# Original Article

# AAV9.I-1c Delivered via Direct Coronary Infusion in a Porcine Model of Heart Failure Improves Contractility and Mitigates Adverse Remodeling

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***Background***—Heart failure is characterized by impaired function and disturbed $Ca^{2+}$ homeostasis. Transgenic increases in inhibitor-1 activity have been shown to improve $Ca^2$ cycling and preserve cardiac performance in the failing heart. The aim of this study was to evaluate the effect of activating the inhibitor (I-1c) of protein phosphatase 1 (I-1) through gene transfer on cardiac function in a porcine model of heart failure induced by myocardial infarction.

***Methods and Results***—Myocardial infarction was created by a percutaneous, permanent left anterior descending artery occlusion in Yorkshire Landrace swine (n=16). One month after myocardial infarction, pigs underwent intracoronary delivery of either recombinant adeno-associated virus type 9 carrying I-1c (n=8) or saline (n=6) as control. One month after myocardial infarction was created, animals exhibited severe heart failure demonstrated by decreased ejection fraction (46.4±7.0% versus sham 69.7±8.5%) and impaired $(dP/dt)_{max}$ and $(dP/dt)_{min}$. Intracoronary injection of AAV9.I-1c prevented further deterioration of cardiac function and led to a decrease in scar size.

***Conclusions***—In this preclinical model of heart failure, overexpression of I-1c by intracoronary in vivo gene transfer preserved cardiac function and reduced the scar size. (*Circ Heart Fail*. 2013;6:310-317.)

**Key Words:** AAV9.I-1c ■ heart failure ■ myocardial infarction ■ SERCA2a

One of the key abnormalities in both human and experimental heart failure (HF) is a defect in sarcoplasmic reticulum (SR) function, resulting in abnormal intracellular $Ca^{2+}$ handling.[1–4] Deficient SR $Ca^{2+}$ uptake during relaxation has been identified in failing hearts from both humans and animal models and has been associated with a decrease in the activity of the SR $Ca^{2+}$-ATPase (SERCA2a), which is at least partially attributable to enhanced phospholamban (PLN) inhibition.[5–7] Restoring SERCA2a levels or reducing PLN inhibition has been shown to improve function, metabolism, and survival in many experimental models of HF.[5] Targeting SERCA2a in experimental models of HF by somatic gene transfer has proven effective in rescuing contractile function while at the same time decreasing ventricular arrhythmias and restoring energetics.

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This has led to the initiation and the recent completion of a first-in-man phase 1 clinical trial of gene therapy for HF, using adeno-associated type (AAV)-1 vector carrying SERCA2a.[8]

The safety profile of AAV gene therapy along with the positive biological signals obtained from this phase 1 trial has led to the initiation of a phase 2 trial of AAV1.SERCA2a in New York Heart Association class III/IV patients.[9] More recently, several studies have shown that by constitutively activating the inhibitor of protein phosphatase 1 (I-1) within the failing heart, there is improvement in SR $Ca^{2+}$ handling, contractility, and most importantly, reversal of adverse remodeling by directly decreasing fibrosis and cardiac hypertrophy.[10–14] In this study, we have chosen to focus on the expression of the constitutively active, truncated form of inhibitor-1, the I-1c protein, because it has the ability to enhance contractility while at the same time decreasing fibrosis in small animal models of HF.[12] Overexpression of I-1c in the heart results in significant enhancement of PLN phosphorylation and cardiac function and abrogates the negative effects of aortic constriction in this model. In fact, beyond the rescue of contractile function, overexpression of I-1c resulted in a significant decrease in fibrosis.[12] However, there have been conflicting reports that I-1c overexpression in a murine model may result in

amplification of the β-adrenergic system and worsening of function when subjected to specific stresses.[15]

Therefore, the specific aim of the study was to evaluate the effects of long-term I-1c expression in a porcine model of HF. We assessed the dynamic changes in ventricular volumes and contractility after gene transfer with AAV9.I-1c in a porcine model of volume overload and infarct after the development of HF.

# Methods

Animal care and all procedures were approved by local institutional committees and were performed in accordance with the Principles of Laboratory Animal Care by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 86-23, revised 1985).

