2). The final plasma Ucn2 level 20 versus 4 months after delivery is 4-fold basal—which is sufficient to provide beneficial effects on LV strain, LV peak +dP/dt, and LV peak −dP/dt.

We previously reported beneficial effects of *Ucn2* gene transfer on Ca^{2+} transients in cardiac myocytes isolated from normal young mice and from young mice with HFrEF.[12–14] However, Ca^{2+} transient measurements are challenging in aged mice because of poor viability of isolated cardiac myocytes and, therefore, were not attempted. Instead, we focused on LV SERCA2a protein expression, a key Ca^{2+} handling protein, which governs cytosolic Ca^{2+} handling in sarcoplasmic reticulum. Previous papers suggest that Ucn2 peptide treatment has beneficial effects on cardiac myocyte Ca^{2+} handling,[24] which increases Ca^{2+} concentrations at the myofilaments during contraction, and consequently increases the force of contraction. We found that LV SERCA2a levels were similar in young and aged mice that received saline. However, in the treatment study, *Ucn2* gene transfer was associated, 4 months later, with a 2.9-fold increase in LV SERCA2a. Increased expression of SERCA2a would be expected to increase cytosolic Ca^{2+} decline, resulting in improved relaxation, and would also contribute to increased systolic function. We previously have shown by telemetry in unsedated untethered mice that mean daily heart rate and basal systolic and diastolic blood

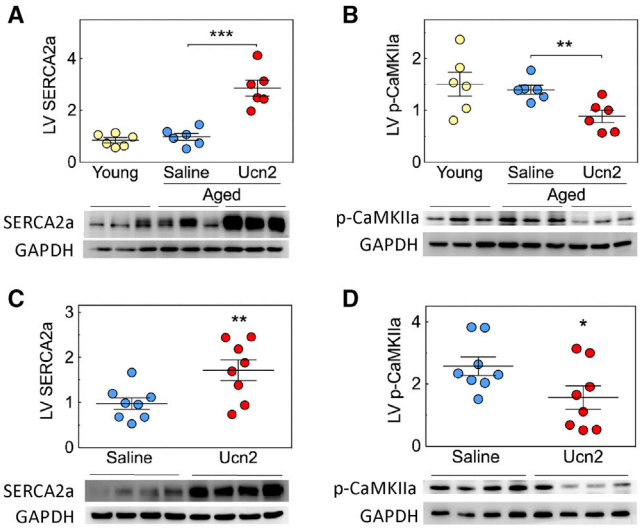

Figure 4. Levels of LV Proteins after Intravenous Delivery of AAV8.*mUcn2* (1.9×10^{13} gc/kg) or Saline

(A) LV sarco/endoplasmic reticulum Ca^{2+} ATPase 2a (SERCA2a) levels were increased in aged mice that received *Ucn2* gene transfer ($p = 0.0004$ versus saline). (B) LV p-CaMKIIa was reduced in AAV8.*mUcn2*-treated aged animals ($p = 0.01$ versus saline). (C and D) LV SERCA2a levels were increased (C) and LV p-CaMKIIa was reduced (D) 20 months after *Ucn2* gene transfer. Data are normalized to GAPDH. Representative immunoblots for the summary data are shown below the graphs. Individual data are indicated (mean ± SE). Young, 3-month-old saline-injected mice; Aged, 2-year-old mice. The p values are from Student's t test (unpaired, two-tailed) with Bonferroni correction for multiple comparisons; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

pressure are unaffected by *Ucn2* gene transfer,[14] underscoring the importance of direct cardiac effects on Ca^{2+} handling as the mechanism for the beneficial effects of *Ucn2* gene transfer on LV diastolic function.

There was no difference between young and aged saline-treated animals in the phosphorylation levels of CaMKIIa in LV samples. However, *Ucn2* gene transfer in aged mice was associated with lower LV levels of p-CaMKIIa, an alteration that can benefit cardiac performance.[25] Increased levels of LV p-CaMKIIa are associated with progressive LV dysfunction, by affecting hypertrophic and inflammatory gene expression, and with arrhythmias.[25] In the prevention study, 20 months after *Ucn2* gene transfer, we saw a 1.9-fold increase in LV SERCA2a and reduced p-CaMKIIa levels. Further studies will be required to elucidate the molecular mechanisms underlying increases in LV SERCA2a and reduced p-CaMKII.

