SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

All experiments were carried out in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act 1986, which conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

Viral Vectors

Adenoviral Vectors

Adenoviral vectors were generated as previously described by our group.[1, 2] The viruses were defrosted from stocks stored at -80^{o}C, suspended in sterile, filtered PBS, and diluted to a concentration of 3.75×10^{9} dnase resistant particles (drp) (Ad.SERCA2a.GFP) and 4.5×10^{9} drp (Ad.GFP) per 50µL. Viral solutions were warmed to 37 ^{o}C within 15 mins of delivery.

Adeno-Associated Viral Vectors

AAV9.SERCA2a vectors were produced using the two-plasmids protocol described by Zolotukhin et al,[3] with the following modifications. 293-T cells (ATCC, Manassas, VA) were grown in triple flasks for 24 h (DMEM, 10% FBS) prior to adding the calcium phosphate precipitate. The human isoform of SERCA2a was introduced under the control of a CMV promoter in the plasmid. 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 concentration and formulation into lactated Ringer's solution (Baxter Healthcare Corporation, Deerfield, IL) using a Vivaspin 20 Centrifugal concentrators 50K MWCO, and stored at –80^{o}C.

***In vivo* Arrhythmia Analysis (Cohort 1)**

HF and Sham ligated rats in cohort 1 received implantable ECG telemetry transmitters (CA-F40 Data Sciences International, Minneapolis, MN) for *in vivo* arrhythmia studies. Animals underwent a baseline (PREGENE) arrhythmia assessment with continuous 24 hour ECG recording for spontaneous ventricular arrhythmias, followed by an *in vivo* intraperitoneal injection of 0.5mg/kg isoproterenol (ISO) for arrhythmia provocation.

Sixteen weeks post infarction, animals underwent a second left thoracotomy for gene delivery. Three 50µL intramyocardial injections of Ad.SERCA.GFP or Ad.GFP were delivered to the failing left ventricle (total dose ~10^{10} dnase resistant particles per heart). Two injections were targeted to the apical and lateral infarct borderzone, and one to the mid posterior left ventricular wall. SHAM cases received three location-matched Ad.GFP injections. Six days post gene delivery, animals underwent repeat 24 hour ECG recording and ISO challenge *in vivo* (POSTGENE). This allows completion of the in vivo and ex vivo studies during the 6-8 day time window post gene delivery when peak gene expression and physiological sequelae of transgene expression are present, and without the confounding effects of the anti-adenoviral immune response.[4] A subgroup of animals in the HF+SERCA2a and HF+GFP cohorts had the complete 7 day recording after gene injection, allowing quantification of daily spontaneous arrhythmia burden.

Upon completion of the *ex vivo* study, planimetric determination of infarct size was performed and SERCA2a protein levels were measured in fluorescent (transfected) and non-fluorescent (untransfected) myocardium samples from each heart by Western blotting.

PREGENE and POSTGENE *in vivo* ECG recordings were acquired using Dataquest ART 3.1 software (Data Sciences International, Minneapolis, MN), and arrhythmia analysis was performed using ECG-Auto 2.4 software (EMKA, France). Ventricular arrhythmias were categorised as initiating events (total ventricular arrhythmias (VAs)) and complex (sustained) arrhythmias with more sustained ventricular electrical activity (couplets, triplets, non-sustained (<30s) and sustained (>30s) VT and VF). The majority (>95%) of total ventricular arrhythmias were ventricular ectopy which displayed a Gaussian distribution after logarithmic transformation. Log total and log sustained arrhythmias were compared between study arms.

***Ex vivo* Ventricular Arrhythmia Threshold Studies (Cohort 1)**

Eight days post myocardial gene injection, and a minimum of 24 hours post *in vivo* ISO challenge, animals were reanaesthetised, hearts were rapidly explanted and mounted upon the constant-pressure Langendorff apparatus. Hearts were perfused with modified Krebs-Henseleit solution: NaCl 118.5mM, KCl 5.9mM, $NaHCO_3$ 25mM, $MgSO_4$ 1.2mM, NaH_2PO_4 1.38mM, Glucose 11mM, buffered with 95%O_2/5%CO_2, and delivered at 36-37^{o}C, pH 7.4 and at a constant pressure of 76-78mmHg (110-110cm H_2O) . All solutions were filtered (5µm pore size) before use.