## AAV9.I-1c Construction and Characterization

There are 2 components of AAV9.I-1c. The first is an active I-1 (GenBank: AAL48321.1) transgene (AA 1–65 with T35D), and the second is the vector, AAV9, which delivers the gene selectively to the heart after intracoronary administration. We used a well-characterized, human, constitutively active I-1 (AA 1–65 with T35D) gene that has been truncated to remove the S67 and T75 protein kinase C (PKC) sites. This truncated gene and the protein it encodes for are designated I-1c. The rationale for removing the PKC sites on I-1 is that their phosphorylation increases PP1 activity, resulting in decreased SERCA and SR Ca-transport and attenuated contractility.[16] Because PKC activity is increased in HF,[11] it is desirable to remove these sites on I-1. In addition to the removal of the PKC sites, the threonine-35 PKA-phosphorylation site of the I-1c gene is constitutively active by replacing threonine with aspartic acid. AAV9 vectors carrying I-1c under control of the cytomegalovirus promoter were produced using the 2-plasmid protocol described by Zolotukhin et al[17] with the following modifications. 293-T cells (ATCC, Manassas, VA) were grown in triple flasks for 24 hours (DMEM, 10% fetal bovine serum) before adding the calcium phosphate precipitate. After 72 hours, the virus was purified from benzonase-treated cell crude lysates over an iodixanol density gradient (Optiprep, Greiner Bio-One Inc., Longwood, FL), followed by heparin-agarose type I affinity chromatography (Sigma-Aldrich Inc., St Louis, MO). Finally, viruses were concentrated and formulated into lactated Ringer's solution (Baxter Healthcare Corporation, Deerfield, IL) using a Vivaspin 20 Centrifugal concentrators 50K MWCO (Vivascience Inc., Carlsbad, CA) and stored at −80°C. Vector stock biochemical purity (>95%) was assessed by silver staining after electrophoresis. Genome containing particles were determined by a real-time polymerase chain reaction (PCR) approach (LightCycler, Roche Diagnostics) using the SYBR Green Taq ReadyMix (Sigma-Aldrich Inc.) and primers cytomegalovirus-F and cytomegalovirus-R.

## Animal Studies and Myocardial Infarction Model

This study, using female Yorkshire Landrace pigs (≈20 kg body weight), was approved by the Institutional Animal Care and Use Committee. All procedures were performed under anesthesia with inhaled isoflurane (0.5–2%). All animals enrolled in the study were prescreened for neutralizing antibodies and had a maximum of 8-fold dilution for detection as described by Rapti et al.[18] For myocardial infarction (MI) generation, we introduced an 8F sheath into the femoral artery and cannulated the left anterior descending artery (LAD) with a 7F hockey stick guiding catheter (Cordis Infiniti, Johnson & Johnson). After injecting 100 μg of nitroglycerin and obtaining a baseline coronary angiogram, we placed a platinum embolic coil (0.035 in., 40 mm length, 5×3-mm diameter; Cook Medical Inc.) using a 4F AR Catheter (Cordis Infiniti, Johnson & Johnson) into the LAD after the takeoff of the first diagonal branch, thus occluding two thirds of the LAD tributary, determined by coronary angiography. Fourteen animals were randomized to receive either control intracoronary injection with saline or AAV9.I-1c, and 3 animals were subject to a sham procedure.

## Intracoronary Antegrade Injection of AAV9.I-1c

The detailed injection procedure has been described previously.[19] Briefly, the solution for intracoronary injection was prepared by adding 2.5×10^{12} AAV9.I-1c vector genomes to a mixture of 10 mL of normal saline and 10 mL of blood. Via the arterial sheath, the left coronary artery was cannulated with a 5F guiding catheter (Cordis Infiniti, Johnson & Johnson). Two hundred micrograms of nitroglycerin were injected into the coronary arteries to enhance coronary perfusion. Two coronary guidewires were introduced in the LAD and the left circumflex artery to stabilize the position of the guiding catheter during the injection. A programmable precision infusion pump (Harvard Apparatus) was used for the injection. Fifteen milliliters of the viral solution was injected into the proximal part of the left coronary artery at 1 mL/min followed immediately by a 5-mL flush of saline/blood mixture. Afterward, the left coronary artery catheters were removed and a 5F Hockey stick guiding catheter (Cordis Infiniti, Johnson & Johnson) was placed in the right coronary artery. Here, 5 mL of viral solution was injected into the right coronary artery at 1 mL/min followed immediately by a 5-mL flush of saline/blood mixture. On the day of gene delivery, some animals (4 control, 5 AAV9.I-1c) underwent subcutaneous implantation of a Medtronic Reveal Insertable Loop Recorder (Minneapolis, MN) at the T4 level to monitor them for arrhythmic events.

## Assessment of Myocardial Function and Structure

We assessed the myocardial function and structure at baseline (ie, before MI generation), 1 month after MI (ie, before application of the virus), and 2 months after application (the 3-month time point). Echocardiography was performed obtaining apical images at 10° intervals with a 5-MHz transthoracic probe (Vivid 7, GE), rotated by software and gated to ECG. Digital images were analyzed on a workstation with custom software. Two stable and well-defined consecutive cardiac cycles were acquired digitally for each measurement.

For hemodynamic catheterization, we accessed the femoral artery and vein with 8F sheaths and placed a 7F Millar Mikro-Tip catheter system (Millar Instruments Inc.) into the aorta and the left ventricle (LV). We determined the following parameters: systolic pressure, end-diastolic pressure, peak LV pressure rate of rise ($dP/dt$)$_{max}$, and tau value (time constant of isovolumic relaxation); ($dP/dt$)$_{max}/P$ was calculated as ($dP/dt$)$_{max}$/(systolic−end-diastolic pressure). The mean of at least 3 consecutive cardiac cycles was calculated for each measurement.

