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Effects of myosin heavy chain manipulation in experimental heart failure

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Abstract

The myosin heavy chain (MHC) isoforms, α - and β -MHC, are expressed in developmental- and chamber-specific patterns. Healthy human ventricle contains ~2-10% α -MHC and these levels are reduced even further in the failing ventricle. While down-regulation of α -MHC in failing myocardium is considered compensatory, we previously demonstrated that persistent transgenic (TG) α -MHC expression in the cardiomyocytes is cardioprotective in rabbits with tachycardia-induced cardiomyopathy (TIC). We sought to determine if this benefit extends to other types of experimental heart failure and focused on two models relevant to human heart failure: myocardial infarction (MI) and left ventricular pressure overload. TG and nontransgenic rabbits underwent either coronary artery ligation at 8 months or aortic banding at 10 days of age. The effects of α -MHC expression were assessed at molecular, histological and organ levels. In the MI experiments, we unexpectedly found modest functional advantages to α -MHC expression. In contrast, despite subtle benefits in TG rabbits subjected to aortic banding, cardiac function was minimally affected. We conclude that the benefits of persistent α -MHC expression depend upon the mechanism of heart failure. Importantly, in none of the scenarios studied did we find any detrimental effects associated with persistent α -MHC expression. Thus manipulation of MHC composition may be beneficial in certain types of heart failure and does not appear to compromise heart function in others. Future considerations of myosin isoform manipulation as a therapeutic strategy should consider the underlying etiology of cardiac dysfunction.

Keywords

myosin heavy chain; heart failure; transgenic rabbits; myocardial infarction; left ventricular pressure overload

1. Introduction

The cardiac ventricle contains two distinct myosin heavy chains (MHCs), α -MHC and β -MHC. The MHCs are expressed in a developmental stage- and species-dependent manner, assembling

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as an α -MHC homodimer or a β -MHC homodimer. Compared to β -MHC, α -MHC has higher ATPase activity and faster maximum velocity of shortening [1], but a lower tension-time integral [2,3]. These mechanical properties are inherent to the myosin molecule, since α -MHC in the *in vitro* motility assay generates faster actin filament velocities [1] but lower average force per myosin molecule when compared to β -MHC [4]. Single molecule studies in the laser trap suggest that differences in the kinetics of cross-bridge cycling for α -MHC and β -MHC are the major determinant of cardiac myosin's mechanical performance [5].

For many years, it was thought that healthy human ventricle expressed only β -MHC. However, a series of reports in 1997 demonstrated that in non-failing ventricles, as much as 30% of the total cardiac MHC RNA pool consisted of α -MHC [6] while α -MHC message levels were consistently down regulated in the failing heart [7]. Later studies confirmed that detectable amounts of α -MHC protein are present in the normal human ventricle, with down regulation of the protein during heart failure (HF) [8]. This is hypothesized to be a pro-survival strategy since the β -MHC isoform, with its lower ATPase activity, is a more economical motor. The functional effect of small amounts of α -MHC in a β -MHC ventricle is not known, but the reverse approach (i.e., small amounts of β -MHC in a α -MHC ventricle) has been performed in mice. In those experiments, replacement of the endogenous (100%) α -MHC with 12% β -MHC resulted in a 31% decrease in $dP/dt_{\max}$ and a 45% increase in $dP/dt_{\min}$. These data suggest that the relationship between contractility and relative isoform content may be non-linear, so small shifts may indeed have important functional consequences [9,10].

The existence of α -MHC in the human ventricle and its consistent down-regulation in HF highlight the importance of determining the functional effects of isoform modulation at the molecular, biochemical and whole organ levels. While the mouse remains the species of choice for the overwhelming majority of TG investigations, the rabbit offers an experimental model with significant advantages for investigation of the MHCs. Like human ventricle, the “slow” β -MHC isoform predominates in mature rabbits and calcium handling mechanisms are similar [11]. Accordingly, we generated TG rabbits with persistent expression of the full-length rabbit α -MHC cDNA driven by the rabbit β -MHC promoter [12]. Use of the β -MHC promoter produces high levels of expression in the ventricles of mature animals and ensures continued transgene expression under conditions of cardiac hypertrophy or failure, when native β -MHC is upregulated.

In our initial studies, we could find no detrimental effects from continued expression of α -MHC under basal conditions. To assess the effects of TG α -MHC expression in stressed myocardium, we studied a model of tachycardia-induced cardiomyopathy (TIC), where a 30 day stepwise increase in ventricular pacing rate consistently produced dilated cardiomyopathy. We found that TG expression of α -MHC was cardioprotective in TIC, with NTG rabbits showing more severely compromised cardiac function [12].

To begin to explore the broader possibilities of altered thick filament composition in the setting of myocardial stress and cardiac disease, we extended our investigations to two dissimilar models of myocardial dysfunction: myocardial infarction (MI) and left ventricular (LV) pressure overload. These models were chosen because of their relevance to clinical human HF, and an extensive body of literature exists detailing the similarities between the rabbit models and human myocardial dysfunction. For example, myocardial infarction in the rabbit produces systolic dysfunction, ventricular remodeling, β -adrenergic receptor (β -AR) down-regulation with functional β -AR uncoupling, increased expression of matrix metalloproteinases and ventricular arrhythmias, all features of human ischemic disease [13-15]. Aortic banding in rabbits is characterized by initial compensatory hypertrophy that shifts to dilation and failure, a transition associated with abnormal glucose uptake, impaired angiogenesis, and activation of signaling cascades evident in human LV pressure overload [16-18]. Taken together, these

two additional models provide insight into the generally applicability of MHC manipulation and the treatment of myocardial dysfunction.

