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Cardiac transgenesis with the tetracycline transactivator changes myocardial function and gene expression

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McCloskey, Diana T., Lynne Turnbull, Philip M. Swigart, Alexander C. Zambon, Sally Turcato, Shuji Joho, William Grossman, Bruce R. Conklin, Paul C. Simpson, and Anthony J. Baker. Cardiac transgenesis with the tetracycline transactivator changes myocardial function and gene expression. *Physiol Genomics* 22: 118–126, 2005. First published March 29, 2005; 10.1152/physiolgenomics.00016.2005.—The cardiac-specific tetracycline-regulated gene expression system (tet-system) is a powerful tool using double-transgenic mice. The cardiac α -myosin heavy chain promoter (α MHC) drives lifetime expression of a tetracycline-inhibited transcription activator (tTA). Crossing α MHC-tTA mice with mice containing a tTA-responsive promoter linked to a target gene yields double-transgenic mice having tetracycline-repressed expression of the target gene in the heart. Using the tet-system, some studies use nontransgenic mice for the control group, whereas others use single-transgenic α MHC-tTA mice. However, previous studies found that high-level expression of a modified activator protein caused cardiomyopathy. Therefore, we tested whether cardiac expression of tTA was associated with altered function of α MHC-tTA mice compared with wild-type (WT) littermates. We monitored *in vivo* and *in vitro* function and gene expression profiles for myocardium from WT and α MHC-tTA mice. Compared with WT littermates, α MHC-tTA mice had a greater heart-to-body weight ratio ($\approx 10\%$), ventricular dilation, and decreased ejection fraction, suggesting mild cardiomyopathy. *In vitro*, submaximal contractions were greater compared with WT and were associated with greater myofilament Ca^{2+} sensitivity. Gene expression profiling revealed that the expression of 153 genes was significantly changed by $>20\%$ when comparing α MHC-tTA with WT myocardium. These findings demonstrate that introduction of the α MHC-tTA construct causes significant effects on myocardial gene expression and major functional abnormalities *in vivo* and *in vitro*. For studies using the tet-system, these results suggest caution in the use of controls, since α MHC-tTA myocardium differs appreciably from WT. Furthermore, the results raise the possibility that the phenotype conferred by a target gene may be influenced by the modified genetic background of α MHC-tTA myocardium.

calcium; trabeculae; tet-system; cardiomyopathy; mouse

CARDIAC-TARGETED conditional gene expression is a powerful tool to control the expression levels of particular genes in the heart. The technique allows transgenes to be turned on or off at specific developmental stages, thus allowing study of genes in

young or adult animals while avoiding potentially adverse effects due to transgene expression during development. Furthermore, after the emergence of a particular phenotype, for example cardiomyopathy (35), target gene expression can be terminated to study the process of recovery.

Conditional expression using a tetracycline-regulated system (tet-system) was described by Furth et al. (12) and has now been widely used. The tet-system was implemented in the mouse heart to study the roles of numerous genes (5, 10, 19, 34–37, 42, 43). Cardiac-specific tetracycline-regulated gene expression uses two lines of transgenic mice. In one line, the cardiac α -myosin heavy chain promoter (α MHC) derived from rat (43) or mouse (36) drives lifetime expression of a transcription activator (tTA). Transactivation by tTA is inhibited by tetracycline, which prevents tTA binding to DNA (12). α MHC-tTA mice are crossed with a second line of mice containing a tTA-responsive promoter linked to a target gene. This cross yields double-transgenic mice having tetracycline-repressed expression of the target gene in the heart. Thus removal of tetracycline from the diet initiates expression of the target gene.

For studies using the tet-system, several different control animals have been used to assess the effect of a target gene expressed in double-transgenic mice, including wild-type (WT) mice (13, 15, 17, 27, 28), single-transgenic tTA mice (2, 10, 27, 34, 35, 37), and single-transgenic mice containing the target gene (5, 7, 22, 27, 31, 42). However, since the first use of the tet-system by Bujard and colleagues, it has been hypothesized that expression of tTA alone could alter gene expression. Indeed, high-level expression of a modified activator protein in the heart caused a lethal cardiomyopathy within 2 mo (36). Thus, despite α MHC-tTA mice appearing overtly normal, there may be myocardial effects that would necessitate use of appropriate controls. Furthermore, because genetic background can affect physiological function (4, 24), significant modification of the background and function of single-transgenic α MHC-tTA mice could potentially also modify the phenotype conferred by a target gene in double-transgenic animals.

Thus, despite the importance and utility of the tet-system, it is not clear whether the introduction of the α MHC-tTA construct modifies the background and function of single-transgenic α MHC-tTA mice compared with WT littermates. Therefore, the goal of the present study was to determine whether myocardium from tTA-expressing mice was different from that of WT littermates. We compared contractions (both *in vivo* and *in vitro*) and gene expression profiles of myocardium from WT

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and α MHC-tTA mice developed by Fishman and coworkers (43) and now used extensively by others (2, 3, 10, 13, 34, 35, 37, 42).

We found that, compared with WT littermates, α MHC-tTA mice had increased heart weight, ventricular dilation, and decreased ejection fraction, suggesting early-stage cardiomyopathy. Furthermore, α MHC-tTA myocardium had increased in vitro contractions, increased myofilament Ca^{2+} sensitivity, and major alterations of gene expression. Thus, for studies using the tet-system, these complex effects of the α MHC-tTA construct on the myocardium may need to be considered for selection of controls and for evaluating the phenotype caused by a target gene.