A silver unipolar sensing electrode was inserted in the right ventricular myocardium, and the earth electrode attached to the aortic cannula. The electrodes were connected to a Bioamplifier and Powerlab (AD Instruments, Sydney, Australia). The

ECG signal was amplified, filtered between 2 and 400Hz, with data acquired at 1000 samples per second, and displayed on a local computer using CHART 5.5 software (AD Instruments, Sydney, Australia).

Paired platinum stimulating electrodes 2mm apart were placed on the posterobasal epicardial surface of the right ventricle and interventricular septum after insertion of the silver sensing electrode into the right ventricle. After five minutes stabilisation in spontaneous sinus rhythm, cardiac pacing commenced, with a further ten minutes stabilisation during pacing prior to PES. Pacing delivered 0.2ms biphasic square-wave stimuli from a custom-made high voltage bipolar stimulator at twice the excitation threshold (~0.2-0.4mV) for 10 min at 150ms basic cycle length (BCL).

Perfused hearts with continuous ECG monitoring were subjected to arrhythmia provocation using programmed electrical stimulation (S1S2 protocol) to assess for susceptibility to reentry arrhythmias. PES protocols consisted of a drive train of nineteen paced S1 beats at 150ms BCL, followed by a premature S2 extrastimulus every twentieth paced beat. The S2 interval started at 90ms, and was successively shortened by 2ms until ventricular refractoriness or sustained ventricular arrhythmia was reached.

Protein Quantification

LV samples were pulverised in a liquid nitrogen cooled percussion mortar and homogenized in a buffer containing 300mM sucrose, 20mM PIPES pH 7.4, 1mM NaF, 1 mM Na_3VO_4 and protease inhibitors (Sigma P8340). The homogenate was centrifuged at 11000 x *g* and the supernatant retained. Protein quantification was measured using the BioRad quantification assay (BioRad Laboratories, CA) with absorbance photospectrometry. For SDS-PAGE analysis, lysates were diluted in Laemmeli buffer and electrophoresed on acrylamide gels (50mV for 30 min, then

140mV for 60 min), and the separated proteins transferred onto activated nitrocellulose or PDVF membranes. Membranes were incubated for 60 min at room temperature with primary antibodies to cardiac SERCA2a 1:2000 (MA 3-919 Affinity Bioreagents, CO), cardiac calsequestrin 1:2000 (PA 1-913 Affinity Bioreagents, CO) and Green Fluorescent Protein 1:2000 (29779 AnaSpec Inc, CA), and blocked with TBS-T and 5% skimmed milk. The 90 minute incubation in the secondary antibodies conjugated with horse radish peroxidase (anti-mouse Fab for SERCA2a and calsequestrin, anti-rabbit Fab for GFP). For GAPDH measurement blots were striped (2% SDS, 50mM Tris-Cl pH 6.8) 30 minutes at 50^{o}C and incubated with a GAPDH antibodies (SC32233 Santa Cruz Biotechnology, CA) for signal normalization.

RyR and PLB levels and phosporylation status were measured from purified membrane preparations from hearts from Cohort 2. LV samples were pulverised in a liquid nitrogen cooled percussion mortar and homogenized in a buffer containing 300mM sucrose, 20mM PIPES pH 7.4, 1mM NaF, 1 mM Na_3VO_4 and protease inhibitors (Sigma P8340). The homogenate was centrifuged at 6500 x *g*, the supernatant retained and the pellet resuspended and homogenised a further two times. The pooled supernatants were centrifuged at 100000 x *g*, and the membrane pellet resuspended in a solution containing 400mM sucrose, 10mM HEPES pH 7.2, 1mM NaF, 1 mM Na_3VO_4 and protease inhibitors.

Proteins were separated by SDS-PAGE and transferred to PVDF membranes before blocking with 5% milk. Primary antibodies used were as follows: RyR2 (ARP 106/1) was from Alan Williams; p-RyR2 (Ser2815) was from Hector Valdivia; p-RyR2(Ser2809), PLB(A1), p-PLB(Ser16) and p-PLB(Thr17) were from Badrilla Ltd (Leeds, UK); cardiac calsequestrin was from Affinity BioReagents (CO). HRP-conjugated secondary antibodies were from Dako (Glostrup, Denmark) or Santa Cruz

(CA). Band densities were quantified on a Gene Genius bio-imaging system (Syngene) using ECL detection reagents (GE Healthcare).