2. Materials and methods

2.1. Myocardial infarction

All surgical procedures were performed under an IACUC-approved protocol in a AALAC certified vivarium. Using a line with ~45% α -MHC replacement [12], 8 month old TG and NTG littermates of mixed gender underwent coronary artery ligation or sham operation. The rabbits were intubated and anesthesia maintained with 2% isoflurane delivered via positive pressure ventilation. The epicardial surface was exposed via left thoracotomy. Using the nomenclature of Podesser et al [19], the marginalis sinistra was identified and encircled with 6-0 prolene suture at the midpoint between the left atrioventricular groove and the ventricular apex. For infarction rabbits, the suture was tied tightly around the coronary artery after two 1-min pre-ischemic periods that provided the dual benefits of visual inspection of the affected myocardium and improved operative survival. For sham rabbits, the suture was placed in the same location but there were no pre-ischemic periods and the suture was not tied. The incision was closed in layers, and the rabbits extubated when sufficiently awake. Buprenorphine was administered subcutaneously for immediate pain management and a 25 μ g fentanyl patch placed for 72 hours of post-operative pain control. The rabbits were monitored daily for signs of pain, infection and dehydration (e.g., poor skin turgor or lethargy) per our institutional standard. Most rabbits demonstrated decreased chow consumption in the 3 days following surgery, but none required supplemental fluids and all rabbits were eating and normally active by 72 hours post surgery.

Two experimental pathways were utilized, with one cohort of myocardial infarction rabbits followed post-surgery for 6 weeks, and a second cohort for 9 months. To assess cardiac function, serial echocardiograms were performed under isoflurane anesthesia 3 and 6 weeks after surgery, then monthly thereafter in the cohort designated for long-term study. Offline measurements and calculations were made by an investigator blinded to genotype and included LV dimensions, interventricular septum (IVS) and LV free wall (LVFW) thickness, shortening fraction (SF) and heart rate-corrected velocity of circumferential fiber shortening (VCF_c).

For invasive hemodynamic assessment, the rabbits were anesthetized with inhaled isoflurane and LV function assessed by echocardiography and Millar catheterization using sterile carotid cutdown as previously described [12]. To assess inotropic reserve, dobutamine (DOB) was infused at increasing doses (10 micrograms/kg/min, increasing every 10 mins) to a maximum dose of 30 mcg/kg/min (indicated DOB0, DOB10, DOB20 and DOB30), repeating the echocardiogram and Millar assessment at each dose. Upon removal of the arterial sheath, the right carotid artery was ligated, the incision closed in layers and the rabbits recovered in a pre-warmed islette until fully awake.

Seven to ten days after the Millar catheterization, the rabbits underwent cardiac MRI (cMRI) using a 3 Tesla Bruker MRI system. ECG-triggered steady state free precession sequences were obtained of the LV short axis in 2 mm slices. Post-processing of LV volume, stroke volume, mass and ejection fraction was performed using the Medis quantification system. After completion of the cMRI, the rabbits were euthanized with pentobarbital and the heart harvested. The entire LVFW and isolated infarcted region were quickly traced for subsequent planimetry using Image J software (NIH). Non-infarcted LVFW myocardium was snap frozen in liquid nitrogen and stored at -80 °C for RNA and protein analyses.

2.2. Aortic banding

Using an ~45% α -MHC replacement line, 10 day old TG and NTG rabbits were anesthetized with intramuscular injection containing a mixture of ketamine (40-50 mg/kg), xylazine (2 mg/kg) and buprenorphine (.025 mg/kg). The rabbits were positioned on a heated surface and given supplemental oxygen via mask. A left thoracotomy was performed and the descending aorta encircled with 2-0 silk suture which was tied down around a blunted 18g needle to provide uniform constriction. The suture left untied in designated sham animals. The incision was closed in layers, air was evacuated from the chest with an angiocatheter and syringe, and the animal allowed to recover in a heated isolette prior to returning to the nesting box. The rabbits were monitored daily for signs of pain, infection and dehydration per AAALAC-approved institutional standards.

We performed weekly echocardiograms until significant left ventricular systolic dysfunction was noted. Offline measurements were made as described above for the MI model. Once an animal was identified as having significant LV systolic dysfunction ($SF \leq 20\%$ or $VCFc \leq 1.0$ circ/sec), cardiac catheterization was performed under isoflurane anesthesia to assess LV peak systolic pressure (LVSP) and end diastolic pressure (LVEDP), $LV \pm dp/dt$, and band gradient (ascending aorta pressure minus descending aorta pressure). Following data acquisition, the rabbits were euthanized with pentobarbital and tissues snap frozen in liquid nitrogen. A subset of animals had tissue harvested and processed for paraffin sections. To provide age-matched controls, non-failing sham operated animals were randomly chosen for invasive hemodynamics and tissue harvest as above.

2.3. Molecular and histological analyses

Transcript levels of α -MHC, β -MHC, brain natriuretic peptide (BNP), sarcomeric endoplasmic reticulum ATPase 2a (SERCA) and phospholamban (PLN) were assessed by semi-quantitative real-time polymerase chain reaction as described [12]. Transgenic α -MHC replacement was determined by SDS-PAGE or Western blotting with BA-G5, an α -MHC specific monoclonal antibody (Abcam, USA). For SDS-PAGE, myofibrils were isolated from atrial and ventricular samples as described [12]. Samples were electrophoresed on 6.5% acrylamide gels with a 100:1 ratio of acrylamide to bis-acrylamide, stained with Sypro Ruby (Biorad, USA), and digitized with a Typhoon imaging system (Molecular Dynamics, USA). For Western blotting, 1 μ g total LVFW protein was electrophoresed on 6% acrylamide gels with a 19:1 ratio of acrylamide to bis-acrylamide then transferred overnight (30 mA at 4 °C) to a PVDF membrane. The membrane was blocked overnight at 4 °C in TBS/1% blocking solution, incubated with 1:500 dilution 1° antibody at room temperature for 1 hour, washed and subsequently incubated with a 1:2500 dilution of goat anti-mouse IgG-HRP conjugated 2° antibody at RT for 30 min. ECLplus (GE Healthcare Bio-Sciences, USA) was used for detection and the blots scanned with the Typhoon imaging system. For both SDS-PAGE and Western blots, Image J software (NIH) was used for MHC band quantification. For histology, 5 μ paraffin sections were stained with either trichrome or hematoxylin & eosin (H&E) as described [12] and photographed at 400 $\times$ magnification.