MATERIALS AND METHODS

Animal methods. This investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publication no. 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committee of the Veterans Affairs Medical Center, San Francisco. WT littermates and mice expressing a tetracycline-controlled transactivator (tTA) under the regulatory control of 2.9 kb of 5'-flanking sequence from the rat α -myosin heavy chain gene (α MHC-tTA) were backcrossed in an FVB/N background for over 10 generations (99.9% congenic) (34, 43). tTA expression in α MHC-tTA mice is able to induce cardiac-restricted expression of tTA-dependent target genes in all chambers of the heart (34, 43). Typically, α MHC-tTA mice are used for experiments involving tetracycline-regulated gene expression, where gene expression is initiated by removal of tetracycline (or the analog doxycycline) from the diet. For this study, all animals were bred and maintained on a diet containing doxycycline (200 mg/kg, Dox diet no. S3888; Bio-Serve, Frenchtown, NJ). Except as noted below, 8-wk-old animals were removed from the Dox diet and experiments performed 2–4 wk later.

In vivo measurements. Systolic blood pressure and heart rate were measured in conscious mice, as previously described (32), using a noninvasive computerized tail cuff system.

Echocardiography in conscious mice was performed as previously described (21). Two-dimensional long-axis images of the left ventricle (LV) were obtained in parasternal long- and short-axis views with guided M-mode recordings at the midventricular level in both views.

Langendorff-perfused hearts. Male or female adult mice (weight 25–30 g) were anesthetized with pentobarbital sodium (1 mg/g; Abbott Laboratories, Chicago, IL) and heparinized (2 U/g; Elkins-Sinn, Cherry Hill, NJ), and hearts were rapidly removed and placed in cold arrest solution. Hearts were mounted on a Langendorff perfusion apparatus and retrogradely perfused with Krebs-Henseleit solution at 37°C as previously described (39). Ventricular pressure was monitored with the use of a fluid-filled balloon placed in the LV. Balloon volume was adjusted to set diastolic pressure to 10 mmHg. Hearts were electrically paced at 6 Hz, since rates closer to physiological (10 Hz) may cause ischemia, using crystalloid perfusate (6).

Right ventricle trabeculae. Thin, unbranched trabeculae were removed from the right ventricle (RV), mounted on a force transducer, and superfused with Krebs-Henseleit solution as previously described (30). Muscles were stretched to a diastolic sarcomere length of 2.1 μ m (monitored with laser diffraction), and measures were made of force, cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) (using fura-2 loaded to the cytosol by iontophoretic injection), and intracellular pH [using the fluorescent pH indicator 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF)]. To minimize rundown of the preparation and washout of fura-2, in vitro experiments were performed at low temperature (22°C) and therefore low pacing rate (0.5 Hz). Steady-state contractions were induced by tetanic stimulation for several

seconds (1). Thus a limitation is that these in vitro conditions are far from physiological temperature and frequency; therefore, caution is required in extrapolating to more physiological conditions.

The width and thickness of trabeculae from WT mice (172 ± 16 and 72 ± 8 μ m, respectively; $n = 18$) were not statistically different ($P > 0.05$) from those from α MHC-tTA mice (162 ± 17 and 85 ± 12 μ m, respectively; $n = 15$).

Western blots. As previously described, we monitored levels of total and phospho-troponin I using Western blots (30). To monitor levels of the sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA) and Na/Ca exchanger (NCX), isolated hearts were rinsed in cold saline and homogenized in RIPA buffer containing protease inhibitors (no. 1836153, Roche) and phosphatase inhibitors (P-2850 and P-5726, Sigma). After low-speed centrifugation of homogenate, samples of supernatant were processed for Western blotting as previously described (30) using 40 μ g of protein per lane run on a 7.5% SDS-PAGE gel (Bio-Rad) and antibodies for SERCA2 ATPase (Cat. MA3-919; Affinity BioReagents, Golden, CO) and NCX (Cat. NCX11-S; Alpha Diagnostic, San Antonio, TX).

Gene expression analysis. Animals were raised on doxycycline-containing chow for 6 wk, and then doxycycline was removed for 8 wk (WT, $n = 3$; α MHC-tTA, $n = 8$). A second cohort of animals was placed back on the Dox diet for 4 wk (WT, $n = 4$; α MHC-tTA, $n = 5$). Animals were anesthetized with 0.02 ml of 2.5% Avertin/g body wt. Chest cavities were opened, and hearts were removed and quickly rinsed in $1 \times$ PBS two times and placed in 1 M KCl. Atria and apex were removed, and hearts were flash frozen in liquid nitrogen (35). From ~ 75 mg of frozen tissue, total RNA was extracted, and 15 μ g of RNA were reverse transcribed and converted to double-stranded cDNA; then biotinylated cRNA was generated by in vitro transcription. For each heart, 15 μ g of fragmented cRNA were hybridized to a separate Affymetrix murine MG-U74Av2 array ($n = 20$). Arrays were hybridized and scanned with a GeneArray Scanner (Hewlett-Packard/Affymetrix). For each array, the ".cel" files were generated with Affymetrix Microarray Suite 5.0 and analyzed with robust microarray analysis (RMA) (20). Array results were similar among animals within each experimental group: for the α MHC-tTA group ($n = 8$), the standard error per gene was 2.4% of the mean expression signal per gene. RMA signal values for each array have been uploaded to the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) and can be retrieved using the series accession number GSE986.

Statistical analysis. Results are presented as means \pm SE. For physiology studies, comparisons between groups were made with Student's *t*-test and two-way ANOVA, where values of $P < 0.05$ were considered statistically significant. For gene expression studies, two-way ANOVA was used to distinguish between the effects of genotype and doxycycline on gene expression. Genes were significantly changed with $P < 0.05$, fold change $> 20\%$, and interaction $P > 0.05$ (interaction $P > 0.05$ indicates that the effects of genotype on gene expression were independent of doxycycline status). Significantly changed genes were annotated with the MAPPFinder program (9).