Pressure-Volume Analysis Protocol (Cohorts 1+2)

Pressure-volume analysis was performed using the 2.0F Mikro-Tip® pressure-volume catheter (SPR 838 Millar Instruments, Houston) via the apical approach following upper midline laparotomy and diaphragm myotomy. Animals were anaesthetised (Isoflurane 1.5%), ventilated (tidal volume 1.5-2.0 ml, ventilation rate 90 cycles/min) and assessment of left ventricular function was performed. After stable steady state recording, evaluation of dynamic load-independent contractility was assessed using transient inferior vena cava (IVC) occlusion. Parallel conductance was measured during 0.1ml hypertonic saline injection. Data analysis was performed using PVAN 3.5 software (Millar Instruments, Houston, TX). Infarcted failing rats without gene transfer (HF) and SHAM or AMC served as controls. Efficiency was calculated as stroke work area (SWA) / stroke work area (SWA) x pressure volume area (PVA) where PVA = SWA + potential energy area. Upon completion of the PV study, HF+Ad.SERCA hearts were perfusion fixed with 4% paraformaldehyde for histological analysis.

Isolated Cardiomyocyte Aftercontraction Provocation Studies (Cohort 2)

Simultaneous contraction and Ca^{2+} transient measurements were performed on freshly isolated cardiomyocytes after loading with the Ca^{2+}-sensitive fluorescent dye, indo-1 (Invitrogen, Paisley, UK). HF+SERCA hearts were rapidly excised and cardiomyocytes enzymatically isolated by Langendorff perfusion method as previously described.[5] Freshly isolated cardiomyocytes were loaded at room temperature with 10 µM of the acetoxymethyl ester (AM) form of the Ca^{2+}-sensitive

fluorescent dye, indo-1 (Invitrogen, Paisley, UK) for 25 min. Once loaded, cells were not used for at least another 30 min to allow the intracellular indo-1-AM to be de-esterified.

Thirty minutes later myocytes were transferred to the recording chamber superfused with Tyrode's solution (in mmol/L: NaCl 140, $CaCL_2$ 2, KCL 6, MgCl 1, HEPES 10 and glucose 10) on mouse laminin (Sigma Aldrich, UK) coated plates. Experiments were performed at 37^{o}C with field stimulation at 0.5Hz at a pulse length of 2ms. Contraction was monitored by video edge detection and concomitant cytoplasmic calcium transients were recorded with excitation of indo-1AM as previously described.[1, 6] Following stabilisation and baseline recording, the incidence of Ca^{2+}-mediated DACs was recorded at baseline and after perfusion with 1 nmol/L ISO and 100 nmol/L ISO, with drug washout between doses.

Cardiomyocyte Intracellular Ca^{2+} Studies (Cohort 2)

Spark studies were performed as previously described by our group.[7] Aliquots of cells were incubated with Fluo-4 AM (10 µM) for 15-20 min. Supernatant was then discarded and substituted with Dulbecco's Modified Eagle's Medium (Gibco BRL, Life Technologies, Paisley, UK). The cells were not used in experiments for at least 30 min to allow de-esterification of the intracellular indicator. The experimental chamber was mounted on the stage of a Nikon TE300 microscope with Bio-Rad Radiance 2000 confocal attachment and myocytes observed through a Nikon Plan-Fluor x 40 oil-immersion lens (N.A. = 1.3). Fluo-4 was excited using the 488 nm line of an argon laser and the emitted fluorescence collected through a 520 nm filter.

Ten thousand line scans were collected (512 pixels wide scanned at 1.3 ms intervals) for analysis with both ImageJ (Scioncorp, Frederick, MD) and LaserPix software (Bio-

Rad, Hemel Hempstead, UK). Detection criteria for Ca^{2+} sparks were set at 3.8 x S.D. and automated counting of sparks was performed using the Sparkmaster plug-in for ImageJ.[8] This is based on the standard algorithm of Ca^{2+} spark analysis developed by Cheng et al.[9]

SR Ca^{2+} content was assessed by measuring the sizes of the Ca^{2+} transients produced by rapid application of 10mM caffeine to the normal superfusate. Prior to the caffeine exposure, myocytes were subjected to a loading protocol consisting of a train of normal stimuli applied at 0.5 Hz. Spark frequency was corrected for SR load from paired cells originating from the same heart.

Total SR leak was measured using the tetracaine-dependent SR leak using the protocol described by Shannon et al.[10] Cardiomyocytes were field stimulated at 1 Hz for a minimum of three minutes to achieve a steady state SR load. Stimulation was then stopped and the solution switched from NT to a $0Na^{+}0Ca^{2+}$ solution (Li replacement and 1mmol/L EGTA) ± tetracaine (1mmol/L) for 30s. $0Na^{+}0Ca^{2+}$ was used to prevent the majority of the movement of Ca^{2+} across the sarcolemma as it inhibits the function of the NCX. Changes in cytosolic Ca^{2+} that occur in $0Na^{+}0Ca^{2+}$ reflect movement of Ca^{2+} between the SR and the cytosol. SR load measured by applying 20mmol/L caffeine in $0Na^{+}0Ca^{2+}$ for ~3s at the end of the ± tetracaine step.