In the treatment study, mRNA expression of proteins related to cardiac hypertrophy and stress showed that LV *β_2AR* expression was increased in aged mice versus young mice. However, *Ucn2* gene transfer in aged mice was associated with reductions in *AT1αR*, *Bcl-2*, *BNP*, and *α-skeletal actin* and increases in *FHL-1* and *Ucn2*. Reductions in *BNP*, *Bcl-2*, and *α-skeletal actin* may reflect reduced stress after *Ucn2* gene transfer, due to improved LV function.[26–29] *SERCA2a* mRNA was unchanged, despite a 2.9-fold elevation in LV SERCA2a protein after *Ucn2* gene transfer—this implies a change in mRNA stability or reduction in SERCA2a protein turnover after *Ucn2* gene transfer. In the prevention study, LV expression of *β_1 adrenergic receptor*, *β_2AR*, and *α-skeletal actin* was reduced, indicating reduction of cardiac stress, and expression of *FHL-1* and murine *Ucn2* were increased. We assessed LV fibrosis using Masson's trichrome, as well as Picrosirius red staining. We did not find age-related increases in the LV collagen deposits. A similar absence of increased LV fibrosis has been reported by others.[30–32]

We are not proposing that *Ucn2* gene transfer be used to prevent the future occurrence of age-related LV dysfunction. However, the two strategies (prevention and treatment) had similar results on multiple measures of LV structure and function and of biochemical alterations in the heart, which serves to embolden the conclusion that *Ucn2* gene transfer affects LV function through beneficial effects on LV SERCA2a and CaMKII. In addition, the absence of deleterious effects on LV structure and function or adverse histological effects, despite nearly 20 months of sustained increased concentrations of plasma Ucn2, indicates not only that the benefits of *Ucn2* gene transfer are prolonged after a single injection but also that sustained increases in plasma Ucn2 are safe.

There are two previous reports of gene transfer treatments for animal models of LV diastolic dysfunction. In aged rats, thoracotomy-based delivery of adenovirus encoding parvalbumin, which may benefit myocardial Ca^{2+} handling, was reported to have a beneficial effect on LV relaxation in anesthetized animals studied 2 days after gene transfer.[33] No subsequent studies were reported. A more recent study examined streptozotocin-treated diabetic mice with diastolic abnormalities detected by echocardiographic analysis of LV filling. Further abnormalities were attenuated 6–8 weeks later by phosphoinositide 3-kinase gene transfer via intravenous delivery of an AAV6 vector.[34] Our present study shows not only prevention of age-related LV diastolic abnormalities 20 months after delivery but also, as shown in the treatment arm, full restoration of normal LV function. Furthermore, the approach that we used—gene transfer of a peptide with paracrine actions that benefit cardiac function—is a novel approach that does not require high transgene expression levels in the heart and can be delivered by an intravenous injection.

There are inherent shortcomings in translating studies in mice to clinical applications, and our findings must be confirmed in larger animal models of HFpEF. Some will criticize our failure to use standard echocardiographic measures of diastolic function in the present studies. However, we used two assessments of diastolic function that are not hampered by the rapid heart rates in mice: LV peak −dP/dt, the peak rate of LV pressure decay during diastole, and LV strain. These two measures provide sensitive and accurate assessments of LV diastolic function that are superior to the usual echocardiographic measurements.[16] Indeed, LV strain has been recently suggested as a standard for the evaluation of diastolic function in clinical HFpEF.[17] Additional insights from our data include a sex difference

in plasma Ucn2 levels unexplained by vector dose or liver expression, underscoring the desirability of including both sexes in preclinical and clinical studies, and the slow decline in AAV8-mediated liver expression over time, likely due to normal hepatocyte turnover. Fortunately, we used a dose of vector sufficiently high to enable a >4-fold elevation in plasma Ucn2 20 months after delivery.

In conclusion, *Ucn2* gene transfer improves both systolic function and diastolic function in mice with age-related LV dysfunction and prevents development of LV dysfunction when given to young mice. The mechanism for these beneficial effects lies, at least in part, in increased LV SERCA2a expression. These findings are potentially relevant for treatment of clinical age-related LV dysfunction. The therapy may be particularly effective for clinical HFpEF, a disease that, to date, has few effective therapies.