We performed coronary angiography at the 1-month and 3-month time points using an Integris H5000 single-plane fluoroscopy system (Philips Medical Systems). All images were acquired and analyzed by an investigator blinded to the study arm. Just before euthanization, a dobutamine stress test was performed to assess the hemodynamic alteration and the potential arrhythmogenicity in response to increased β-stimulation. Intravenous dobutamine infusion was started at 5 μg/kg per minute and gradually increased up to 40 μg/kg per minute. Continuous pressure and electrocardiographic recording were obtained during the stress test.

The animals were euthanized by intravenous injection of Euthasol (pentobarbital, phenytoin; 1 mL/4.5 kg). Then the hearts were removed, the right ventricle was resected, and the LV was cut along the short axis into 6 slabs of the same thickness. We visualized the viable myocardium by staining 5 of these slices with 2,3,5-triphenyltetrazolium chloride and quantified scar volume using unbiased stereology.

## Real-Time Quantitative PCR Detection of I-1c mRNA

Total RNA was isolated with TRIzol reagent (Invitrogen) followed by DNase I treatment (Qiagen). Total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, ABI) according to the manufacturer's protocol. Quantitative PCRs were performed with Power SYBR Green Master Mix using an ABI Prism 7500 Real-Time PCR System. The real-time quantitative PCR assay detects a 125-bp sequence unique to AAV9.I-1c, and the number of copies of AAV9.I-1c was quantified using serial dilutions of a plasmid containing the target sequences as standards. Primers for the I-1c were
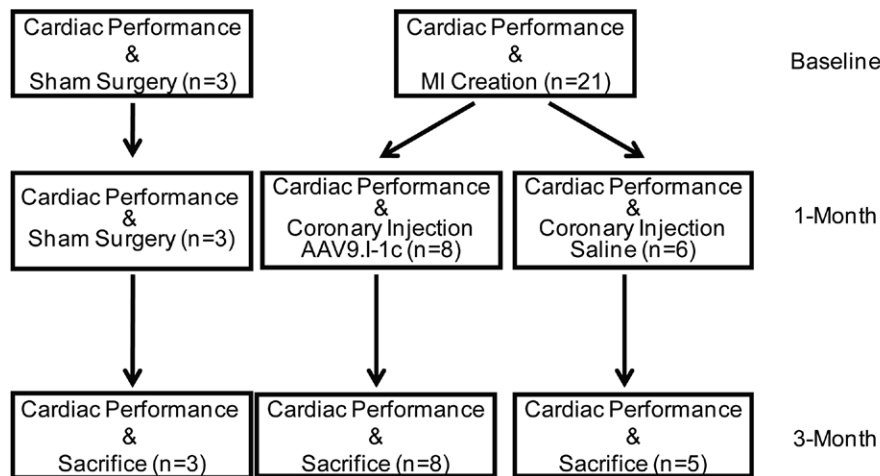
Figure 1. Study design. Twenty-one pigs underwent myocardial infarction (MI) and 14 survived 1 month after MI. Eight pigs received AAV9.I-1c, whereas 6 pigs received saline. All animals receiving AAV9.I-1c survived until euthanization after 3 months, whereas there was 1 death in the control group. Referenced time points are indicated on the right.

designed with the Primer Express software based on the human protein sequence (NP 006732.3; forward: CGTGCCCCTGCTGGAA, reverse: TCCGGTCCTCGTCGATCTC). Relative quantitation is the I-1c copies detected relative to the number of lowest number of I-1 copies detected in the treatment group. Because the actual number of cycles required for detection was relatively high, determination of vector genomes per gram of tissue may not be justified.

## Western Blot

Cardiac whole homogenate samples (50 μg) in sodium dodecyl sulfate sample buffer were run on an sodium dodecyl sulfate-polyacrylamide gel electrophoresis gradient (4–20%, Bio-Rad), and proteins in the gel were transferred to nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% skim milk and incubated overnight with an antibody directed against I-1c (provided by Dr Kranias) and GAPDH (Sigma-Aldrich Inc.). The membrane was then incubated with an antirabbit IgG secondary antibody conjugated to horseradish peroxidase (Cell Signaling) and developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

## Statistical Analyses

Investigators quantified observations independently from one another and in a blinded manner. Numeric data are presented as mean±SD unless otherwise indicated. Pairwise statistical comparisons were performed statistically between saline versus I-1c and sham versus I-1c groups using GraphPad software. Within-group comparisons were carried out using the paired *t* test, and between-group comparisons were carried out using the 2-independent sample Student *t* test. Probability values are 2-sided, and there was no adjustment for multiple comparisons because of the nature of the study. The α value was set at 0.05.

## Results

Twenty-one pigs underwent MI as described below and 14 survived 1 month after MI. During the first month, the pigs died mainly from ventricular arrhythmias or sudden cardiac death. Eight pigs received AAV9.I-1c, whereas 6 pigs received saline (Figure 1). The 8 pigs receiving AAV9.I-1c survived, whereas 1 of the pigs in the saline group died. The pigs were then euthanized 2 months after gene transfer. Three animals were subject to sham procedure only.