SUPPLEMENTAL RESULTS

Data Supplement Figure S1: Restoration of myocardial SERCA2a protein levels in the failing heart following three focal myocardial injections of Ad.SERCA2a.GFP.

A. Western blots comparing SERCA2a levels (corrected for calsequestrin (CSQ)) in the fluorescent left ventricular myocardium from gene injected hearts, with quantitative comparison of SERCA2a protein levels in the injected (fluorescent (FL)) and remote (non-fluorescent (NFL)) myocardial regions. SERCA2a protein levels were increased in both injected and remote ventricular myocardium following SERCA2a transduction of failing hearts. B. CSQ levels are unchanged across study arms, serving as an appropriate SR loading control protein. N=6 per study arm, *$p<0.05$.

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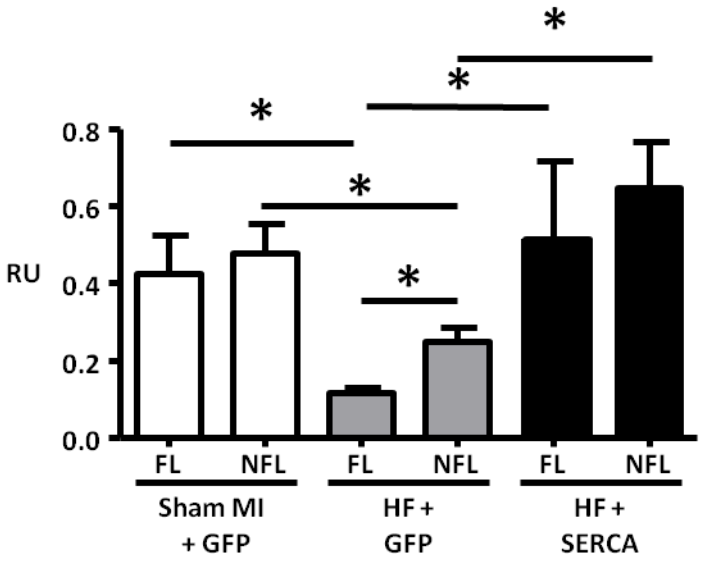
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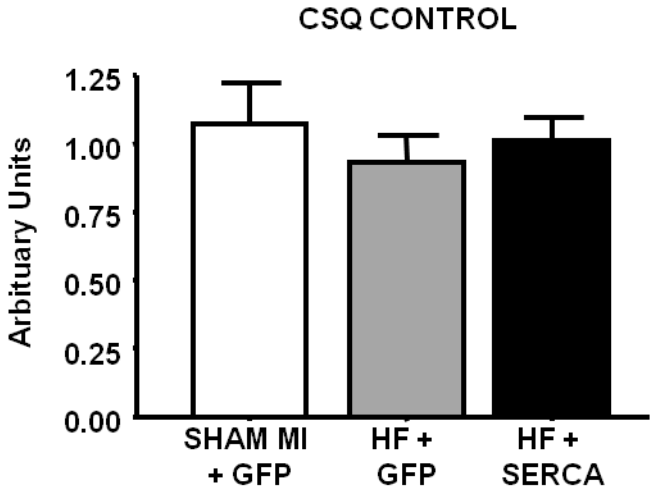
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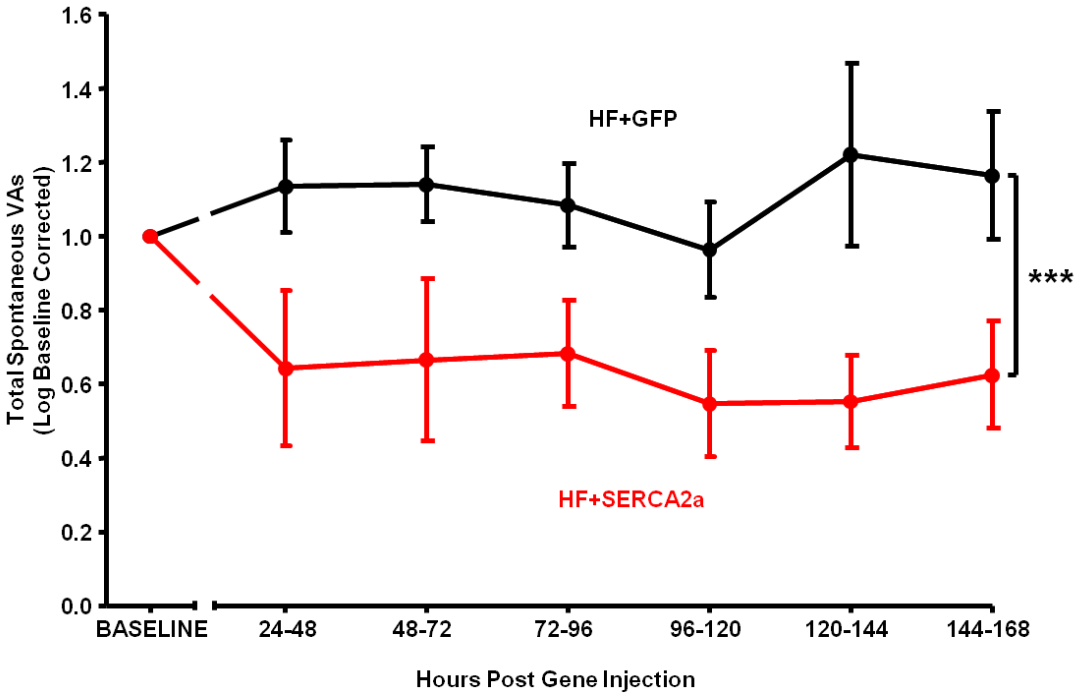
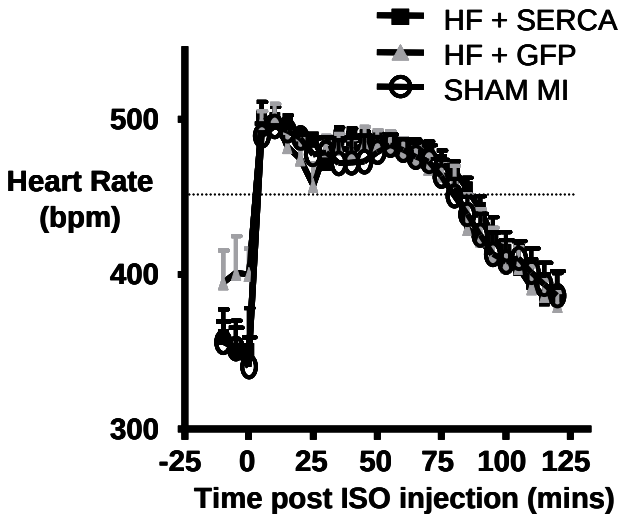
Data Supplement Figure S2: Spontaneous ventricular arrhythmia generation for each 24 hour time phase for the first six days after gene injection, corrected for baseline in vivo arrhythmia burden pre injection. Injection was performed between 0900 and 1400 on day 0, and the first complete 24 hour time bin was measured from 0700 on day 1 post op, spanning 24 hours to 0700 on day 2. HF n=6, HF+SERCA2a n=4. Two way ANOVA ***p<0.0001.