RESULTS

Except as noted below, all experiments were performed in the absence of doxycycline.

In vivo α MHC-tTA heart function. Table 1 shows that, for α MHC-tTA mice, blood pressure, heart rate, and cardiac output did not differ from WT. However, α MHC-tTA hearts had increased LV mass (114% of WT), were dilated (end-diastolic volume 127% of WT), and had reduced fractional shortening and ejection fraction (89 and 94% of WT, respectively). These abnormalities suggest mild cardiomyopathy.

α MHC-tTA hearts have increased in vitro function. Consistent with the in vivo assessment, Table 2 indicates that α MHC-

Table 1. Echocardiography in conscious WT and α MHC-tTA mice

	WT (n = 6)	α MHC-tTA (n = 6)	α MHC-tTA/WT, %	P Value
<i>Tail cuff</i>				
Systolic blood pressure, mmHg	107 \pm 4	111 \pm 2	103	0.4
<i>Echocardiography</i>				
LV end-diastolic dimension, mm	3.48 \pm 0.05	3.80 \pm 0.05	109	0.002
LV end-systolic dimension, mm	1.91 \pm 0.04	2.27 \pm 0.04	119	<0.0001
End-diastolic septal wall thickness, mm	0.98 \pm 0.03	1.01 \pm 0.02	103	0.4
End-diastolic posterior wall thickness, mm	0.99 \pm 0.03	1.00 \pm 0.02	101	0.8
Heart rate, beats/min	651 \pm 26	615 \pm 15	95	0.3
Fractional shortening, %	45.3 \pm 0.5	40.2 \pm 0.7	89	0.0002
LV end-diastolic volume, μ l	43.5 \pm 1.9	55.1 \pm 2.4	127	0.003
LV end-systolic volume, μ l	11.4 \pm 0.5	17.0 \pm 0.8	150	0.0002
Stroke volume, μ l	32.1 \pm 1.5	38.1 \pm 1.9	119	0.03
Ejection fraction, %	73.8 \pm 0.7	69.0 \pm 1.2	94	0.006
Cardiac output, ml/min	20.8 \pm 1.2	23.5 \pm 1.6	113	0.2
LV mass, mg	88 \pm 3	101 \pm 4	114	0.04

WT and α MHC-tTA values are means \pm SE. WT, wild type; MHC, myosin heavy chain promoter; tTA, tetracycline-inhibited transcription activator; LV, left ventricle. Boldface nos. indicate $P < 0.05$.

tTA hearts had mild hypertrophy (heart-to-body wt ratio was 10% greater than for WT). Other organs weights (kidney or liver) were not significantly changed.

In contrast to decreased indexes of contraction in vivo, α MHC-tTA hearts in vitro had increased (128% of WT) LV developed pressure (systolic minus diastolic). Furthermore, the maximum rates of tension rise and fall for α MHC-tTA hearts were greater than for WT (even after normalizing these measures to the developed pressure). Faster contraction and relaxation kinetics for α MHC-tTA hearts were also reflected in a shorter time to peak pressure and time from peak to half relaxation (Table 2).

In some experiments, we interrupted pacing stimulation with a brief rest and used the postrest contraction as an index of maximum pressure development (33). For α MHC-tTA hearts compared with WT, there was not a significant increase in postrest systolic pressure (291 \pm 5 mmHg, $n = 16$, vs. 276 mmHg \pm 7, $n = 14$; $P > 0.05$). Thus increased submaximal pressure for α MHC-tTA hearts did not involve increased

maximum pressure. Instead, the data indicate that, compared with WT, α MHC-tTA hearts were relatively more activated during pacing.

α MHC-tTA trabeculae have increased myofilament Ca^{2+} sensitivity. To investigate the basis for increased pressure development in vitro for α MHC-tTA hearts, we monitored force and Ca^{2+} transients using ventricular trabeculae (Fig. 1). Increased submaximal contraction for α MHC-tTA myocardium was also evident in trabeculae. Figure 1 and the pooled data show that at 1.5 mM bath $[Ca^{2+}]_i$, force development for α MHC-tTA trabeculae was appreciably greater than for WT myocardium (40 \pm 1 vs. 11 \pm 3 mN/mm²; $P < 0.001$, $n = 6$ /group). For α MHC-tTA trabeculae, there was a trend toward an increase in systolic cytosolic $[Ca^{2+}]_i$ ($[Ca^{2+}]_c$) vs. WT (829 \pm 67 vs. 617 \pm 113 nM; $P > 0.05$, $n = 6$ per group), which became significant over a wider range of bath $[Ca^{2+}]_i$ (see below). The time to peak $[Ca^{2+}]_c$ for α MHC-tTA trabeculae was not different from WT (65 \pm 10 vs. 67 \pm 9 ms; $P > 0.05$, $n = 6$ /group). However, the time from peak to 50%

Table 2. Body and organ weights for WT and α MHC-tTA mice and in vitro Langendorff heart function