We used a pig model of MI, which consists of placement of an occlusive coil in the LAD. Chronic HF models of swine are suitable preclinical models because they share anatomic and pathophysiological similarities to humans. One month after infarct generation, AAV9.I-1c or saline was administered intracoronary. We determined the effect on cardiac function at baseline, time of gene transfer (1 month), and at 3 months.

Cardiac output increased in all groups, 1 month after MI was created (Figure 2). In the control group only receiving intracoronary saline, cardiac output deteriorated in the 2 months (from 6.32±1.9 to 5.0±0.96 L/min at 3 months), indicating severe progression of HF. In contrast, cardiac output in animals treated with AAV9.I-1c was preserved and did not significantly differ from sham procedure animals (7.1±1.1 versus 7.2±0.5 L/min).

Likewise, the ejection fraction remained stable after animals were treated with AAV9.I-1c 1 month after MI (from 46.4±5.8% to 50.5±3.5% at 3 months) while continuously deteriorating in control animals from 44% to 37.4% at 3 months (Figure 3). In parallel, hemodynamic catheterization showed increased $(dP/dt)_{max}/P$, indicating improved myocardial contractility (Figure 4; Table 1). Although sham animals showed almost no change over time (1.6±3.6), animals with HF that received saline had a pronounced decrease in systolic function measured by $(dP/dt)_{max}/P$ (−41.7±14.2). In contrast, gene therapy with AAV9.I-1c preserved function with a smaller decrease of −4.9±19.8 (*P*<0.05 to saline). The time constant of isovolumic relaxation (tau) was measured
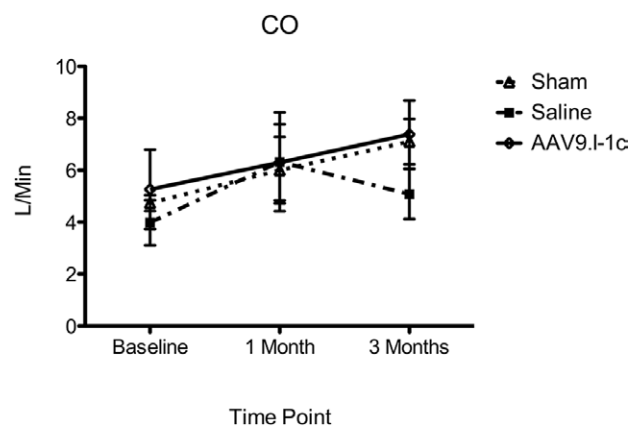


Figure 2. Cardiac output. Cardiac output was measured before myocardial infarction was created, at the time of gene transfer and before euthanization at 3 months.
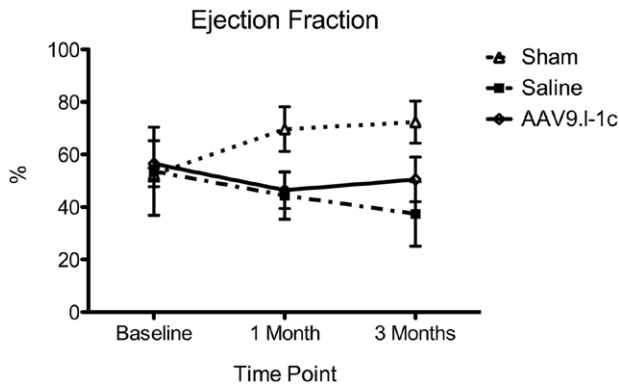
## Ejection Fraction



**Figure 3.** Ejection fraction. Global functional analysis shows long-term improvements in AAV9.I-1c–treated animals as determined by ejection fraction.

to assess the diastolic LV function. Diastolic dysfunction was prominent after 1 month and remained impaired after 3 months in animals that underwent MI and received saline injection (1.3±18.8). Gene transfer of I-1c average tau change (−13.3±27.0 s) suggests improved diastolic dysfunction in the treated animals after 1 month, although it failed to reach statistical significance when compared with saline.

At the end of the study period fractional shortening, determined by echocardiography, was significantly higher in I-1c–treated animals (25.3±5.2%) than in control animals (18.8±6.1%, $P<0.05$; Table 2), consistent with improved contractility. In summary, the presented data suggest that gene therapy with I-1c may induce functional improvements after MI in swine.

To evaluate the arrhythmogenicity of I-1c gene transfer to the porcine myocardium, 4 of the control animals and 5 of the treatment group animals were implanted with loop recorders for long-term monitoring. No events attributable to ventricular tachycardia were detected in the 2-month period from the time of injection to euthanization. Furthermore, we subjected the animals to a dobutamine challenge before euthanization. In both arms of the study, we detected 2 ventricular tachycardia events in each group. Therefore, while the animal model itself might be considered arrhythmogenic, the AAV9.I-1c delivery did not seem to enhance this effect.

Adjusted for body weight, I-1c–overexpressing animals had lower average LV muscle mass (2.9±0.08 mg/kg versus 3.1±0.05 mg/kg; $P=0.11$) relative to the saline group (Figure 5), but these differences did not reach statistical significance. In addition, the right ventricular muscle mass was not statistically different from the control group.