MATERIALS AND METHODS

AAV8.*mUcn2* Vector

HEK293T cells were transfected with the pRep2/Cap8 and pAd-Helper plasmids,[14] for the production of an AAV8 vector encoding murine *Ucn2* driven by a chicken β-actin (CBA) promoter (AAV8.CBA.*mUcn2*; Figure 1A). Plasmid pRep2/Cap8 was obtained from the University of Pennsylvania Vector Core. Virus vectors were then purified and concentrated as previously described.[13,35] Real-time PCR with virus genome DNA prepared from purified virus was used to quantify virus titers.

Animal Use

The Animal Use and Care Committee of the Veterans Affairs San Diego Healthcare System approved the studies. In this study, to answer our first hypothesis, we used 10 male mice (C57BL/6J; Jackson Laboratories, Bar Harbor, ME, USA) 10.7 ± 0.6 weeks old (treatment group, young), weighing 27.8 ± 0.5 g, and 23 male mice (C57BL/6J) 102 weeks old (treatment group, aged), weighing 33.4 ± 0.5 g (Figure 1Bi). To address our second hypothesis, we used 29 male mice (C57BL/6J) 11.9 ± 0.4 weeks old, weighing 26.4 ± 0.5 g, and 19 female mice (C57BL/6J) 10.2 ± 0.1 weeks old (prevention group), weighing 18.5 ± 0.2 g (Figure 1Bii). From those 48 mice in the prevention group, 5 that received saline and 6 that received AAV8.m*Ucn2* died before the terminal study. All of the mice were provided (*ad libitum*) a cereal-based diet (Harlan Teklad Laboratory, Madison, WI, USA) and tap water. Mice were housed (20°C–21°C) with lights off from 6 p.m. to 6 a.m. daily.

Ucn2 Gene Transfer

Under anesthesia (1.5% isoflurane via nose cone), the jugular vein was exposed. AAV8.m*Ucn2* (1.9×10^{13} gc/kg in 100 μL) or a similar volume of saline was delivered using a syringe with a 31G needle.

Echocardiography

Echocardiography was performed as previously described,[12] using a Vevo 3100 ultrasound system with an MX550s transducer and VevoLab v3.1 analysis software (VisualSonics, Toronto, ON, Canada), to document LV function and to record LV chamber dimensions. LV strain was calculated by VevoLab v3.1 analysis software, from planimetered LV wall changes during diastole and systole on a long-axis view.

LV Function

Using a 1.4-F micromanometer catheter (SPR 839, Millar Instruments, Houston, TX, USA), the LV pressure signal was recorded, stored digitally, and then processed (IOX v2.9.5, Emka Technologies, Christchurch, VA, USA) as previously reported.[36] The first derivatives of LV pressure development (LV +dP/dt) and decline (LV −dP/dt) were used to define LV systolic function and diastolic function, respectively.

Plasma Ucn2

In the terminal study under anesthesia, the carotid artery was bled, and blood was collected (with EDTA) and centrifuged (1,600 × *g*, 15 min). Plasma was collected and stored at −80°C. Plasma Ucn2 levels were measured using a mouse urocortin 2 enzyme immunoassay (EIA) kit (Kamiya Biomedical, Seattle, WA, USA).

RT-PCR, PCR, and Immunoblotting

LV and liver samples were collected and stored at −80°C. Total RNA was isolated and reverse transcribed into cDNA that was used in qPCR as previously described.[12] Additionally, *Ucn2* gene mRNA was reverse transcribed into cDNA that was amplified and quantified (copies per microgram of cDNA) using an ABI TaqMan 7700 System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. All gene-specific primers used in PCR are listed in Table S1. Immunoblotting was performed as described previously.[12] The following antibodies were used: phospho-CaMKII (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and SERCA2a (Enzo Life Sciences, Farmingdale, NY, USA).

Necropsy and Histology

Body, liver, lung, and LV weight (including interventricular septum) were recorded. Samples of liver and transmural LV were formalin fixed and paraffin embedded. Five-micron sections were mounted and counterstained with H&E and with Masson's trichrome. Slides were then scanned using an Axio Scan.Z1 slide scanner (Zeiss, Oberkochen, Germany) and analyzed with ImageJ v1.49 software (Bethesda, MD, USA).