	WT (n = 44)	α MHC-tTA (n = 28)	α MHC-tTA/WT, %	P Value
<i>Weights</i>				
Body weight, g	28.6 \pm 0.6	27.4 \pm 0.8	96	0.2
Ventricular weight, mg	117 \pm 3	124 \pm 5	106	0.18
Ventricular weight/body weight, mg/g	4.13 \pm 0.08	4.53 \pm 0.13	110	0.01
Liver weight/body weight, g/g $\cdot 10^{-2}$	4.86 \pm 0.17	4.63 \pm 0.1	95	0.24
Kidney weight/body weight, mg/g	1.32 \pm 0.07	1.21 \pm 0.03	91	0.12
<i>Langendorff heart function</i>				
Coronary flow rate, ml \cdot min ⁻¹ \cdot g ⁻¹	24.3 \pm 1.2	25.4 \pm 1.7	104	0.6
End-diastolic pressure, mmHg	8.8 \pm 0.2	9.0 \pm 0.2	103	0.3
LV systolic pressure, mmHg	121 \pm 2	153 \pm 3	127	<0.001
Developed pressure, mmHg	112 \pm 2	144 \pm 3	128	<0.001
Maximum +dP/dt, mmHg/s	4,082 \pm 94	5,721 \pm 154	140	<0.001
(+dP/dt _{max})/developed pressure, s ⁻¹	35.7 \pm 0.6	39.9 \pm 0.5	112	<0.001
Maximum -dP/dt, mmHg/s	-3,216 \pm 84	-4,235 \pm 115	134	<0.001
(-dP/dt _{max})/developed pressure, s ⁻¹	-28.1 \pm 0.5	-29.6 \pm 0.61	106	0.045
Time to peak pressure, ms	56.4 \pm 0.6	51.6 \pm 1.6	92	0.002
Time from peak to 50% relaxation, ms	40.8 \pm 0.6	38.9 \pm 0.7	96	0.045

WT and α MHC-tTA values are means \pm SE. dP/dt_{max}, maximum change in pressure over time. Boldface nos. indicate $P < 0.05$.

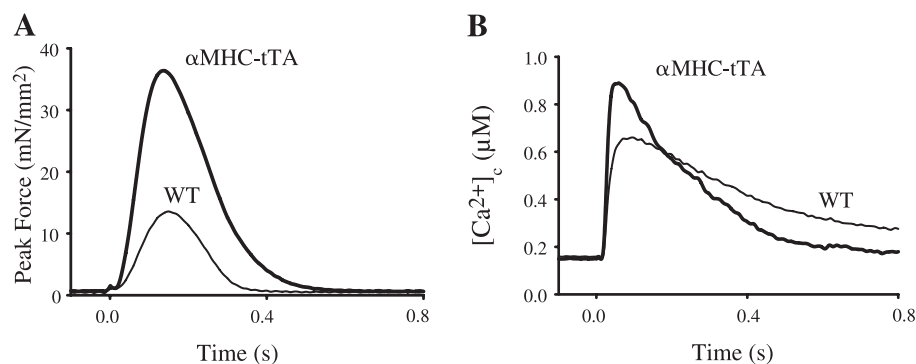


Fig. 1. α MHC-tTA trabeculae have increased sub-maximal force and Ca^{2+} transients. Representative recordings of force (A) and $[\text{Ca}^{2+}]_c$ (B) during contractions of WT (thin trace) and α MHC-tTA (bold trace) trabeculae at 1.5 mM bath Ca^{2+} . MHC, myosin heavy chain promoter; tTA, tetracycline-inhibited transcription activator; $[\text{Ca}^{2+}]_c$, cytosolic Ca^{2+} concentration; WT, wild type.

decline was considerably faster than for WT (130 ± 21 vs. 218 ± 26 ms; $P < 0.05$, $n = 6/\text{group}$) (see below).

We monitored force and Ca^{2+} transients over the full range of activation by varying extracellular $[\text{Ca}^{2+}]_e$ (Fig. 2). Figure 2A shows the effect of increasing $[\text{Ca}^{2+}]_e$ on peak twitch force. Similar to previous studies, WT mouse myocardium required high levels of extracellular Ca^{2+} to fully activate (14, 29, 30). In contrast, similar to our previous study (2), Fig. 2A shows that α MHC-tTA myocardium was close to fully activated at 2 mM $[\text{Ca}^{2+}]_e$. Like the perfused heart, for trabeculae at full activation, maximum force developed was similar for α MHC-tTA and WT myocardium. Figure 2B shows corresponding $[\text{Ca}^{2+}]_c$ at peak systole. For contractions between 0.75 and 2 mM $[\text{Ca}^{2+}]_e$, systolic $[\text{Ca}^{2+}]_c$ for α MHC-tTA myocardium was $\approx 30\%$ higher than that for WT ($P < 0.05$, ANOVA).

Figure 3 shows the relationship between peak systolic $[\text{Ca}^{2+}]_c$ and force for the experiments described in Fig. 2. The data for α MHC-tTA myocardium were shifted to the left of the data for WT, suggesting that α MHC-tTA myocardium had increased myofilament Ca^{2+} sensitivity. To confirm this suggestion, we monitored the steady-state relationship between $[\text{Ca}^{2+}]_c$ and force using tetanization (during which $[\text{Ca}^{2+}]_c$ and force reach steady-state levels) (1). Figure 4 shows the relationship between steady-state tetanic force and $[\text{Ca}^{2+}]_c$ for contractions performed at various $[\text{Ca}^{2+}]_e$. Again, the data for α MHC-tTA were shifted to the left of the data for WT, indicating that α MHC-tTA had increased myofilament Ca^{2+} sensitivity.

To investigate the basis for increased myofilament Ca^{2+} sensitivity, we monitored intracellular pH using the fluorescent probe BCECF. Intracellular alkalinization is one factor that can increase myofilament Ca^{2+} sensitivity (38). However, intracel-

lular pH for α MHC-tTA was not different from that for WT (7.33 ± 0.05 , $n = 5$, vs. 7.32 ± 0.06 , $n = 7$; $P > 0.05$). Therefore, we also monitored the phosphorylation status of troponin I (TnI) using phospho-specific antibodies and Western blots (30). Decreased phospho-TnI has been associated with increased myofilament Ca^{2+} sensitivity (for review see Ref. 26). For quiescent myocytes, there was no detectable level of phospho-TnI (data not shown). However, after stimulation with isoproterenol or forskolin, myocytes from α MHC-tTA hearts had less phospho-TnI compared with WT (Fig. 5).