Examination of slices (4–6 mm) of explanted hearts by vital staining with tetrazolium chloride, which highlights scar area in white and viable tissue in pink (Figure 6), revealed that hearts of I-1c group had significantly smaller fibrotic scar areas at 2 months after intracoronary gene transfer.

To evaluate the effectiveness of our gene transfer method, we examined the expression of I-1c by RT-PCR and Western blotting. As shown in Figure 7A, I-1c expression was present in LV tissue (anterior wall) from animals that had received AAV9.I-1c, whereas Figure 7B shows the presence of I-1c genomes.

## Discussion

There are several key conclusions from our porcine model. First, intracoronary gene transfer of I-1c leads to expression of the constitutive protein as indicated by our quantitative PCR and Western blot analyses with a significant amount of variability. Although the endogenous total I-1 remains relatively high in both animal groups studied, the Western blot analysis clearly demonstrates ≈20% additional inhibitor present because of gene transfer. Thus, our data suggest the translational potential of I-1c therapy for the failing heart in a clinically relevant large animal ischemia injury model. This adds another potential option to consider in addition to the now well-documented SERCA2a therapy being evaluated in clinical trials.[9] It is notable that I-1c therapy could be considered either as an alternative or as a potential enhancement to other proven therapies.

Although the I-1c protein levels are significantly higher in our treatment group, these values need to be kept in context of the constitutive I-1 levels, including both phosphorylated and unphosphorylated forms. It is notable that I-1c imparts constitutive inhibition while the endogenous I-1 could be phosphorylated rendering it inactive against protein phosphatase. El-Armouche et al[20] showed that the phosphorylated I-1 levels in the failing human heart are elevated with concomitant decreases in phosphorylated PLN,[20,21] with these overall changes negatively affecting calcium handling. Our goal was to alter the balance between phosphorylated and unphosphorylated 28 kDa I-1 by overexpression of the constitutively active I-1c. Our data suggest that the expression levels achieved positively impacted cardiac function. These results support the conclusion that even modest transfection of I-1c can have beneficial effects to the failing heart.

Our biodistribution studies clearly demonstrated that though expression levels are variable, there is clear transfection of

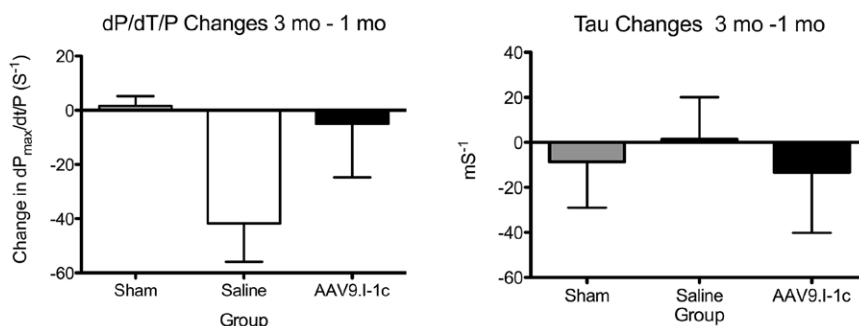



**Figure 4.** Differences in the adjusted peak left ventricular pressure rate of rise and the time constant isovolumic relaxation. $(dP/dt)_{max}$ as a measure of cardiac contractility was normalized to the average pressure: $(dP/dt)_{max}/P$.

**Table 1.  Hemodynamic Data**

| | Control (n=6) | | | AAV.I-1c (n=8) | | |
|---|---|---|---|---|---|---|
| | Baseline | 1 mo | 3 mo | Baseline | 1 mo | 3 mo |
| $(dP/dt)_{max}$, mm Hg/s | 1441±131 | 1914±310 | 1266±357 | 1316±426 | 1635±396 | 1675±325* |
| EDP, mm Hg | 11.8±2.0 | 10.9±0.9 | 11.9±2.0 | 12.4±1.1 | 12.3±1.51 | 12.6±1.9 |
| SBP, mm Hg | 97.8±14.3 | 104.6±20.1 | 87.8±56.4 | 87.2±11.1 | 98.2±13.8 | 87.8±40.8 |
| HR, bpm | 91.4±19.1 | 98.2±10.8 | 61.1±33.5 | 87.3±9.4 | 81.8±12.9 | 69.7±31.8 |

At baseline, 1 mo and 3 mo after myocardial infarction cardiac catheterization was performed, left ventricular peak pressure rate of rise ($dP/dt_{max}$, end-diastolic pressure (EDP), systolic blood pressure (SBP), and heart rate (HR). The average of 3 measurements was used for analysis. Results are given as mean±SD.

bpm indicates beats per minute.

*$P<0.05$ vs control group.