2.4. Statistical analyses

InStat3.1a (GraphPad Software, USA) was used for statistical analyses. For tests requiring comparisons among multiple experimental groups (e.g., echocardiography, cardiac catheterization, RNA quantification and Western blot) data were analyzed using ANOVA with Tukey-Kramer multiple comparisons test (parametric data) and Kruskal-Wallis with Dunn multiple comparisons test (non-parametric data). To compare the means of two groups unpaired two-tailed *t*-tests were used for parametric data and Mann-Whitney for nonparametric data. Statistical significance was set at $P \leq 0.05$. All results are reported as mean $\pm$ standard deviation.

3. Results

3.1. Myocardial infarction model

A total of 19, 8 month old rabbits (9 TG and 10 NTG) with significant LVFW infarcts (% infarct $32 \pm 3\%$ TG versus $31 \pm 4\%$ NTG, $p = 0.95$) survived for study. An additional 8 rabbits (4 TG and 4 NTG) underwent sham operations. All animals had an echocardiogram performed immediately preceding surgery, then three and six weeks post-surgery. The NTG rabbits were significantly heavier than the TG group ($4.7 \pm .6$ kg NTG versus $3.7 \pm .5$ kg TG, $p = .002$, likely due to a preponderance of females in the NTG group), so echocardiographic variables affected by body mass, including LV dimensions, wall thickness and mass were indexed to body weight. As previous studies have shown no gender-related differences in echocardiography parameters ([12] and unpublished data) and the number of rabbits relatively small, data were not analyzed for potential gender-related effects. The pre-surgery data from the TG and NTG shams were combined as, aside from aortic ejection time (ET) and VCFc, the differences between the two sham groups failed to reach statistical significance. We found subtle baseline differences between genotypes, with the TG rabbits having higher IVS, LVFW and LVEDD compared to NTG (Table 1). The ET was consistently shorter in TG animals, likely a manifestation of the faster crossbridge cycling rate inherent to the α -MHC [20]. Accordingly, VCFc, a preload independent measure of contractility with ET in the equation's denominator, was also increased.

Based on our previous results with the TIC model, we hypothesized that significant differences between TG and NTG would likely present early (i.e., 6 weeks following coronary artery ligation). However, we found no significant difference in LV morphometry, and functional parameters were not strikingly different aside from a significantly shorter ET and higher VCFc in the infarcted TG rabbits (Table 1). Additionally, we performed Millar catheterization and tissue harvest in all shams and a subset of infarcted animals (3 TG and 2 NTG) at 6 weeks post-surgery and found no significant differences between TG and NTG (data not shown).

We posited that perhaps 6 weeks was too early in the compensatory process for MHC alteration to exert an affect on ventricular structure and function, and thus elected to follow the remaining animals (8 TG and 6 NTG) with serial echocardiograms until 9 months post-surgery. Even 9 months after coronary artery ligation, the effects of genotype on cardiac function remained subtle. Serial echocardiography over the follow-up period demonstrated persistent differences in ET and VCFc (Fig. 1A and Table 1) and the TG rabbits had mildly elevated LV dimensions 9 months post-infarct (Table 1). Cardiac catheterization 9 months after coronary ligation showed a slight elevation in peak LV systolic pressure at baseline in TG rabbits (75 ± 11 mmHg TG versus 63 ± 8 mmHg NTG, $p = 0.02$). However, the NTG rabbits were able to achieve identical peak LV pressure at DOB10, and there were no significant differences between genotypes at the higher infusion rates of DOB20 and DOB30. Interestingly, we noted mildly elevated LV end diastolic pressure (LVEDP) in TG rabbits compared to NTG at all points in the Millar protocol, with LVEDP of 12 ± 3 mmHg in the TG rabbits versus 9 ± 2 mmHg in the NTG animals ($p = 0.02$). There was no significant difference in $\pm dP/dt$ between TG and NTG rabbits, with both groups showing comparable measurements at baseline and in response to DOB infusion (Fig. 1B).

As was the case with the serial echocardiograms, in the final echo studies the only difference between TG and NTG MI rabbits was in the VCFc, which was slightly increased at baseline in the TG rabbits ($1.70 \pm .27$ circ/s TG versus $1.42 \pm .15$ circ/s NTG, $p = 0.03$). The disparity between genotypes was most apparent at DOB10, when the VCFc in the TG rabbits increased to $2.25 \pm .18$ circ/s TG versus $1.64 \pm .21$ circ/s NTG ($p = 0.0001$). In TG rabbits, escalating DOB above $10 \mu\text{g/kg/min}$ did not result in any further increase in VCFc. In the NTG group, the VCFc increased slightly between DOB0 and DOB10, with the greatest increase occurring

between DOB10 and DOB20 (from $1.64 \pm .21$ circ/sec to $2.01 \pm .20$ circ/sec). Since there was no significant difference between genotypes in heart rate or SF at any DOB dose (data not shown), the observed VCFc differences between TG and NTG can be accounted for by the shorter ET in the TG rabbits.

Seven to ten days after cardiac catheterization, rabbits underwent cMRI as a terminal procedure (Table 2). The advantages of cMRI include the evaluation of those portions of the LV not assessed by echocardiography (i.e., the infarcted LV lying below the mid-cavity papillary muscles), quantification of ventricular volumes and cardiac output and assessment of regional wall motion abnormalities. While the LV end-diastolic volume, LV end-systolic volume and ejection fraction were not significantly different, the infarcted TG rabbits had a significantly higher cardiac index compared to infarcted NTG (26 ± 8 mL/min/kg TG versus 16 ± 5 mL/kg/min NTG, $p = 0.02$).