Statistical Analysis

Data acquisition and analysis were done without knowledge of group identity. Group sizes were determined by power calculations. We used only male mice, because aged female mice were not available from the vendor. The hypothesis was that *Ucn2* gene transfer would effectively treat LV diastolic dysfunction in aged mice. First, young versus aged mice that received intravenous saline were compared to establish that LV diastolic dysfunction was present in aged mice. Subsequently, aged mice that received *Ucn2* gene transfer versus saline were compared to determine whether *Ucn2* gene transfer in aged mice was associated with increased LV diastolic function. To test for group differences, Student's t test (unpaired, two-tailed) was

used. Bonferroni correction was used for multiple testing. Analyses were performed using GraphPad Prism v6.07 (GraphPad Software, San Diego, CA, USA). The null hypothesis was rejected when $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.ymthe.2019.10.003.

AUTHOR CONTRIBUTIONS

Conceptualization, D.G., M.H.G., N.C.L., and H.K.H.; Experiments, D.G., M.H.G., and N.C.L.; Data Analysis, D.G., M.H.G., N.C.L., T.G., and H.K.H.; Writing, D.G, M.H.G., N.C.L., W.M.B., E.A.L.B., and H.K.H.

CONFLICTS OF INTEREST

H.K.H. is a founder, board member, and unpaid consultant for Renova Therapeutics. Renova played no role in the studies. The remaining authors declare no competing interests.

ACKNOWLEDGMENTS

This work was supported by an NIH grant (P01 HL66941) and by merit grants from the Department of Veterans Affairs (1101 BX001515 and 1I01 BX003774).

REFERENCES

1. Lloyd-Jones, D., Adams, R.J., Brown, T.M., Carnethon, M., Dai, S., De Simone, G., Ferguson, T.B., Ford, E., Furie, K., Gillespie, C., et al.; American Heart Association Statistics Committee and Stroke Statistics Subcommittee (2010). Executive summary: heart disease and stroke statistics–2010 update: a report from the American Heart Association. Circulation *121*, 948–954.

2. Borlaug, B.A., and Paulus, W.J. (2011). Heart failure with preserved ejection fraction: pathophysiology, diagnosis, and treatment. Eur. Heart J. *32*, 670–679.

3. Ouzounian, M., Lee, D.S., and Liu, P.P. (2008). Diastolic heart failure: mechanisms and controversies. Nat. Clin. Pract. Cardiovasc. Med. *5*, 375–386.

4. Maeder, M.T., and Kaye, D.M. (2009). Heart failure with normal left ventricular ejection fraction. J. Am. Coll. Cardiol. *53*, 905–918.

5. Shah, A.M., Claggett, B., Sweitzer, N.K., Shah, S.J., Anand, I.S., Liu, L., Pitt, B., Pfeffer, M.A., and Solomon, S.D. (2015). Prognostic importance of impaired systolic function in heart failure with preserved ejection fraction and the impact of spironolactone. Circulation *132*, 402–414.

6. Bale, T.L., Hoshijima, M., Gu, Y., Dalton, N., Anderson, K.R., Lee, K.F., Rivier, J., Chien, K.R., Vale, W.W., and Peterson, K.L. (2004). The cardiovascular physiologic actions of urocortin II: acute effects in murine heart failure. Proc. Natl. Acad. Sci. USA *101*, 3697–3702.

7. Rademaker, M.T., Charles, C.J., Ellmers, L.J., Lewis, L.K., Nicholls, M.G., and Richards, A.M. (2011). Prolonged urocortin 2 administration in experimental heart failure: sustained hemodynamic, endocrine, and renal effects. Hypertension *57*, 1136–1144.

8. Chan, W.Y., Frampton, C.M., Crozier, I.G., Troughton, R.W., and Richards, A.M. (2013). Urocortin-2 infusion in acute decompensated heart failure: findings from the UNICORN study (urocortin-2 in the treatment of acute heart failure as an adjunct over conventional therapy). JACC Heart Fail. *1*, 433–441.

9. Gheorghiade, M., Greene, S.J., Ponikowski, P., Maggioni, A.P., Korewicki, J., Macarie, C., Metra, M., Grzybowski, J., Bubenek-Turconi, S.I., Radziszewski, W., et al. (2013). Haemodynamic effects, safety, and pharmacokinetics of human stresscopin in heart failure with reduced ejection fraction. Eur. J. Heart Fail. *15*, 679–689.

