2. Methods

2.1. Generation of transgenic Lys104Glu (Tg-MUT) mice

All animal studies were conducted in accordance with institutional guidelines. The University of Miami has an Animal Welfare Assurance (A-3224-01, effective November 23, 2011) on file with the Office of Laboratory Animal Welfare (OLAW), National Institutes of Health.

We have generated transgenic mouse models for the wild-type human ventricular RLC (GenBank accession no. **P10916**) [1] and the Lys104Glu-mutated RLC. The cDNA of the mutant was cloned into the unique SalI site of the plasmid, α-MHC clone 26 (generously provided by Dr. J. Robbins, Cincinnati Children's Hospital Medical Center, Cincinnati, OH). The resulting constructs contained about 5.5 kb of the mouse α-MHC promoter, including the first two exons and part of the third, followed by the Lys104Glu cDNA (498 bp) and a 630 bp 3' untranslated region from the human growth hormone transcript as described earlier for Tg-WT mice [1]. All of the founders were bred to non-transgenic B6SJL mice. Multiple crosses of Tg-mice with B6SJL/F1 mice were performed before the animals were used for experiments.

2.1.1. Analysis of transgenic protein expression

The α-MHC-driven expression of the human ventricular WT RLC or Lys104Glu -RLC proteins in mouse hearts was determined as described previously [1-4], but instead of heart extracts the atrial and ventricular myofibrils were used. Briefly, the hearts from Tg-MUT L2, L3 and L7, Tg-WT L2 and non-transgenic (NTg) mice were excised and the ventricles and atria were immediately separated and frozen in liquid nitrogen. Prior to the experiment, ventricles and atria were thawed in CMF (cardiac myofibril) buffer consisting of 5 mM NaH_2PO_4, 5 mM Na_2HPO_4 (pH 7.0), 0.1 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 5 mM ATP, 5 nM microcystin, 0.1% Triton X-100, 20 mM NaF (phosphatase inhibitor), 5mM DTT and 1 μl/ml protease inhibitor cocktail. Tissue samples were homogenized for 2 min in a Mixer-Mill MM301 at 30 Hz, chilled on ice and homogenized again for 2 min. Homogenates were then centrifuged for 4 min at 1800 g and the supernatants were discarded. The pellets were re-suspended in the CMF buffer and the myofibrils were subsequently dissolved in SDS-PAGE sample buffer and loaded on 15% SDS-PAGE at 30 μg per lane for Coomassie staining and at 20 μg per lane for Western blotting. The expression level was assessed on the basis of the faster gel mobility of the human ventricular RLC (18.789 kDa) vs. mouse atrial RLC (19.450 kDa) [1] detected utilizing a rabbit polyclonal RLC CT-1 antibody (produced in this lab [2]) followed by a secondary goat anti-rabbit antibody conjugated with the fluorescent dye, IR red 800 [1, 2]. The myosin ELC was used as a loading control and detected with the monoclonal ab680 antibody (Abcam) followed by a secondary goat anti-mouse antibody conjugated with the fluorescent dye, Cy 5.5. Blots were scanned and respective bands were quantified using the Odyssey Infrared Imaging System (LI-COR Inc.).

2.1.2. Analysis of transcript expression by real-time PCR

Analyses of *MYL2* expression in the ventricles and atria of Tg-Lys104Glu mouse lines were performed as described previously [5]. Briefly, total RNA was isolated from ventricles and atria of 4-6 month-old Tg- Lys104Glu (L2, L3 and L7), Tg-WT L2 and NTg mice (control), and 3 μg samples were converted to double stranded cDNAs using Random Primers and a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The reverse

transcription was performed in MJ Research PTC-200 [5]. Obtained cDNA samples were stored at -80°C until used for the quantitative PCR, conducted using SYBR Green I (Applied Biosystems) chemistry with the following gene-specific Quantitect Primer Sets (Qiagen): human *MYL2* (myosin RLC, ventricular; NCBI #NM_000432) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase; NCBI #NM_008084). All reactions were performed in duplicate and were run twice in an Bio-Rad CFX connect Real-time system with the following cycle parameters: 1 cycle at 95°C for 30 s followed by 40 cycles at 95°C for 5 s followed by 60°C for 30 s. Raw data were converted to the relative quantities by plotting the standard curves for *MYL2* and *GAPDH* genes. The results were expressed as a ratio of *MYL2* to *GAPDH* (housekeeping gene).