α MHC-tTA hearts have faster Ca^{2+} removal from the cytosol. As noted above, the decline phase of the Ca^{2+} transient was appreciably faster for α MHC-tTA myocardium compared with WT. Figure 6 shows the relationship between the time to half decline of the Ca^{2+} transient and systolic $[\text{Ca}^{2+}]_c$, where systolic $[\text{Ca}^{2+}]_c$ was varied using different levels of $[\text{Ca}^{2+}]_e$. The slope of this relationship was steeper for α MHC-tTA myocardium compared with WT ($P < 0.01$). Thus, at higher levels of activation, clearance of Ca^{2+} from the cytosol was faster for α MHC-tTA myocardium than for WT myocardium. Faster Ca^{2+} transient decline was not associated with changes in the abundance of SERCA, phospho-phospholamban, or NCX by Western blot analysis (data not shown).

For mouse myocardium, Ca^{2+} removal from the cytosol is mediated predominantly by SERCA and NCX. To investigate the mechanism of improved Ca^{2+} removal in α MHC-tTA myocardium, we inhibited SERCA using ryanodine (1 μM) and cyclopiazonic acid (100 nM) and monitored $[\text{Ca}^{2+}]_c$ decline after brief tetanization (2 mM bath $[\text{Ca}^{2+}]_e$) (1). With SERCA inhibited, there was no difference in the initial rate of $[\text{Ca}^{2+}]_c$ decline between α MHC-tTA vs. WT myocardium (92 ± 14 nM/s, $n = 6$, vs. 107 ± 19 nM/s, $n = 5$; $P > 0.05$). This suggests that the more rapid $[\text{Ca}^{2+}]_c$ decline in α MHC-

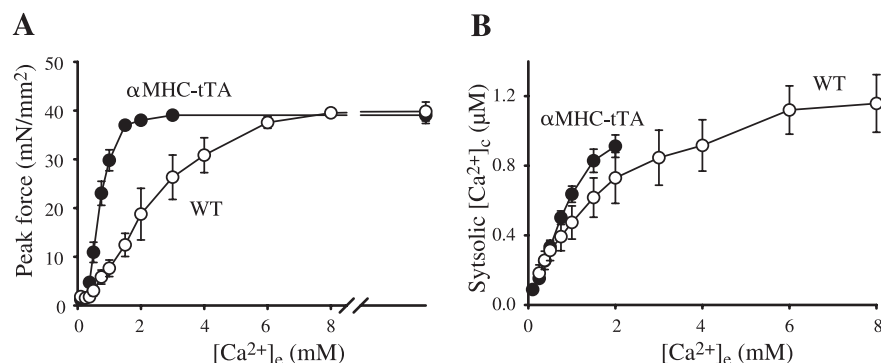


Fig. 2. α MHC-tTA trabeculae have increased responsiveness to $[\text{Ca}^{2+}]_e$. Pooled data for force (A) and systolic $[\text{Ca}^{2+}]_c$ (B) during contractions at varied $[\text{Ca}^{2+}]_e$ for WT ($n = 6$, \circ) and α MHC-tTA ($n = 6$, \bullet). Force levels were normalized to the maximum for each experiment and scaled to the maximum for all experiments (shown at right of break in x-axis). To avoid injury, $[\text{Ca}^{2+}]_e$ did not exceed that producing maximum force. Data are presented as means \pm SE. $[\text{Ca}^{2+}]_e$, extracellular $[\text{Ca}^{2+}]_e$.

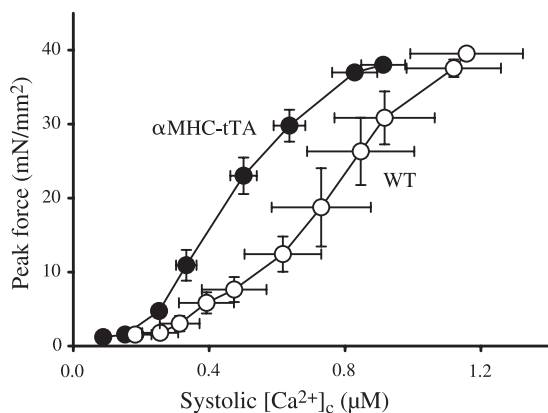


Fig. 3. α MHC-tTA trabeculae have increased myofilament Ca^{2+} sensitivity. Relationship between peak force vs. peak systolic $[\text{Ca}^{2+}]_c$ during contraction of WT and α MHC-tTA trabeculae (data from Fig. 2).

tTA myocardium is mediated by SERCA function. Furthermore, with SERCA inhibited, Ca^{2+} removal by the remaining NCX function was not different between α MHC-tTA and WT myocardium.

α MHC-tTA hearts have altered gene expression. We used DNA microarrays to assess gene expression profiles in WT and α MHC-tTA myocardium. Figure 7 shows results of an ANOVA among the treatment groups to identify significant differences in gene expression attributable to the different genotypes and doxycycline treatment (with fold changes $>20\%$, $P < 0.05$, and interaction $P > 0.05$). The greatest difference in gene expression was associated with the different genotypes (75 genes upregulated and 78 genes downregulated), with fewer differences in gene expression attributable to doxycycline (18 genes upregulated and 4 genes downregulated). These findings suggest that the presence of the α MHC-tTA construct caused modification of the genetic background.