10. Stirrat, C.G., Venkatasubramanian, S., Pawade, T., Mitchell, A.J., Shah, A.S., Lang, N.N., and Newby, D.E. (2016). Cardiovascular effects of urocortin 2 and urocortin 3 in patients with chronic heart failure. Br. J. Clin. Pharmacol. *82*, 974–982.

11. Patel, K., Rademaker, M.T., Kirkpatrick, C.M., Charles, C.J., Fisher, S., Yandle, T.G., and Richards, A.M. (2012). Comparative pharmacokinetics and pharmacodynamics of urocortins 1, 2 and 3 in healthy sheep. Br. J. Pharmacol. *166*, 1916–1925.

12. Gao, M.H., Lai, N.C., Miyanohara, A., Schilling, J.M., Suarez, J., Tang, T., Guo, T., Tang, R., Parikh, J., Giamouridis, D., et al. (2013). Intravenous adeno-associated virus serotype 8 encoding urocortin-2 provides sustained augmentation of left ventricular function in mice. Hum. Gene Ther. *24*, 777–785.

13. Lai, N.C., Gao, M.H., Giamouridis, D., Suarez, J., Miyanohara, A., Parikh, J., Hightower, S., Guo, T., Dillmann, W., Kim, Y.C., et al. (2015). Intravenous AAV8 encoding urocortin-2 increases function of the failing heart in mice. Hum. Gene Ther. *26*, 347–356.

14. Giamouridis, D., Gao, M.H., Lai, N.C., Tan, Z., Kim, Y.C., Guo, T., Miyanohara, A., Blankesteijn, W.M., Biessen, E., and Hammond, H.K. (2018). Effects of urocortin 2 versus urocortin 3 gene transfer on left ventricular function and glucose disposal. JACC Basic Transl. Sci. *3*, 249–264.

15. Valero-Muñoz, M., Backman, W., and Sam, F. (2017). Murine models of heart failure with preserved ejection fraction: a "fishing expedition." JACC Basic Transl. Sci. *2*, 770–789.

16. Kalam, K., Otahal, P., and Marwick, T.H. (2014). Prognostic implications of global LV dysfunction: a systematic review and meta-analysis of global longitudinal strain and ejection fraction. Heart *100*, 1673–1680.

17. Kosmala, W., Przewlocka-Kosmala, M., Rojek, A., Mysiak, A., Dabrowski, A., and Marwick, T.H. (2018). Association of abnormal left ventricular functional reserve with outcome in heart failure with preserved ejection fraction. JACC Cardiovasc. Imaging *11*, 1737–1746.

18. Palakodeti, V., Oh, S., Oh, B.H., Mao, L., Hongo, M., Peterson, K.L., and Ross, J., Jr. (1997). Force-frequency effect is a powerful determinant of myocardial contractility in the mouse. Am. J. Physiol. *273*, H1283–H1290.

19. Gao, M.H., Giamouridis, D., Lai, N.C., Walenta, E., Paschoal, V.A., Kim, Y.C., Miyanohara, A., Guo, T., Liao, M., Liu, L., et al. (2016). One-time injection of AAV8 encoding urocortin 2 provides long-term resolution of insulin resistance. JCI Insight *1*, e88322.

20. Davidoff, A.M., Ng, C.Y., Zhou, J., Spence, Y., and Nathwani, A.C. (2003). Sex significantly influences transduction of murine liver by recombinant adeno-associated viral vectors through an androgen-dependent pathway. Blood *102*, 480–488.

21. Nathwani, A.C., Rosales, C., McIntosh, J., Rastegarlari, G., Nathwani, D., Raj, D., Nawathe, S., Waddington, S.N., Bronson, R., Jackson, S., et al. (2011). Long-term safety and efficacy following systemic administration of a self-complementary AAV vector encoding human FIX pseudotyped with serotype 5 and 8 capsid proteins. Mol. Ther. *19*, 876–885.

22. Magami, Y., Azuma, T., Inokuchi, H., Kokuno, S., Moriyasu, F., Kawai, K., and Hattori, T. (2002). Cell proliferation and renewal of normal hepatocytes and bile duct cells in adult mouse liver. Liver *22*, 419–425.