2.1.3. Analysis of protein phosphorylation

RLC phosphorylation was determined in the ventricles of ~3 month-old (young) and 17-18 month-old (old) Tg-MUT vs. Tg-WT mice. Ventricular myofibrils were prepared as described in detail in 2.1.1. Myofibrillar samples were dissolved in SDS-PAGE sample buffer and loaded on 15% SDS-PAGE. Phosphorylated RLC was detected with phospho-specific RLC antibodies (produced in this laboratory [4]), which recognize the phosphorylated form of the RLC followed by a secondary goat anti-rabbit antibody conjugated with the fluorescent dye, IR red 800. The total RLC protein was detected with CT-1 and served as a loading control.

2.1.4. Analysis of mitochondrial content

The hearts from young (~4 months) and old (~14 months) Tg-WT and Tg-MUT mice were homogenized for 2 min in a Mixer-Mill MM301 at 25 Hz in a buffer containing 8 M urea, 10 mM Tris-HCl, pH 7.0, 1% SDS, 1% β-mercaptoethanol (β-BME), 1 mM EDTA, 1 mM PMSF and protease inhibitor cocktail (PIC) (Sigma-Aldrich). At least two mice per group were used to prepare heart extracts. The heart extracts were clarified by centrifugation at 18,000 g x 5 min and quantified by a Coomassie-Plus Assay (Pierce). Supernatants were loaded at 20 μg per lane, subjected to 15% SDS-PAGE and electrophoresed for Western blotting. The mitochondrial Voltage-dependent anion channel (VDAC/porin) protein was detected using anti-VDAC antibodies (V2139, Sigma-Aldrich) followed by a secondary goat anti-rabbit antibody conjugated with the fluorescent dye, IR red 800. The myosin ELC was used as a loading control (detected as described above). Respective protein bands were quantified using the Odyssey Infrared Imaging System (LI-COR Inc.) [4]. The normalized VDAC / ELC band intensity ratio was used to assess the relative mitochondrial content in Tg-WT and Tg-MUT hearts.

2.2. *In vivo* study

2.2.1. Echocardiography assessment

Mice were anesthetized in a Plexiglas box with isoflurane 4%, weighted and placed on a heating pad to maintain body temperature of 37°C monitored with a rectal thermometer. Ventilation and anesthesia were provided via a nose cone and isoflurane was reduced to 1.0-1.5% in order to minimize any effect on cardiac function. Heart rate was monitored with ECG recording.

In vivo cardiac function, pulse-wave Doppler images and Tissue Doppler Imaging (TDI) were assessed in 6 month-old Tg-MUT (n=10 mice) and age matched Tg-WT (n=10) male mice. Images were obtained using a Vevo 2100 ultrasound machine (Visualsonics Inc.) with a 40 MHz transducer, which was mounted on the Vevo Imaging Station (Visualsonics Inc.) for improved micromanipulation. Anterior and posterior wall thicknesses, left ventricular (LV)

end-diastolic and end-systolic dimensions were measured in short axis (SAX) at papillary muscle level using M-mode images. LV mass, LV volumes and ejection fraction (EF) were calculated from these measurements [6, 7]. Apical four chamber view was used for Pulsed-Wave (PW) Doppler measurement of mitral inflow, and TDI measurements at the annular level of the septal wall. With PW-Doppler we measured early (E) and late (A) mitral inflow and deceleration time and with TDI we measured systolic velocity s' and early diastolic velocity e'. We evaluated diastolic function with E/A-ratio, deceleration time, e' and E/e' [8].

Cardiac morphology and function were also assessed in 27 adult Tg-WT (n=15) and Tg-MUT (n=12) mice (>13 month old) using a Vevo 770 echocardiography system. The echocardiography was performed using the methods described above but without PW- and TDI measurements.