For α MHC-tTA vs. WT myocardium, the most highly regulated genes are summarized in Table 3. Genes for the myofilament proteins β -tropomyosin and skeletal α -actin were upregulated. Both genes were significantly upregulated with or without doxycycline; however, expression levels were also affected by doxycycline (hence significant interaction P values). Previously, β -tropomyosin was associated with increased

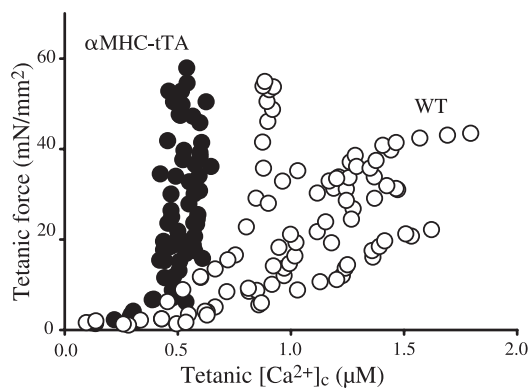


Fig. 4. α MHC-tTA trabeculae have increased steady-state myofilament Ca^{2+} sensitivity during tetanization. Each data point represents a single tetanic contraction during which force and systolic $[\text{Ca}^{2+}]_c$ achieved a steady state for several seconds for α MHC-tTA ($n = 6$, ●) and WT trabeculae ($n = 6$, ○).

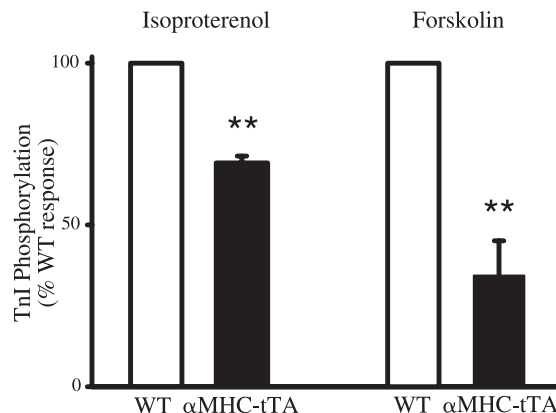


Fig. 5. α MHC-tTA myocytes have decreased phospho-TnI. Phospho-TnI levels in α MHC-tTA and WT myocytes after stimulation with isoproterenol or forskolin. Phospho-TnI levels were normalized to total TnI levels and scaled relative to the WT response. TnI, troponin I. $**P < 0.01$ ($n = 3$ per group).

myofilament Ca^{2+} sensitivity (41), and skeletal α -actin was upregulated with hypertrophy (25).

To relate all 153 significantly regulated genes to biologically relevant groupings, we used the Gene Ontology (GO) (16) Consortium (9) terms that describe biological processes, cellular components, and molecular functions of genes. We also used MAPPFinder, a software program that links gene expression data to the GO hierarchy (9). The 153 most affected genes were distributed among a number of different biological processes (Table 4). Compared with WT, for α MHC-tTA myocardium there was an upregulation of genes involved in intracellular transport, intracellular signaling cascade, and kinase, transferase, and heat shock protein activities; furthermore, there was a downregulation of genes involved in lipid, carbohydrate, and protein metabolism and mitochondria and transporter activity.

Influence of doxycycline on the phenotype of α MHC-tTA hearts. The transcription activator tTA is inhibited by doxycycline. To investigate whether the phenotype of α MHC-tTA hearts was influenced by doxycycline, in a separate set of experiments, mice were not removed from the doxycycline diet. In the presence of doxycycline, there was a greater heart weight-to-body weight ratio for α MHC-tTA vs. WT hearts (4.64 ± 0.19 , $n = 7$, vs. 3.98 ± 0.23 , $n = 7$; $P < 0.05$), similar

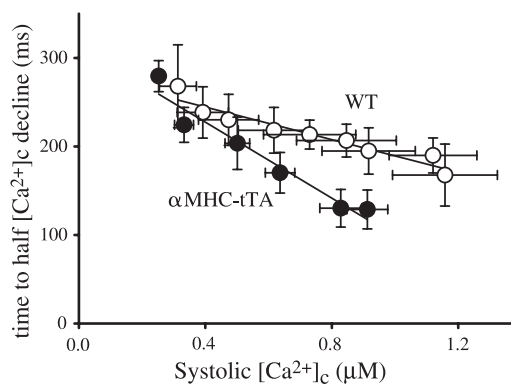


Fig. 6. α MHC-tTA hearts have faster Ca^{2+} transient decline. Summary of the relationship between the time to half decline of the Ca^{2+} transient and systolic $[\text{Ca}^{2+}]_c$ for α MHC-tTA ($n = 6$, ●) and WT trabeculae ($n = 6$, ○). Data are presented as means \pm SE.

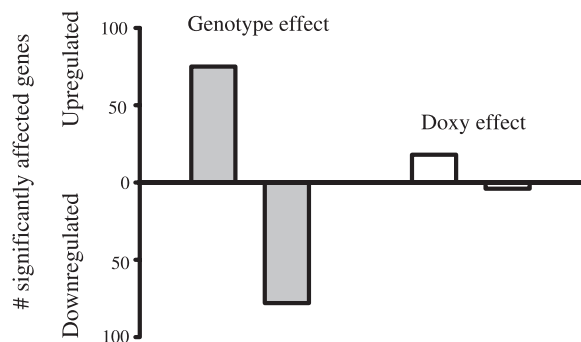


Fig. 7. α MHC-tTA hearts have altered gene expression. No. of genes with statistically significant differences in expression attributable to genotype and doxycycline (determined using 2-way ANOVA, where differences in expression had fold change >20%, $P < 0.05$, and interaction $P > 0.05$).

to the data without doxycycline (Table 2). Furthermore, with doxycycline there was greater LV systolic pressure for α MHC-tTA vs. WT hearts (142 ± 6 mmHg, $n = 7$, vs. 122 ± 3 mmHg, $n = 11$; $P < 0.01$), similar to the data without doxycycline (Table 2). Finally, as noted above, significant effects on gene expression were attributable to the α MHC-tTA genotype rather than to doxycycline. Together, these findings suggest that, compared with WT, alterations in the physiology and gene expression of α MHC-tTA hearts were independent of doxycycline.