Using semi-quantitative PCR, we compared the relative LVFW expression of α -MHC, β -MHC, BNP, SERCA and PLN among 4 groups: NTG and TG sham (6 weeks post-surgery) and NTG and TG MI (9 months post-surgery), normalizing to GAPDH and expressed as X-fold NTG sham 6 weeks post-surgery (Fig. 2A). Comparable results were obtained with samples derived from MI rabbits 6 weeks post-surgery (data not shown). The only significant change noted was in α -MHC expression, which was increased both at baseline and after MI in the TG rabbits (sham and MI TG 4.3-fold and 5.7-fold compared to 6 week post-surgery NTG sham rabbits, $p \leq 0.002$ for both). The increase in α -MHC expression was confirmed using SDS-PAGE (data not shown), with α -MHC comprising $42 \pm 8\%$ of total MHC in the LVFW of TG sham rabbits, increasing to $63 \pm 5\%$ in TG MI rabbits ($p = 0.03$). In contrast, α -MHC composition of LVFW samples from NTG sham rabbits was $4 \pm 3\%$ and $4 \pm 1\%$ in NTG MI rabbits ($p = \text{NS}$).

Light microscopy was performed on samples of viable LVFW 9 months post-surgery to assess for changes in ventricular architecture that might be associated with genotype. We found no striking differences between TG and NTG MI hearts using H&E and trichrome staining (Fig. 2B).

3.2. Aortic banding model

The different outcomes noted between myocardial dysfunction models (i.e., a conclusive benefit from persistent α -MHC expression in TIC versus a subtle, if any benefit in MI) suggested that the effects of α -MHC replacement on ventricular function and remodeling might be dependent upon the mode of cardiac stress. Accordingly, we created LV pressure overload in 10 day old rabbits by placing an initially non-obstructive suture around the descending aorta. We chose 10 day old rabbits for surgery as their size at that age enhanced surgical survival and yet was early enough to take advantage of the rapid increase in size that rabbits experience in the first 6 weeks of life. Compared to transverse aortic constriction commonly performed in mature mice, in neonatal aortic banding the suture becomes increasingly more obstructive with growth, resulting in escalating LV pressure overload with time. This model allows for compensatory ventricular remodeling stimulated by progressive obstruction, comparable to that experienced by humans with conditions such as coarctation of the aorta or aortic stenosis.

Surgeries were performed on day-of-life (DOL) 10 with tissue taken at the time of operation for subsequent genotyping. Only three rabbits required supplemental (intraperitoneal) fluids in the first 24 hours post-surgery, with complete recovery by 72 hours. Our cohort consisted of 61 banded animals (16 TG and 45 NTG, designated TG-B and NTG-B, respectively) with an additional 17 rabbits randomly selected for sham operations (8 TG and 9NTG, TG-S and NTG-S, respectively). Since we ultimately found no detectable anatomic or functional differences between TG-S and NTG-S at the ages studied in this model, TG-S and NTG-S echocardiography and catheterization data were combined for comparison to banded rabbits.

The 3 groups had similar weights on DOL 10 (Table 3) and comparable weight gains over the course of the study (data not shown). Serial echocardiography demonstrated a highly reproducible phenotype with LV hypertrophy and fairly well preserved systolic function persisting in both TG-B and NTG-B animals up to three weeks post-surgery (Fig. 3A). Thereafter, LV dimensions increased as systolic performance worsened. While the banded rabbits were clearly compromised compared to sham operated controls, there were no significant differences between TG-B and NTG-B in echocardiographic parameters, including LV systolic and diastolic dimensions, SF and septal and LVFW thickness. As we observed previously [12], at most time points the TG-B rabbits had a significantly shorter LV ET ($p \leq 0.02$) compared to NTG-B (data not shown), but this did not translate into a consistently higher VCFc in the TG-B cohort.

Rabbits were removed from the cohort for terminal invasive hemodynamic assessment and tissue harvest once the SF dropped to $\leq 20\%$ or VCFc ≤ 1.0 circ/sec. Sham operated animals were randomly selected for cardiac catheterization each week until the last of the banded animals went into failure, at which time all remaining control rabbits underwent cardiac catheterization and tissue harvest. Due to unexpected death in some banded animals during the course of the study, we collected complete invasive hemodynamic data on 38 NTG-B, 10 TG-B and 12 sham controls. Time to failure was not different between genotypes (Fig. 3B), nor was the peak pressure gradient between ascending and abdominal aorta (Table 3). Banded rabbits of both genotypes had significantly higher LVSP ($p < 0.05$ NTG-B versus sham and $p < 0.001$ TG-B versus sham) and higher LVEDP ($p < 0.001$ NTG-B versus sham and $p < 0.05$ TG-B versus sham). Between the banded groups, the TG-B LVSP was significantly higher (120 ± 19 mmHg TG-B versus 103 ± 119 mmHg NTG-B, $p < 0.05$), but there was no genotype-dependent difference in LVEDP. Both NTG-B and TG-B showed significantly depressed $dp/dt_{\max}$ compared to shams ($p < 0.001$ NTG-B versus sham and $p < 0.01$ for TG-B versus sham), but the difference between NTG-B and TG-B rabbits was not significant. Interestingly there was a significant difference in $dp/dt_{\min}$ with NTG-B showing impairment in this measure of diastolic performance compared to both TG-B and sham (-2394 ± 1000 mmHg/sec for NTG-B, -3229 ± 747 mmHg/sec for TG-B and 3171 ± 717 mmHg/sec for sham, $p < 0.05$ NTG-B versus both sham and TG-B, $p = \text{NS}$ for TG-B versus sham).