2.2.2. Invasive hemodynamics

Surgical preparation and instrumentation: Six month-old Tg-MUT and Tg-WT animals were used in this study. After the animal had been placed in a Plexiglas box with isoflurane 4% for several seconds, the chest and neck were shaved and a tracheostomy was performed. Anesthesia was maintained with mixture of oxygen 100% and isoflurane 1-1.5% and mechanical ventilation was provided with a MiniVent ventilator for mice (Harvard apparatus). For a 30g intubated mouse we used a respiratory rate of 130-135/min and tidal volume of 180µl. The mouse was placed on a heating pad to maintain the body temperature at 37 °C, monitored with a rectal thermometer. The left jugular vein was accessed and canulated with a 30G needle and a constant infusion of albumin 12.5% in normal saline was administered at 5µl/min after a 50µl bolus to counteract peripheral vasodilatation and hypotension induced by anesthesia. The heart was accessed via a subxyphoid incision and diaphragm exposure. Then, the LV apex was punctured with a 26G needle and a pressure-volume catheter (Millar Instruments) was introduced into the LV cavity aligned with the LV longitudinal axis [9, 10]. Baseline pressure and volume signals were recorded and consecutive pressure-volume loops under varying loading conditions were taken with progressive compression of the inferior vena cava (IVC) with a cotton tip. Since the animal was heavily sedated with an open chest, an intra-cardiac injection of 50 mg/kg sodium pentobarbital was administered for euthanasia (AVMA Guidelines for Euthanasia).

Catheter Calibration: Pressure calibration was performed before starting the experimental procedure according to the manufacturer's guidelines.

Hemodynamic measurements: LV pressure and volume signals were recorded during the experiment. All hemodynamic parameters along with indices of systolic and diastolic function were calculated with Labchart 7 Pro software (AdInstruments). Systolic indices consist of maximum derivative of LV pressure (dp/dtmax), the ratio of dp/dtmax to pressure at dp/dtmax [(dp/dtmax)/(P@dp/dtmax)] and Preload Recruitable Stroke Work (PRSW) calculated as the slope of the linear correlation between Stroke Work (SW) and End-Diastolic Volume (EDV). Diastolic indices consist of minimum dp/dt (dp/dtmin) and time constant (tau) representing the isovolumic relaxation time.

2.2.3. Histology and electron microscopy

For histological assessment of heart morphology Tg-MUT and Tg-WT mice of various ages (~4, ~8 and ~15 month-old) were used (5-6 mice per group). After sacrificing, the hearts were excised and immersed in 10% buffered formalin. Slides of whole hearts were prepared by American Histolabs Inc. The paraffin-embedded longitudinal

sections of the hearts stained with H&E (haematoxylin and eosin) and Masson's trichrome were examined for overall morphology and fibrosis using a Dialux20 microscope, ×40/0.65 NA (numerical aperture) Leitz Wetzlar objective and an AxioCam HRc camera (Zeiss).

Transmission EM imaging was conducted in the EM Core Facility at the University of Miami Miller School of Medicine. After euthanasia, the hearts of ~6 month old Tg-WT or Tg-MUT mice were excised and immediately perfused in a solution containing 4% paraformaldehyde, 1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4 overnight at 4°C. The hearts were then fixed with 2% glutaraldehyde and subsequently separated into the left ventricle and septum. Left ventricles from Tg-MUT and Tg-WT mice were sectioned longitudinally for imaging. The slides were examined using a Philips CM-10 electron microscope with 10,500x magnification.

2.3. Studies on skinned papillary muscle strips

The papillary muscles of the left ventricles from 5-6 month-old transgenic mice were isolated**,** dissected into muscle bundles in the buffer containing pCa 8 solution (10^{-8} M [Ca^{2+}], 1 mM free [Mg^{2+}] (total MgPr (propionate) =3.88 mM), 7 mM EGTA, 2.5 mM [Mg-ATP^{2-}], 20 mM MOPS, pH 7.0, 15 mM creatine phosphate and 15 units/ml of phosphocreatine kinase, ionic strength =150 mM adjusted with KPr), 15% glycerol and 30 mM BDM. Muscle bundles were then skinned in 50% pCa 8 solution and 50% glycerol containing 1% Triton X-100 for 24hr at 4ºC. Muscle bundles were then transferred to the same solution without Triton X-100 and stored at -20°C for experiments within ~5 days [2].

2.3.1. Steady-state force development

Small muscle strips of approximately 1.4 mm in length and 100 μm in diameter were isolated from a batch of glycerinated skinned mouse papillary muscle bundles and attached by tweezer clips to a force transducer. The strips were placed in a 1 ml cuvette and freshly skinned in 1% Triton X-100 dissolved in pCa 8 buffer for 30 min to remove the remaining membrane and extracellular matrix proteins. Then they were rinsed 3 times x 5 min in pCa 8 buffer and their length was adjusted to remove the slack. This procedure resulted in sarcomere length ~ 2.1 μm as judged by the first order optical diffraction pattern as described in [11, 12]. Then the strips were tested for maximal steady state force development in pCa 4 solution (composition is the same as pCa 8 buffer except the [Ca^{2+}] =10^{-4} M). Maximal tension readings (in pCa 4) were taken before and after the force-pCa curve, averaged and expressed in kN/m^2. The cross sectional area of the muscle strip was assumed to be circular.