I-1c in the treatment group myocardial border zone, scar tissue, and a the more remote inferior zone. Furthermore, we did detect some off-target transfection. The variability in transfection and expression levels can be because of many sources, including the inherent intra-animal variability we experience in our porcine models that at least partially recapitulates the variability seen in the clinic. Notably, there can be significant variability in infarct size and in coronary artery architecture of the porcine heart,[22] which is also expected in the human heart.[23] Thus, while the observed variability increases the uncertainty in determining differences between experimental groups, it does serve to represent some of the variability expected in translation to clinical trials.

There are several measurements of cardiac performance that suggest overall cardioprotection from the progression of HF or potentially reverse remodeling. There is preservation in the native increase in cardiac output compared with the sham group as shown in Figure 2. Systolic function recovers from a negative trajectory after model creation because there is a leveling off of the ejection fraction decline determined in the control group as shown in Figure 3. In the treatment group, ejection fraction measures suggest improved cardiac performance. Furthermore, the scar size is significantly smaller in the treated animals relative to the controls. Taken together, these results support the potential of I-1c gene transfer to HF patients.

PLN has been shown to be the major regulator of basal contractility, a key mediator of the inotropic and lusitropic effects of β-agonists in the mammalian heart. Dysregulation of the PLN phosphorylation state is one of the insults contributing to the depressed SR $Ca^{2+}$-transport function in HF. Indeed, the SR protein phosphatase 1 activity is increased in patients with end-stage HF, which coupled with the downregulation of β-receptors[10,12,13,24] would contribute to decreased PLN phosphorylation and increased inhibition of SERCA2a's $Ca^{2+}$ affinity. Thus, regulation of the phosphatase activity in SR may provide another mechanism by which the cell achieves increases in PLN phosphorylation resulting in increases in basal contractile parameters and their responses to β-agonists.[10,12,13,24] β-adrenergic stimulation results in cAMP-dependent increased phosphorylation of the I-1 protein, effectively inhibiting the SR phosphatase activity resulting in stimulatory effects through unopposed PLN phosphorylation.[25,26]

However, in HF, the I-1 is dephosphorylated because of decreased β-adrenergic drive, and this results in diminished inhibition of the type 1 phosphatase and dephosphorylation of PLN.[27] Ablation of I-1 resulted in increased protein phosphatase activity, which was associated with decreases in PLN phosphorylation status and thus, increased inhibition of SERCA2a's $Ca^{2+}$ affinity and myocardial contractility. Overexpression of the constitutively active I-1 had opposite effects than its ablation.[10] Phosphorylation at Ser67 of I-1 may be associated with diminished inhibitor activity and increased PP1 activity. Indeed, our preliminary studies in human failing hearts show that the phosphorylation of Ser67 in I-1 is increased (55%) and PLN phosphorylation (pSer16; pThr17) is decreased, in agreement with previous reports on enhanced PP1 activity. Furthermore, we have recently identified an additional PKC phosphorylation site on I-1, Thr75.[13]

**Table 2.  Echocardiographic Data**

| | Control (n=6) | | | AAV.I-1c (n=8) | | |
|---|---|---|---|---|---|---|
| | Baseline | 1 mo | 3 mo | Baseline | 1 mo | 3 mo |
| IVSTd, mm | 0.7±0.07 | 0.4±0.05# | 0.42±0.12# | 0.7±0.06 0 | 0.38±0.05# | 0.43±0.14# |
| FS, % | 36.4±13.2 | 22.8±5.9# | 18.8±6.1# | 30.3±5.5 | 26.3±7.0 | 25.3±5.2* |
| SV, mL | 53.8±14.5 | 76.0±18.9# | 82.4±26.0 # | 50.6±11.2 | 60.9±23.7 | 101.6±23.7# |
| ESV, mL | 41.6±15.7 | 97.5±34.8# | 146.6±64.6# | 36.5±4.7 | 75.2±24.7# | 108.3±59.4# |
| EDV, mL | 91.4±20.2 | 174.3±44.7# | 229±61.0# | 87.3±11.4 | 143.5±40.9# | 222.6±59.8# |
| E/Ea | 0.05±0.004 | 0.06±0.007 | 0.07±0.013 | 0.05±0.010 | 0.06±0.012 | 0.05±0.019 |

At baseline, 1 mo and 3 mo after myocardial infarction, interventricular septum thickness (IVSTd), fractional shortening (FS), stroke volume (SV), end-systolic volume (ESV), end-diastolic volume (EDV), and E/Ea were measured at the mid-ventricular level. The average of 3 measurements was used for analysis. Results are given as mean±SD.

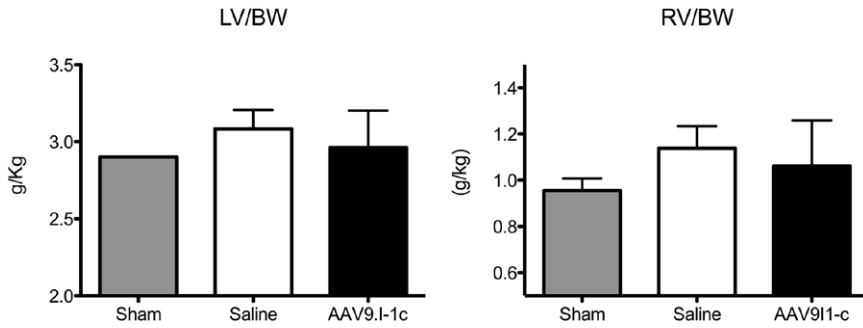*$P<0.05$ vs control group.