At the time of tissue harvest, pericardial and pleural effusions, left atrial enlargement and ascites were common observations in both TG-B and NTG-B rabbits. However, we found no striking differences by light microscopy in the histological appearance of TG-B and NTG-B hearts with qualitatively equivalent myocyte size and interstitial fibrosis (Fig. 4A).

Molecular markers of hypertrophy were assessed by semi-quantitative real time PCR. To determine relative expression of α - and β -MHC at the time of surgery, left ventricular RNA was isolated from 10 day old TG and NTG rabbits and used in real-time experiments. At this age, the TG rabbits demonstrated 3.9-fold overexpression of α -MHC compared to NTG, with both genotypes showing equivalent expression of β -MHC (Fig. 4B). Transcript levels of ANF, SERCA2a and PLN were not significantly different between genotypes, indicating that at the time of operation no differences existed in the molecular phenotype aside from the expected upregulation of α -MHC in TG rabbits.

Real-time PCR analyses were likewise performed on RNA isolated from left ventricular tissue harvested at the time of terminal study (Fig. 4C). The levels of α -MHC, β -MHC, SERCA2a, PLN and brain natriuretic peptide (BNP) were normalized to GAPDH expression. Comparisons were made among TG-B, NTG-B, TG-S and NTG-S using one-way ANOVA and the Tukey multiple comparisons post test. Values are expressed as X-fold relative to NTG-S samples. The only difference between the two sham genotypes was in α -MHC expression, with 3.4-fold overexpression in TG-S compared to NTG-S ($P \leq 0.05$).

As expected, the greatest α -MHC levels were found in TG-B rabbits, with 4.8-fold overexpression compared to NTG-S ($P \leq 0.001$), while in TG-S α -MHC was upregulated 3.4-fold. NTG-B rabbits had essentially non-detectable levels of α -MHC at 0.04-fold compared to NTG-S ($P \leq 0.01$ versus NTG-S and ≤ 0.001 versus TG-B). We found no difference among the groups in β -MHC or PLN expression. SERCA2a was significantly downregulated in both TG-B and NTG-B rabbits compared to NTG-S ($P \leq 0.01$), but there was no genotype-dependent difference between the banded or sham groups. Not unexpectedly given the severity of cardiac dysfunction, BNP expression was markedly increased in both TG-B and NTG-B animals compared to NTG-S, but again no genotype dependent differences presented.

One explanation for the lack of a dramatic phenotypic difference between pressure-overloaded TG and NTG rabbits is that transgenic overexpression of α -MHC at the RNA level may not directly translate into protein accumulation in young rabbits during developmental stage modulation of endogenous MHC isoforms. While we have consistently found excellent correlation between MHC transcript and protein levels in older rabbits, the MHC isoform composition of the ventricles varies significantly in the first few months of post-natal life as the fetal gene program is down-regulated [21]. To quantify accumulation of α -MHC we used an isoform-specific antibody for Western blot analysis of LV protein extracts from 6 week old NTG-B, TG-B, NTG-S and TG-S rabbits (Fig. 4D). NTG-S LA samples were used as a representation of 100% α -MHC. Unexpectedly, we found relatively high levels of α -MHC accumulation in 6 week old NTG-S animals ($35 \pm 1\%$), exceeding the typical 5 – 10% α -MHC in mature rabbits and in contrast to the relatively low α -MHC message level determined by real-time PCR. The 6 week old TG-S rabbits (with 3.4-fold α -MHC RNA) message showed $45 \pm 9\%$ LV α -MHC accumulation compared to $62 \pm 6\%$ in TG-B rabbits (4.9-fold α -MHC RNA). NTG-B rabbits had significantly less but generally still detectable α -MHC ($8 \pm 6\%$), even though the α -MHC message level as determined by real-time PCR was barely detectable. Taken together, the lack of perfect congruence between message and protein levels in 6 week old NTG groups is likely due the persistence of α -MHC protein synthesized earlier in life and not yet replaced by β -MHC.

4. Discussion

The concept of MHC diversity in the cardiac sarcomere has been appreciated for over 30 years [22], but the role such diversity plays in tuning cardiac sarcomere function remains ambiguous. To study the functional effects of α -MHC in a predominantly β -MHC heart (the scenario present in human ventricle), we created TG rabbits in which expression of the full length rabbit α -MHC cDNA was driven by the rabbit β -MHC promoter, then studied these animals under conditions that resulted in cardiac disease. Our initial studies compared TG and NTG cardiac function in tachycardia-induced cardiomyopathy and demonstrated that TG rabbits fared better than NTG, with superior systolic function and ventricular morphology [12]. However, if MHC isoform manipulation is to be used as an effective therapy, its safety and efficacy in other heart disease models should be determined. To explore the general feasibility of MHC manipulation in systolic dysfunction, we studied two other models of cardiac stress: MI and LV pressure overload, both experimental conditions with direct correlates in human cardiac disease. In both models we found subtle benefits with persistent α -MHC expression but not a functional rescue. Rather surprisingly, in neither model did we find evidence of compromise.

Coronary artery ligation resulted in ~32% LVFW infarction and depressed LVEF in both TG and NTG rabbits. Assessment of cardiac structure and function revealed modestly increased LV dimensions and higher LVEDP in the TG MI rabbits. Although the cardiac index and VCFc were slightly better in TG rabbits, other measures of cardiac function such as dP/dt were not affected. In these experiments, the lack of a consequential phenotype cannot be attributed to

failure to accumulate significant amounts of α -MHC in the ventricles as α -MHC replacement in TG MI rabbits achieved levels comparable to those of the TG rabbits in the TIC studies.