Data Supplement Figure S3: *In vivo* Isoproterenol-Induced Heart Rate Changes.

ISO-induced a sinus rate increase which remained above the upper limit of spontaneous physiological heart rate (HR) *in vivo* (450 beats per minute - horizontal dotted line), for the 60 minute analysis window. There was no significant difference in *in vivo* ISO-induced heart rate between study arms.



Data Supplement Table S1.

Biometric Data, Infarct Size and Ex vivo Coronary Flow Rate in Study Animals from Cohort 1.

	SHAM/AMC	HF +/- Ad.GFP	HF + Ad.SERCA2a	HF + AAV9.SERCA2a
HW (g)	1.7±0.0	2.4±0.1**	2.1±0.1$^{\#}$	1.9±0.1$^{\#\#}$
HW:BW Ratio (g/kg)	4.2±0.2	5.9±0.3*	5.3±0.3$^{\#}$	4.3±0.3$^{\#\#}$
Infarct Size (%LV)	N/A	44±3	39±4	-
Ex vivo CFR (ml/min/g LV tissue) (paced)	11.8±0.3	9.1±0.3*	10.9±0.8$^{\#}$	-

CFR = Coronary Flow Rate

*p<0.05, **p<0.01 vs AMC/SHAM MI

# p<0.05, ## p<0.01 vs HF

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