#$P<0.05$ vs baseline.

**Figure 5.** Heart weight to body weight ratio. Weight of the left ventricle (LV) was determined after explanation at euthanization and the ratio to animal's body weight was calculated. BW indicates body weight; and RV, right ventricle.

The expression of a constitutively phosphorylated I-1 at either S67D or T75D in isolated rat cardiomyocytes is associated with significantly depressed contractile performance.

Given these in vitro and in vivo findings, potent and specific inhibition of PP1 activity might improve cardiac function and arrest the progression of hypertrophy and subsequent HF.[10,28]

These experiments suggest that I-1 is a molecular inotrope that regulates cardiac β-adrenergic responses through PLN phosphorylation. Indeed, overexpression of the active truncated I-1 in vivo was associated with enhanced cardiac function and protection against pressure-overload hypertrophy.[12] In addition, gene delivery of this activated molecule in failing rat hearts resulted in rescue of the depressed cardiac function and partial restoration of cardiac remodeling. Importantly, the effects of I-1 in vivo seemed to be mediated by specific alterations only in PLN phosphorylation while there were no alterations in either ryanodine receptor or troponin I phosphorylation levels, and the activity of the L-type $Ca^{2+}$-channel remained unaltered.[12]

More recently, an inducible transgenic mouse model was generated that enabled temporally controlled expression of active I-1.[29] Active I-1 expression in the adult heart elicited significant enhancement of contractile function, associated with preferential PLN phosphorylation and enhanced SR $Ca^{2+}$-transport. During ischemia/reperfusion-induced injury, active I-1 expression augmented contractile function and recovery. Further examination revealed that the infarct region and apoptotic and necrotic injuries were significantly attenuated by enhanced I-1 activity. These cardioprotective effects were associated with suppression of the endoplasmic reticulum stress response.

The protective role of I-1c was challenged by recent reports from El-Armouche et al[20,28] who found that I-1 deletion exhibited less susceptibility to acute isoprenaline-induced deaths, associated with a lesser extent of ventricular arrhythmias and also protected against chronic isoprenaline-induced hypertrophy, dilatation, and fibrosis. More recently, the same group found that mice with conditional cardiomyocyte-restricted expression of I-1c on an I-1–deficient background exhibited enhanced cardiac contractility but exaggerated contractile dysfunction and ventricular dilation on catecholamine infusion.[15] It is not clear why different groups have such different results, however different genetic backgrounds (FVB/N versus C57Bl/6J) are known to alter morphology, function, and survival in transgenic mice.

For this reason, our studies in a relevant preclinical model of HF are quite important to specifically address the issue of I-1c overexpression on contractile measures. Using gene transfer methodologies targeting the heart, our studies clearly showed that I-1c overexpression improved contractile function, mitigated the adverse remodeling after MI, and led to a decrease in infarct size. We did not observe any negative effects of I-1c overexpression in this preclinical model of HF.

There are some limitations to this study that can be addressed in subsequent studies to further assess the benefits of I-1c gene transfer as a therapeutic approach to HF. Although we have chosen the $2.5 \times 10^{12}$ vector genome dose in our treatment groups, it is unclear what the optimal dose is and furthermore, if there would be any negative effects at elevated doses. Therefore, determination of dose dependence on cardiac performance would be beneficial for determining the safety and efficacy. Although we have clearly demonstrated myocardial transfection of I-1c, a more comprehensive myocardial distribution pattern study would be beneficial so that we could better understand the correlation of I-1c levels to cardiac function. In addition, while our study design included evaluating the arrhythmogenicity of gene transfer by dobutamine challenges just before euthanization, determination of PLN phosphorylation state was precluded. Now that we have determined that there are no significant increases in rates of arrhythmia, future more extensive studies will focus on determining the PLN phosphorylation and more quantitative determination of I-1c mRNA. In addition, while some studies have demonstrated long-term efficacy[9] on AAV administration, our 3-month study may not be predictive of long-term I-1c gene delivery
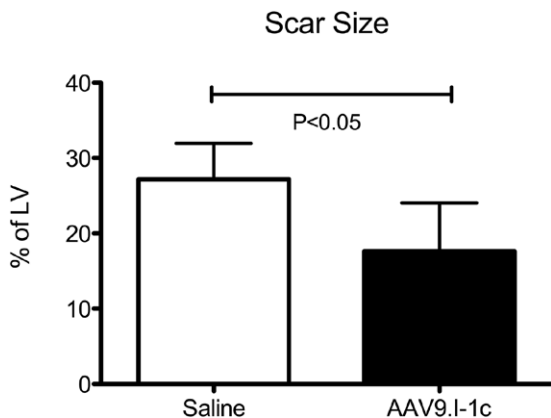