Neonatal aortic banding resulted in a predictable sequence of compensatory hypertrophy with preserved systolic function transitioning to ventricular dilation and severely depressed function. Similar to our other models of myocardial dysfunction, we achieved LV α -MHC replacement of ~60% in the failing TG rabbits but found no genotype-dependent difference in time to failure or degree of ventricular remodeling. As before, there were no differences in expression of molecular markers of hypertrophy aside from α -MHC accumulation in the TG cohort. Interestingly, while NTG-B rabbits had low levels of α -MHC message and α -MHC protein, Western blotting of NTG-S samples demonstrated more α -MHC than would be predicted given the low level of α -MHC message. This may be due to the relatively long half life of MHC *in vivo* [23,24].

This study is limited by the lack of overt HF in the MI model. We found that while we could produce a larger visualized LVFW infarct during surgery, left coronary artery ligation at a more proximal location was accompanied by higher rates of intraoperative and perioperative death. This factor also contributed to the relatively low numbers of rabbits in the MI studies due to experimental animal loss. Nevertheless, the decreased LVEF documented by cMRI and alterations in MHC isoform ratios confirm that significant cardiac dysfunction was achieved in those experiments. The LV pressure overload model results are somewhat complicated by the presence of significant α -MHC protein in young NTG rabbits, which might result in a relative resistance to failure in this group. Placing a tight band in older rabbits would clear this molecular hurdle, but induce an acute and thus non-physiological increase in aortic pressure, a rare circumstance in clinical practice and thus not representative of typical human LV pressure overload and subsequent compensatory responses. Finally, interpretation of functional parameters are problematic when variables such as preload and afterload cannot be completely controlled as is the case in intact preparations. Thus, some caution must be applied to the interpretation of differences in measures such as LV ET, $\pm dP/dt$ and even blood pressure as load is not exactly controlled or even precisely measurable in an intact animal.

In summary, TG α -MHC expression was conclusively beneficial in TIC, but essentially neutral in MI and LV pressure overload. The advantages of α -MHC in TIC (with obligatory elevated heart rate) might be explained by the inherent physical properties of α -MHC [20]. For example, LV muscle strips from non-stressed TG rabbits with 40% α -MHC had a higher velocity of loaded shortening, a characteristic that would support blood ejection over a shorter period of time as would be the case in experimental tachycardia. Similarly, the significantly shorter t_{on} (reflecting a faster off-rate of the acto-myosin crossbridge) and lower elastic and viscous moduli (lower sarcomeric stiffness and drag) would favor sarcomeric shortening and relengthening and thus prove beneficial in a tachycardic state.

We originally hypothesized that TG MI rabbits would fare poorly compared to NTG MI, so the conserved cardiac function in TG rabbits subjected to the procedure was surprising to us. Our prediction was based on the superior economy of contraction present in β -MHC and the general understanding of failing myocardium as “energy starved [25-27].” Unexpectedly, we did not detect any detrimental effects related to persistent expression of α -MHC in the TG rabbits. LV pressure overload and TIC resulted in severe cardiac enlargement, depressed ventricular function and florid evidence of HF in both NTG and TG animals. In these latter cases, the rabbits with α -MHC as the predominant motor performed at least as well or better than those with β -MHC, providing evidence for rejecting the hypothesis that the down-regulation of α -MHC in HF is a required pro-survival compensatory mechanism.

Taken together, our data suggest that persistent expression of α -MHC may be beneficial in certain clinical circumstances and, importantly, is unlikely to be detrimental. Therapeutic strategies aimed at enhancing crossbridge cycling kinetics should possess a favorable safety profile while providing benefit to at least a subset of cardiac patients. Taking this concept a step further, the dissimilar experimental responses to MHC isoform manipulation emphasize that while the end manifestations of HF may be phenotypically identical (i.e., cardiac enlargement with depressed function), the stressors preceding decompensation are different. Thus, while contemporary management of HF commonly includes β -AR blockade, afterload reduction, diuresis and Na^+/K^+ ATPase pump inhibition, future improvement in HF morbidity and mortality may derive from the development of etiology-specific therapies targeting specific points in the HF pathway unique to the mode of cardiac stress.

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References

1. VanBuren P, Harris DE, Alpert NR, Warshaw DM. Cardiac V1 and V3 myosins differ in their hydrolytic and mechanical activities in vitro. *Circ Res* 1995;77:439–44. [PubMed: 7614728]
2. Alpert NR, Mulieri LA. Determinants of energy utilization in the activated myocardium. *Fed Proc* 1986;45:2597–600. [PubMed: 2944770]
3. Alpert NR, Mulieri LA. Functional consequences of altered cardiac myosin isoenzymes. *Med Sci Sports Exerc* 1986;18:309–13. [PubMed: 2941668]
4. Harris DE, Work SS, Wright RK, Alpert NR, Warshaw DM. Smooth, cardiac and skeletal muscle myosin force and motion generation assessed by cross-bridge mechanical interactions in vitro. *J Muscle Res Cell Motil* 1994;15:11–9. [PubMed: 8182105]
5. Sugiura S, Yamashita H. Functional characterization of cardiac myosin isoforms. *Jpn J Physiol* 1998;48:173–9. [PubMed: 9689146]
6. Lowes BD, Minobe W, Abraham WT, Rizeq MN, Bohlmeier TJ, Quaife RA, et al. Changes in gene expression in the intact human heart. Downregulation of alpha-myosin heavy chain in hypertrophied, failing ventricular myocardium. *J Clin Invest* 1997;100:2315–24. [PubMed: 9410910]
7. Nakao K, Minobe W, Roden R, Bristow MR, Leinwand LA. Myosin heavy chain gene expression in human heart failure. *J Clin Invest* 1997;100:2362–70. [PubMed: 9410916]
8. Miyata S, Minobe W, Bristow MR, Leinwand LA. Myosin heavy chain isoform expression in the failing and nonfailing human heart. *Circ Res* 2000;86:386–90. [PubMed: 10700442]
9. Herron TJ, McDonald KS. Small amounts of alpha-myosin heavy chain isoform expression significantly increase power output of rat cardiac myocyte fragments. *Circ Res* 2002;90:1150–2. [PubMed: 12065316]
10. Tardiff JC, Hewett TE, Factor SM, Vikstrom KL, Robbins J, Leinwand LA. Expression of the beta (slow)-isoform of MHC in the adult mouse heart causes dominant-negative functional effects. *Am J Physiol Heart Circ Physiol* 2000;278:H412–9. [PubMed: 10666070]
11. Bers DM. Calcium fluxes involved in control of cardiac myocyte contraction. *Circ Res* 2000;87:275–81. [PubMed: 10948060]
12. James J, Martin L, Krenz M, Quatman C, Jones F, Klevitsky R, et al. Forced expression of alpha-myosin heavy chain in the rabbit ventricle results in cardioprotection under cardiomyopathic conditions. *Circulation* 2005;111:2339–46. [PubMed: 15867177]
13. Burton FL, McPhaden AR, Cobbe SM. Ventricular fibrillation threshold and local dispersion of refractoriness in isolated rabbit hearts with left ventricular dysfunction. *Basic Res Cardiol* 2000;95:359–67. [PubMed: 11099162]