2.3.2. The Ca^{2+} dependence of force development

After the initial steady state force was determined, muscle strips were relaxed in pCa 8 buffer and exposed to solutions of increasing Ca^{2+} concentrations from pCa 8 to pCa 4 [13]. The level of force was measured in each "pCa" solution. Data were analyzed using the Hill equation [14], where "$[Ca^{2+}]_{50}$ or pCa_{50}" is the free Ca^{2+} concentration which produces 50% of the maximal force and n_H is the Hill coefficient. The pCa_{50} represents the measure of Ca^{2+} sensitivity of force and the n_H is the measure of myofilament cooperativity.

2.3.3. Muscle relaxation kinetics

To monitor the muscle relaxation rate, a photolabile derivative of BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid), diazo-2, was used. Diazo-2 is able to rapidly chelate Ca^{2+} upon photolysis converting from a low affinity (K_d=2.2μmol/l) to a high affinity (K_d=0.073 μmol/l) for Ca^{2+}. The experiment was performed as

described in Kazmierczak *et al.*[5]. Briefly, after testing the steady state force, papillary muscle strips were immersed in the solution of 2 mM diazo-2, 0.5 mM $CaCl_2$, 60 mM TES, pH 7.0, 5 mM MgATP, 1 mM [Mg^{2+}] and 10 mM creatine phosphate along with 15U/ml creatine phospho kinase, ionic strength = 200 mM. At the ratio of total added Ca^{2+} to diazo-2 given above, the resulting average initial force was around 80% of the maximum (determined in pCa 4 solution). When the force level reached equilibrium, the strips were exposed to a UV flash from a Xenon lamp. The photolysis-induced relaxation isotherms were fitted to a double exponential decay equation (Sigma Plot 11.0) yielding the relaxation rates in (s^{-1}) for Tg-MUT vs. Tg-WT muscles.

2.3.4. Passive force measurements

The measurement of passive force (in pCa 8 solution) in response to muscle stretch was performed as described in Kazmierczak *et al.*[5]. The strip was first released and stretched until it began generating tension. This point was set as zero for both the passive force and starting length of the muscle strip. Then, the strip was stretched by 10% of its length x 4 consecutive times, and the passive force per cross-section of muscle (in kN/m^2) was determined.

2.4. Fluorescence polarization anisotropy study

All chemicals were from Sigma-Aldrich (St Louis, MO). Cross-bridges were labeled with SeTau-647-mono-maleimide dye (SETA BioMedicals, Urbana, IL). Composition of solutions used in kinetic experiments was described in Midde *et al.* [15]. Myofibrils were prepared from the left ventricles of ~6 month old Tg-Lys104Glu and Tg-WT mice as described earlier [16, 17]. Briefly, the ventricles were washed 3 times with ice-cold EDTA-rigor solution (50 mM KCl, 10 mM Tris-HCl pH 7.5, 5 mM EDTA) for 30 min in order to remove ATP present in the glycerinating solution without causing contraction. They were then washed thoroughly in rigor solution (50 mM KCl, 10 mM Tris-HCl pH 7.5, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$) and homogenized with a Cole-Palmer LabGen 125 homogenizer for 10 s followed by further 10 s homogenization after a cool down period of 30 s.

Chicken recombinant ELC containing a single cysteine residue (Cys178) was expressed and purified as described previously [15]. The ELC was labeled at position 178 with a 5 molar excess of SeTau overnight on ice. After initial purification by dialysis against 50 mM KCl, 10 mM phosphate buffer pH 7.0, labeled ELC was passed through a Sephadex G50 LP column to eliminate free dye. Myofibrils from Tg-MUT and Tg-WT mice were immobilized without affecting their ATPase by cross-linking with a water-soluble cross-linker 1-ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide (EDC) as described earlier [18-21]. Myofibrils at the concentration of 1 mg/ml were incubated with 20 mM EDC in Ca-rigor solution for 20 min at room temperature. The reaction was stopped by adding 20 mM DTT. 1 mg/ml of freshly prepared myofibrils were exchanged with 10 nM SeTau-ELC in an exchange solution [22] under very mild conditions for 20 min at 30°C [23]. As a result, the native myofibrillar ELC was exchanged with 10 nM SeTau-ELC. Images were acquired on a PicoQuant Micro Time 200 confocal lifetime microscope. The sample was excited with a 640 nm pulsed laser and observed through a LP 650 nm filter.