DISCUSSION

The major finding of this study was that the presence of the α MHC-tTA construct caused significant effects on myocardial gene expression and function. Compared with WT, hearts from α MHC-tTA mice had mild hypertrophy and were dilated, and in vivo measures showed decreased ejection fraction but with normal cardiac output. In vitro measures showed that, compared with WT, α MHC-tTA myocardium had increased sub-

maximal contractions and enhancement of both myofilament Ca^{2+} sensitivity and Ca^{2+} handling. Finally, gene expression analysis revealed that α MHC-tTA myocardium had wide-ranging changes in gene expression compared with WT.

The significance of these findings is, first, that they suggest that studies using the tet-system may require careful selection of appropriate control animals, since single-transgenic α MHC-tTA mouse hearts differ markedly from true WTs. Thus, using the tet-system, WT animals may not be suitable controls. Instead, single-transgenic α MHC-tTA mice are preferred controls for double-transgenic mice containing the α MHC-tTA and target gene constructs. Second, genetic background can profoundly influence the phenotype conferred by genetic manipulation (4, 24). Thus, using the tet-system, the phenotype conferred by a target gene may be influenced by the modified background caused by the α MHC-tTA construct. Finally, the findings underscore the concept that genetic manipulation can cause unintended side effects on gene expression and function (18).

The findings of this study are limited to the particular α MHC-tTA line studied (43). However, this line has now been widely used by others. Other lines used for conditional gene expression would have to be separately evaluated.

Abnormalities of α MHC-tTA myocardium. Previously, Sanbe et. al. (36) found that high-level expression of a modified activator protein in the heart caused a lethal cardiomyopathy within 2 mo. Furthermore, a low level of expression of tTA protein was sufficient for inducible gene expression but was not associated with disease (36). Here we extend their findings to show that significant myocardial abnormalities exist in α MHC-tTA mice that otherwise appear phenotypically normal. Failure to appreciate these abnormalities of α MHC-tTA myocardium could lead to erroneous conclusions in studies using the tet-system to control the expression of a target gene in the heart.

Table 3. *Most highly regulated genes in α MHC-tTA myocardium vs. WT myocardium*

UnigeneID	GeneSymbol	Title	Fold Change	Fold Change P value	Interaction P value
<i>Upregulated genes</i>					
Mm.646	<i>Tpm2</i>	β -tropomyosin	2.873	<0.001	0.044
Mm.89137	<i>Acta1</i>	skeletal α -actin	2.256	<0.001	0.035
Mm.142594	<i>Clu</i>	clusterin	2.105	<0.001	0.574
Mm.68889	<i>Gnb3</i>	G protein β -polypeptide	1.541	<0.001	0.745
Mm.43831	<i>Lgals1</i>	galectin-1	1.530	<0.001	0.416
Mm.5567	<i>Pdlim1</i>	PDZ and LIM domain 1	1.475	<0.001	0.992
Mm.9440	<i>Ptdss1</i>	phosphatidylserine synthase 1	1.411	<0.001	0.473
Mm.6818	<i>Itgb1bp1</i>	integrin β -binding protein 1	1.380	<0.001	0.455
Mm.34828	<i>Hsp105</i>	heat shock protein 105 kDa	1.338	0.001	0.721
Mm.1843	<i>Hsp86-1</i>	heat shock protein 86 kDa	1.309	0.001	0.665
<i>Downregulated genes</i>					
Mm.88078	<i>Ces3</i>	carboxylesterase 3	-2.913	<0.001	0.344
Mm.2422	<i>Pah</i>	phenylalanine hydroxylase	-1.874	0.005	0.978
Mm.16537	<i>Acta2</i>	vascular smooth muscle α -actin	-1.818	<0.001	0.110
Mm.1008	<i>Ptgds</i>	prostaglandin D2 synthase	-1.673	<0.001	0.179
Mm.30072	<i>Cox7a21</i>	cytochrome <i>c</i> oxidase 7a polypeptide	-1.649	<0.001	0.246
Mm.1334	<i>Ryr2</i>	cardiac ryanodine receptor	-1.605	<0.001	0.076
Mm.21579	<i>H2bfs</i>	H2B histone family member S	-1.578	<0.001	0.222
Mm.154057	<i>Cx43</i>	connexin-43	-1.555	<0.001	0.677
Mm.2740	<i>Nppb</i>	natriuretic peptide precursor type B	-1.500	0.003	0.088
Mm.13445	<i>Oxct</i>	3-oxoacid CoA transferase	-1.423	<0.001	0.469

Boldface nos. indicate $P < 0.05$.