14. Lindsey ML, Gannon J, Aikawa M, Schoen FJ, Rabkin E, Lopresti-Morrow L, et al. Selective matrix metalloproteinase inhibition reduces left ventricular remodeling but does not inhibit angiogenesis after myocardial infarction. *Circulation* 2002;105:753–8. [PubMed: 11839633]
15. White DC, Hata JA, Shah AS, Glower DD, Lefkowitz RJ, Koch WJ. Preservation of myocardial beta-adrenergic receptor signaling delays the development of heart failure after myocardial infarction. *Proc Natl Acad Sci U S A* 2000;97:5428–33. [PubMed: 10779554]
16. Friehs I, Cao-Danh H, Nathan M, McGowan FX, del Nido PJ. Impaired insulin-signaling in hypertrophied hearts contributes to ischemic injury. *Biochem Biophys Res Commun* 2005;331:15–22. [PubMed: 15845351]
17. Friehs I, Moran AM, Stamm C, Choi YH, Cowan DB, McGowan FX, et al. Promoting angiogenesis protects severely hypertrophied hearts from ischemic injury. *Ann Thorac Surg* 2004;77:2004–10. discussion 2011. [PubMed: 15172254]
18. Stamm C, Friehs I, Cowan DB, Moran AM, Cao-Danh H, Duebener LF, et al. Inhibition of tumor necrosis factor-alpha improves postischemic recovery of hypertrophied hearts. *Circulation* 2001;104:1350–5. [PubMed: 11568081]
19. Podesser B, Wollenek G, Seitelberger R, Siegel H, Wolner E, Firtas W, et al. Epicardial branches of the coronary arteries and their distribution in the rabbit heart: the rabbit heart as a model of regional ischemia. *Anat Rec* 1997;247:521–7. [PubMed: 9096792]
20. Suzuki T, Palmer B, James J, Wang Y, Chen Z, VanBuren P, et al. Effects of cardiac myosin isoform variation on myofilament function and cross-bridge kinetics in transgenic rabbits. In press.
21. James J, Sanbe A, Yager K, Martin L, Klevitsky R, Robbins J. Genetic manipulation of the rabbit heart via transgenesis. *Circulation* 2000;101:1715–21. [PubMed: 10758055]
22. Hoh JF, McGrath PA, Hale PT. Electrophoretic analysis of multiple forms of rat cardiac myosin: effects of hypophysectomy and thyroxine replacement. *J Mol Cell Cardiol* 1978;10:1053–76. [PubMed: 722801]
23. Martin AF, Rabinowitz M, Blough R, Prior G, Zak R. Measurements of half-life of rat cardiac myosin heavy chain with leucyl-tRNA used as precursor pool. *J Biol Chem* 1977;252:3422–9. [PubMed: 863888]
24. Morkin E, Kimata S, Skillman JJ. Myosin synthesis and degradation during development of cardiac hypertrophy in the rabbit. *Circ Res* 1972;30:690–702. [PubMed: 4260249]
25. Hayashi Y, Takeuchi M, Takaoka H, Hata K, Mori M, Yokoyama M. Alteration in energetics in patients with left ventricular dysfunction after myocardial infarction: increased oxygen cost of contractility. *Circulation* 1996;93:932–9. [PubMed: 8598084]
26. Katz AM. Cardiomyopathy of overload. A major determinant of prognosis in congestive heart failure. *N Engl J Med* 1990;322:100–10. [PubMed: 2403651]
27. Ingwall JS, Weiss RG. Is the failing heart energy starved? On using chemical energy to support cardiac function. *Circ Res* 2004;95:135–45. [PubMed: 15271865]

Fig 1.

Left ventricular functional parameters following myocardial infarction. (A) Assessment of post-infarction left ventricular function by serial echocardiography, including shortening fraction (top), aortic valve ejection time (middle) and VCFc (bottom). (B) Invasive hemodynamics 9 months after myocardial infarction. Measurements were made under anesthesia prior to dobutamine infusion and repeated at increasing doses of dobutamine to assess responsiveness to β -adrenergic stimulation. Left ventricular systolic and end-diastolic pressures (top), $dP/dt_{\max/\min}$ (middle) and VCFc (bottom). VCFc = velocity of circumferential shortening, $dP/dt_{\max}$ = maximal change in pressure over time (mmHg/sec), $dP/dt_{\min}$ = minimal change in pressure over time (mmHg/sec). ● TG, □ NTG, * $p \leq 0.05$, # $p \leq 0.01$, † $p \leq 0.001$; TG versus NTG.

Fig. 2.

Molecular and histological consequences of coronary artery ligation. (A) Semi-quantitative real-time PCR analysis comparing relative expression of the MHC isoforms and molecular markers of hypertrophy 9 months after coronary ligation (normalized to GAPDH expression and compared to sham animals 6 weeks after MI, with NTG sham expression set at 1.0). (B) H&E (upper panels) and trichrome (lower panels) staining of viable left ventricle 9 months following coronary artery ligation. All 400 $\times$ magnification; TG = transgenic, NTG = non-transgenic, $*p \leq 0.05$.