2.5. Actin-activated Tg-mouse myosin ATPase activity assays

Myosins isolated from the hearts of 6-11 months old Tg-mice were purified as described previously [24, 25]. Rabbit skeletal F-actin was prepared according to Kazmierczak *et al.* [25]. Actin-activated myosin ATPase activity was measured as a function of actin concentration and the data analyzed as described in detail in [25]. Briefly, 0.5 μM myosin dissolved in 0.4 M KCl (in monomeric form) was added to the 96-well microplate containing increasing

concentrations of rabbit skeletal F-actin (in μM): 0.1, 0.5, 3, 5, 7.5, 10, 15. The assay was performed in a 120 μl reaction volume in a buffer consisting of 25 mM imidazole, pH 7.0, 4 mM $MgCl_2$, 1 mM EGTA, and 1 mM DTT. The final KCl concentration was 107 mM. Protein mixtures were first incubated on ice for 10 min and then for another 10 min at 30°C. The reactions (run in triplicate) were initiated with the addition of 2.5 mM ATP with mixing in a Jitterbug incubator shaker (Boekel), allowed to proceed for 15 min at 30°C and then terminated by the addition of 4% trichloroacetic acid. Precipitated proteins were cleared by centrifugation and the inorganic phosphate was determined using the Fiske Subbarow method as described in [4]. Data were analyzed using the Michaelis–Menten equation yielding the V_{max} and K_m parameters [26].

2.6. Frictional loading *in vitro* motility assays

To assess the functionality of Tg mouse purified cardiac myosins, a frictionally-loaded *in vitro* motility assay was performed using Tg-MUT vs. Tg-WT myosin preparations. *In vitro* motility assays were performed as previously described [27] with some modifications. Before motility experiments, actin was mixed with myosin in an approximately equal molar concentration and incubated in the absence of ATP for 5 min at room temperature. Then ATP was added to 1.5 mM and the mixture incubated for 60-90 s to allow the "active" heads to release the actin. Inactive actomyosin complexes were removed by centrifugation at ~135,000 g x 25 min (30 psi Airfuge; Beckman-Coulter, Inc.). The concentration of myosin in the resulting supernatant was determined using a Bradford Assay (Bio-Rad, Hercules, CA) and adjusted to ~100 μg/ml with high salt buffer containing: 300 mM KCl, 25 mM Imidazole, 1 mM EGTA, 4 mM $MgCl_2$, 10 mM, DTT, pH 7.3.

To examine the impact of increased load on the motility of actin filaments the actin binding protein α-actinin was added, in varying amounts, to the diluted myosin. Sliding velocity of unregulated actin was calculated using a plugin for imageJ called wrMTrck (Jesper Søndergaard Pedersen: http://www.phage.dk/plugins/wrmtrck.html), which is an automated centroid-based filament-tracking program. For analysis, movies were generated at 5 frames per second and a threshold was applied to improve contrast. The wrMTrck algorithm identifies filaments by position and tracks the motion by determining the closest filament in successive images. Therefore a maximum velocity threshold was set to prevent tracks from switching between different filaments from one frame to the next. To remove background noise particles smaller than <0.5 μm were removed. Because of variation of filament centroid calculation between frames, filaments that moved less than one quarter pixel were considered stalled. Only filaments that moved greater than 75% of the total frames in a movie were quantified. Filaments from 4 preparations were pooled (average 5 movies per experiment, 3-4 experiments per α-actinin,0-6 μg/ml, while 7 and 8 μg/ml had 2 and 1 experiments, respectively) and were averaged to determine the relationship between velocity and frictional load applied by α-actinin.

2.7. Statistical analysis

All values are shown as means ±SEM (standard error of the mean) or SD (standard deviation). Statistically significant differences between two groups (WT and Lys104Glu) were determined using an unpaired Student's *t*-test (Sigma Plot 11; Systat Software, San Jose, CA), with significance defined as $P<0.05$. Comparisons between multiple groups were performed using ANOVA and/or ANOVA for repeated measures using IBM® SPSS® Statistics 21 software (Chicago, IL). Actin sliding velocity curves were fit using Prism 5 (GraphPad7825, La Jolla, CA 92037

USA) and the quality of the fit was determined based on the R^2 value derived from the fit. Significance between the curves was determined using the extra sum-of squares F-test.

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