Table 4. Analysis of GO terms representing transcripts differentially regulated by genotype (α MHC-tTA vs. WT myocardium)

GO Name	GO Type	No. Changed	No. Measured	No. in GO	Permuted <i>P</i> Value
<i>Upregulated GO terms</i>					
Intracellular transport	P	7	268	439	0.004
Kinase activity	F	9	438	656	0.008
Lipid binding	F	3	63	88	0.017
Transferase activity	F	12	727	1,130	0.022
Heat shock protein activity	F	2	26	37	0.024
Amino acid derivative metabolism	P	2	34	46	0.029
Intracellular signaling cascade	P	7	363	579	0.034
Cytoskeleton	C	8	448	649	0.040
Purine nucleotide binding	F	14	1,010	1,524	0.046
Angiogenesis	P	2	40	46	0.047
<i>Downregulated GO terms</i>					
Lipid metabolism	P	8	206	309	<0.001
Organic acid metabolism	P	7	165	227	0.001
Transporter activity	F	17	787	1,323	0.001
Behavior	P	3	46	80	0.010
Carbohydrate metabolism	P	5	169	244	0.012
Lyase activity	F	3	78	118	0.028
Mitochondrion	C	7	344	508	0.030
Chromatin assembly/disassembly	P	2	35	56	0.031
Protein metabolism	P	3	1,088	1,658	0.032

Upregulated and downregulated genotype-dependent transcripts (see Fig. 7) were analyzed by MAPPFinder (9). Gene Ontology (GO) terms describe biological processes (P), cellular components (C), and molecular functions of genes (F). Top nonredundant GO terms are shown with permuted *P* < 0.05.

For α MHC-tTA hearts, the phenotype of modest hypertrophy, dilation, and decreased ejection fraction suggests a mild cardiomyopathy. In vitro function, assessed using Langendorff hearts and RV trabeculae, showed that compared with WT, α MHC-tTA myocardium had increased submaximal contractions but maximal contractions were unchanged. It is not clear why α MHC-tTA hearts had decreased indexes of contraction in vivo but increased contraction in vitro. Possibly the in vitro measures are more reflective of intrinsic myocardial properties without regulatory influences exerted by the whole animal. Nevertheless, for this study, a central finding was that α MHC-tTA hearts differed substantially from WT hearts in terms of function (in vivo or in vitro) and gene expression.

Compared with WT, α MHC-tTA myocardium had increased myofilament Ca^{2+} sensitivity. Interestingly, there is growing evidence for increased Ca^{2+} sensitivity with heart failure (2, 30, 40). Thus increased Ca^{2+} sensitivity for α MHC-tTA myocardium may reflect the cardiomyopathic state suggested by our in vivo studies. On the other hand, as noted above, in vitro function was not impaired; moreover, the issue of Ca^{2+} sensitivity in heart failure remains unclear, because other studies report that, with heart failure, Ca^{2+} sensitivity was unchanged or even decreased (reviewed in Ref. 8).

The mechanism for increased myofilament Ca^{2+} sensitivity in α MHC-tTA myocardium did not involve increased intracellular pH. However, α MHC-tTA had increased expression of β -tropomyosin, which previous studies found to play a role in increased myofilament Ca^{2+} sensitivity and cardiomyopathy (41). In addition, we found that stimulation of Gs signaling in quiescent myocytes was associated with decreased phosphorylation of TnI for α MHC-tTA myocytes compared with WT. Because contracting myocardium will likely have some basal Gs tone, decreased TnI phosphorylation for α MHC-tTA myocardium could also contribute to increased myofilament Ca^{2+}

sensitivity compared with WT. Previous studies found decreased TnI phosphorylation and increased Ca^{2+} sensitivity in human heart failure (2, 30, 40).

There were 153 genes that were significantly changed >20% in α MHC-tTA hearts compared with WT hearts. These genes were linked to multiple GO terms. Interestingly, upregulation of heat shock genes and downregulation of genes involved in metabolism may relate to the better protection against ischemic injury that we have also observed in α MHC-tTA myocardium (L. Turnbull and A. J. Baker, unpublished observations).

Some changes in gene expression observed for α MHC-tTA myocardium are consistent with the observed phenotype and with previous studies of cardiomyopathy. Other changes in gene expression do not fit such a pattern. For α MHC-tTA myocardium, we found mild hypertrophy and upregulation of skeletal α -actin gene expression. Upregulation of skeletal α -actin gene expression was previously associated with cardiac myocyte hypertrophy (25). In contrast, two other hypertrophy genes (natriuretic peptide precursor type B and vascular smooth muscle α -actin) (11) were downregulated rather than upregulated. For α MHC-tTA myocardium, we found cardiomyopathy and increased Ca^{2+} sensitivity. As noted above, the β -tropomyosin gene was upregulated in α MHC-tTA myocardium, and increased levels of this myofilament protein have been associated with cardiomyopathy and increased Ca^{2+} sensitivity. α MHC-tTA myocardium had downregulation of genes for connexin-43 and the ryanodine receptor. Similar changes were associated with some forms of cardiomyopathy (16, 23) and could lead to impaired excitation-contraction coupling. However, α MHC-tTA myocardium also had a faster Ca^{2+} transient decline, which suggested improved SERCA function.

Compared with WT, α MHC-tTA hearts manifested alterations in gene expression and physiology (mild hypertrophy, higher systolic pressure in vitro) that were independent of the

presence of doxycycline. Because doxycycline inhibits the interaction of tTA with DNA, our findings suggest that altered gene expression for α MHC-tTA myocardium does not depend on the conformational change in the DNA-binding domain that occurs when tTA binds doxycycline. Possibly altered gene expression in α MHC-tTA myocardium could arise because tTA contains a VP16 domain that is a powerful transcription activator that interacts with a large number of other transcription factors. Alternatively, effects on DNA caused by integration of the α MHC-tTA construct at a particular site(s) could play a role.

In conclusion, controlling gene expression in the mouse heart using the tet-system can be complicated by unintended gene expression and physiological effects caused by introduction of the α MHC-tTA construct. This suggests that caution is required in the selection of control animals and in interpreting the effects of expression of a target gene.

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