Fig. 3.

Left ventricular parameters and survival following aortic banding at 10 days of age. (A) Serial echocardiographic assessment of left ventricular size (top) and systolic performance (shortening fraction and VCFc, middle and bottom panels, respectively). Studies were performed weekly with rabbits removed for cardiac catheterization when the shortening fraction was $\leq 20\%$ or the VCFc ≤ 1.0 circ/sec. (B) Kaplan-Meier curve comparing survival of banded TG and NTG rabbits to sham operated animals. The loss of sham operated animals was intentional to allow evaluation of ventricular function in rabbits of various ages. ● TG, ■ NTG, △ sham. * $p \leq 0.05$, # $p \leq 0.01$, † $p \leq 0.001$, band versus sham.

Fig. 4.

Effects of aortic banding. (A) Representative histology of failing 6 week old TG (i, ii) and NTG (iii, iv) banded rabbits with H&E (i, iii) and trichrome (ii, iv) staining. All images 400× magnification. (B) Comparison of hypertrophy marker expression by semi-quantitative real time PCR in 10 day old TG and NTG rabbits. (C) Expression of molecular markers of hypertrophy at 6 weeks of age determined by semi-quantitative real time PCR comparing NTG-Sham to TG-Sham, TG-band and NTG-band. (D) α-MHC replacement. Upper panel representative Western blot comparing NTG left atrium (considered 100% α-MHC) to LV protein isolates from NTG-B, TG-B, NTG-S and TG-S rabbits. * $p \leq 0.05$, # $p \leq 0.01$, † $p \leq 0.001$

Table 1

Echocardiographic assessment of cardiac structure and function following coronary artery ligation.

	Pre-surgery			6 weeks post-surgery			9 months post-surgery		
	NTG N = 14	TG N = 13	NTG-MI N = 10	TG-MI N = 9	NTG-Sham N = 4	TG-Sham N = 4	NTG N = 8	TG N = 6	
IVS	.76 ± .16	.90 ± .17*	.74 ± .27	.99 ± .24	.85 ± .13	1.0 ± .13	.9 ± .2	1.1 ± .3	
LVFW	.76 ± .14	.97 ± .15 [#]	.75 ± .20	.97 ± .17	.90 ± .22	.98 ± .19	.8 ± .1	.9 ± .2	
LVEDD	4.0 ± .6	4.7 ± .6 [#]	4.0 ± .6	4.7 ± .6	4.1 ± .8	4.7 ± .4	3.1 ± .6	3.9 ± .5*	
LVESD	2.6 ± .4	2.8 ± .3	2.9 ± .4	3.3 ± .4	2.9 ± .5	3.3 ± .3	2.1 ± .4	2.7 ± .4*	
LVM	1.6 ± .3	1.8 ± .5	1.9 ± .6	2.2 ± .4	1.8 ± .5	2.1 ± .3	1.9 ± .4	2.0 ± .5	
SF	30 ± 3	32 ± 4	29 ± 3	30 ± 3	28 ± 5	29 ± 4	33 ± 4	32 ± 5	
ET	129 ± 13	100 ± 11 [†]	122 ± 7	101 ± 10 [#]	113 ± 14	103 ± 11	120 ± 14	98 ± 10 [#]	
VCFc	1.32 ± .14	1.71 ± .23 [†]	1.19 ± .18	1.51 ± .20*	1.30 ± .35	1.44 ± .18	1.42 ± .15	1.70 ± .27*	

IVS = interventricular septum (mm/kg), LVFW = left ventricular free wall (mm/kg), LVEDD = left ventricular end-diastolic dimension (mm/kg), LVESD = left ventricular end-systolic dimension (mm/kg), LVM = left ventricular mass (gm/kg), ET = ejection time (ms), VCFc = heart rate-corrected velocity of circumferential fiber shortening (cm/s).

* $p \leq 0.05$,

[#] $p \leq 0.01$,

[†] $p \leq .001$, TG or Sham versus NTG at the given time.

Table 2

Cardiac MRI measurements 9 months after coronary artery ligation

	TG (n=5)	NTG (n=8)	<i>p</i>
Gender	3M, 2F	2M, 6F	----
Wt (kg)	4.0 ± .7	5.2 ± .5	.003
LVEDV (ml/kg)	1.3 ± .4	.9 ± .3	.07
LVESV (ml/kg)	.7 ± .2	.6 ± .2	.38
LVEF (%)	42 ± 6	35 ± 7	.10
Cardiac index (ml/min/kg)	26 ± 8	16 ± 5	.02

Values are mean ± standard deviation. Wt = weight, LVEDV = left ventricular end diastolic volume, LVESV = left ventricular end systolic volume, LVEF = left ventricular ejection fraction.

Table 3

Aortic banding invasive hemodynamics

	NTG – B	TG – B	Sham
	N = 26	N = 9	N = 7
Weight on DOL 10 (gms)	158 ± 27	158 ± 25	176 ± 25
LVSP (mmHg)	103 ± 19	120 ± 19 [*]	80 ± 9 [#]
LVEDP (mmHg)	32 ± 8	29 ± 10	4 ± 2 [#]
Gradient (mmHg)	50 ± 17	57 ± 11	-9 ± 10 [#]
dP/dt _{max} (mmHg/sec)	1730 ± 601	2292 ± 648	3425 ± 841 [#]
dP/dt _{min} (mmHg/sec)	-2394 ± 1000	-3229 ± 747 [*]	-3171 ± 717

All values given as mean ± SD.

^{*}
 $p < 0.05$ TG-B versus NTG-B.

[#]
 $p < 0.05$ sham versus both TG-B and NTG-B.