23. Malato, Y., Naqvi, S., Schürmann, N., Ng, R., Wang, B., Zape, J., Kay, M.A., Grimm, D., and Willenbring, H. (2011). Fate tracing of mature hepatocytes in mouse liver homeostasis and regeneration. J. Clin. Invest. *121*, 4850–4860.

24. Domínguez-Rodríguez, A., Mayoral-Gonzalez, I., Avila-Medina, J., de Rojas-de Pedro, E.S., Calderón-Sánchez, E., Díaz, I., Hmadcha, A., Castellano, A., Rosado, J.A., Benitah, J.P., et al. (2018). Urocortin-2 prevents dysregulation of Ca2+ homeostasis and improves early cardiac remodeling after ischemia and reperfusion. Front. Physiol. *9*, 813.

25. Swaminathan, P.D., Purohit, A., Hund, T.J., and Anderson, M.E. (2012). Calmodulin-dependent protein kinase II: linking heart failure and arrhythmias. Circ. Res. *110*, 1661–1677.

26. Battistoni, A., Rubattu, S., and Volpe, M. (2012). Circulating biomarkers with preventive, diagnostic and prognostic implications in cardiovascular diseases. Int. J. Cardiol. *157*, 160–168.

27. Buzello, M., Boehm, C., Orth, S., Fischer, B., Ehmke, H., Ritz, E., Mall, G., and Amann, K. (2003). Myocyte loss in early left ventricular hypertrophy of experimental renovascular hypertension. Virchows Arch. *442*, 364–371.

28. Black, F.M., Packer, S.E., Parker, T.G., Michael, L.H., Roberts, R., Schwartz, R.J., and Schneider, M.D. (1991). The vascular smooth muscle alpha-actin gene is reactivated during cardiac hypertrophy provoked by load. J. Clin. Invest. *88*, 1581–1588.

29. Copeland, O., Nowak, K.J., Laing, N.G., Ravenscroft, G., Messer, A.E., Bayliss, C.R., and Marston, S.B. (2010). Investigation of changes in skeletal muscle alpha-actin expression in normal and pathological human and mouse hearts. J. Muscle Res. Cell Motil. *31*, 207–214.

30. Wang, J., Song, Y., Li, H., Shen, Q., Shen, J., An, X., Wu, J., Zhang, J., Wu, Y., Xiao, H., and Zhang, Y. (2016). Exacerbated cardiac fibrosis induced by β-adrenergic activation in old mice due to decreased AMPK activity. Clin. Exp. Pharmacol. Physiol. *43*, 1029–1037.

31. Ito, T., Hanahata, Y., Kine, K., Murakami, S., and Schaffer, S.W. (2018). Tissue taurine depletion induces profibrotic pattern of gene expression and causes aging-related cardiac fibrosis in heart in mice. Biol. Pharm. Bull. *41*, 1561–1566.

32. Marques, F.Z., Chu, P.Y., Ziemann, M., Kaspi, A., Kiriazis, H., Du, X.J., El-Osta, A., and Kaye, D.M. (2018). Age-related differential structural and transcriptomic responses in the hypertensive heart. Front. Physiol. *9*, 817.

33. Schmidt, U., Zhu, X., Lebeche, D., Huq, F., Guerrero, J.L., and Hajjar, R.J. (2005). In vivo gene transfer of parvalbumin improves diastolic function in aged rat hearts. Cardiovasc. Res. *66*, 318–323.

34. Prakoso, D., De Blasio, M.J., Qin, C., Rosli, S., Kiriazis, H., Qian, H., Du, X.J., Weeks, K.L., Gregorevic, P., McMullen, J.R., and Ritchie, R.H. (2017). Phosphoinositide 3-kinase (p110α) gene delivery limits diabetes-induced cardiac NADPH oxidase and cardiomyopathy in a mouse model with established diastolic dysfunction. Clin. Sci. (Lond.) *131*, 1345–1360.

35. Xiao, X., Li, J., and Samulski, R.J. (1998). Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. J. Virol. *72*, 2224–2232.

36. Gao, M.H., Lai, N.C., Roth, D.M., Zhou, J., Zhu, J., Anzai, T., Dalton, N., and Hammond, H.K. (1999). Adenylylcyclase increases responsiveness to catecholamine stimulation in transgenic mice. Circulation *99*, 1618–1622.