**Figure 6.** Left ventricular (LV) scar size. Quantification of scar volume by tetrazolium chloride staining.
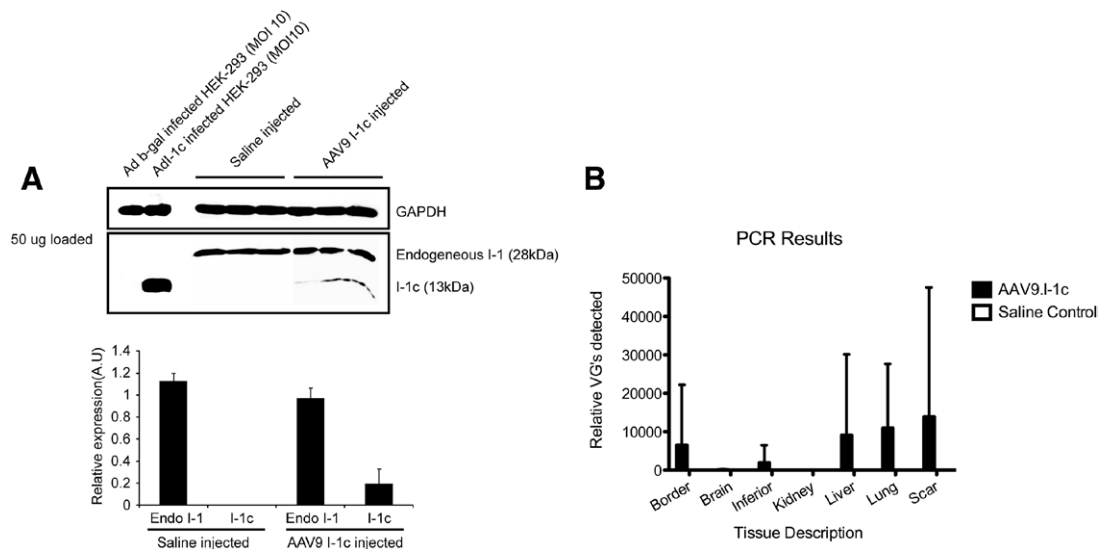
**Figure 7.** Expression of I-1c. **A,** Western blot for I-1 and I-1c in left ventricular tissues after gene transfer of I-1c or injection with saline. **B,** RT-PCR of I-1c in left ventricular tissues after gene transfer of I-1c. Border: Viable tissue adjacent to scar tissue. Inferior: Myocardium supplied by right coronary artery at midpapillary muscle level.

effects, especially regarding the potential immunogenicity of this approach. We have taken the precaution of screening our animals for neutralizing antibodies to mitigate this effect. Therefore, long-term follow-up would be beneficial. In addition, scar sizes before gene therapy were not realized in this study. Measurement by MRI will be beneficial in future studies. Finally, while we have shown statistically significant differences between the AAV9.I-1c and control groups, future studies would benefit from groups with higher populations, thus enhancing our ability to measure more subtle cardiac performance changes and alterations in signaling pathways.

## Conclusion

In this porcine model of MI, we found that once LV dysfunction was established for 1 month, AAV9.I-1c injection into the coronary arteries induced improvements that reversed the adverse remodeling of the LV, which could be detected 2 months later. The beneficial effects were specifically on contractility (d$P$/d$t$/$P$) and ejection fraction. In addition, there was evidence that I-1c overexpression halted infarct expansion.

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## Disclosures

Drs Kranias, Jude Samulski, and Hajjar are scientific cofounders of NanoCor Therapeutics. NanoCor is planning to commercialize I-1c for future therapeutic use. The other authors have no conflicts to report.

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## CLINICAL PERSPECTIVE

Congestive heart failure is a major cause of morbidity and mortality in the United States. Although progress in conventional treatments is making steady and incremental gains to reduce heart failure mortality, there is a critical need to explore new therapeutic approaches. Gene therapy was initially applied in the clinical setting for inherited monogenic disorders. It is now apparent that gene therapy has broader potential in diseases such as congestive heart failure. Improvement in our understanding of the molecular mechanisms of heart failure, along with the development of novel and safer vectors for gene delivery, has led to the identification of novel targets that are difficult to manipulate pharmacologically but may be more amenable to gene therapy. Over the past few years, calcium cycling abnormalities and specifically deficiencies in sarcoplasmic reticulum calcium uptake have been hallmarks of advanced heart failure. The complex of SERCA2a-phospholamban-protein phosphatase 1 has been difficult to target pharmacologically. However, the encouraging results from the CUPID Trial in which AAV1.SERCA2a gene transfer was found to be safe and demonstrated benefit in clinical outcomes, symptoms, functional status, NT-proBNP, and cardiac structure in a phase 2 study have once again validated calcium cycling as being an important target for heart failure treatment. For this reason, I-1c with its additional benefits is emerging as an important and valid target for the treatment of heart failure. In this research report, we describe positive impacts of I-1c delivery using the AAV9 vector to a clinically relevant large animal model of heart failure.