Journal of the American College of Cardiology
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Published by Elsevier Inc.

Vol. 44, No. 11, 2004
ISSN 0735-1097/04/$30.00
doi:10.1016/j.jacc.2004.09.005

Induction and Reversal of Cardiac Phenotype of Human Hypertrophic Cardiomyopathy Mutation Cardiac Troponin T-Q92 in Switch On–Switch Off Bigenic Mice

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OBJECTIVES The aim of this study was to establish reversibility of cardiac phenotypes in hypertrophic cardiomyopathy (HCM) by generating bigenic mice in which expression of the mutant transgene could be turned on and off as needed.

BACKGROUND Advances in molecular therapeutics could ultimately lead to therapies aimed at correcting the causal mutations. However, whether cardiac phenotypes, once established, are permanent, or could be reversed, if expression of the mutant protein is turned off, is unknown.

METHODS We generated ligand-inducible bigenic mice, turned on and off expression of cardiac troponin T-Q92 (cTnT-Q92), responsible for human HCM, and characterized molecular, histologic, and functional phenotypes.

RESULTS We established six lines and in dose-titration studies showed that treatment with 1,000 μg/kg of mifepristone consistently switched on cTnT-Q92 expression in the heart. Short-term (16 days) induced expression enhanced myocardial systolic function without changing myocardial cyclic adenosine monophosphate levels. Levels of PTEN, a regulator of cardiac function, phospho-protein kinase C-Zλ-Thr538 and phosphor-protein kinase D-Ser744-748 were reduced, whereas messenger ribonucleic acid (mRNA) levels of *NPPA*, *NPPB*, and sarcoplasmic reticulum calcium adenine triphosphatase 2 (*ATP2A2*) (hypertrophic markers) and procollagen *COL1A1*, *COL1A2*, and *COL3A1* were unchanged. Long-term (70 days) induced expression increased *COL1A1* and COL1A3 mRNAs levels and collagen volume fraction and reduced levels of *NPPA* and *NPPB*. Switching off expression of the cTnT-Q92 reversed functional, molecular, and histologic phenotypes completely.

CONCLUSIONS The initial phenotype induced by cTnT-Q92 is enhanced myocardial systolic function followed by changes in signaling kinases and interstitial fibrosis. Established phenotypes in HCM reverse upon turning off expression of the mutant protein. These findings provoke pursuing specific therapies directed at correcting the underlying the genetic defect in HCM. (J Am Coll Cardiol 2004;44:2221–30) © 2004 by the American College of Cardiology Foundation

Human hypertrophic cardiomyopathy (HCM) is a genetic disease characterized by unexplained cardiac hypertrophy, myocyte disarray, interstitial fibrosis, and increased left ventricular ejection fraction (LVEF) (1–3). Mutations in at least 10 different sarcomeric proteins cause HCM (2), and those in cardiac troponin T (cTnT) account for approximately 15% of HCM cases (2). Hypertrophic cardiomyopathy resulting from cTnT mutations is generally characterized by an increased LVEF, minimal or mild hypertrophy, severe disarray, fibrosis, and a high incidence of sudden cardiac death (4,5).

Elucidation of the molecular genetic basis of HCM has shifted the focus toward elucidation of the pathogenesis of HCM and developing therapies aimed at reversing or attenuating the evolving phenotypes and ultimately, correcting the primary genetic defects. Currently, it is unknown whether turning off expression of the mutant protein could lead to reversal of the established phenotypes; or the phenotypes, once established, are permanent. Proving the reversibility of the HCM phenotypes is fundamental and prerequisite to pursuing specific molecular therapies directed at correcting the underlying genetic mutation in HCM. Thus, to determine the reversibility of the evolving phenotypes and gain insight into the pathogenesis of HCM, we generated an inducible bigenic mouse model in which expression of cardiac troponin T-Q92 (cTnT-Q92), known to cause HCM in humans (4), could be switched on and off as needed. The inducible bigenic model is based on ligand-dependent activation of an inactive tripartite regulatory protein containing the ligand-binding domain of a truncated human progesterone receptor (PR) that upon activation with mifepristone binds to the deoxyribonucleic acid

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Manuscript received July 6, 2004; revised manuscript received August 9, 2004, accepted September 2, 2004.

Abbreviations and Acronyms

ANOVA	= analysis of variance
ATPase	= adenosine triphosphatase
cAMP	= cyclic adenosine monophosphate
cDNA	= complementary deoxyribonucleic acid
cTnT	= cardiac troponin T
cTnT-Q92	= cardiac troponin T-Q92
CVF	= collagen volume fraction
HCM	= hypertrophic cardiomyopathy
LV	= left ventricular
LVEF	= left ventricular ejection fraction
mRNA	= messenger ribonucleic acid
MyHC	= myosin heavy chain
NTG	= non-transgenic
PCR	= polymerase chain reaction
PKC	= protein kinase C
PR	= progesterone receptor
RT-PCR	= reverse transcription-polymerase chain reaction
UAS	= upstream activating sequences

(DNA) sequences in the target transgene and induces expression of the target gene (6).

METHODS

The switch on–switch off (inducible) cTnT-Q92 bigenic mice. The Animal Subjects Committee of Baylor College of Medicine approved the experiments. The regulator is a tripartite protein composed of the ligand-binding domain of a C-terminal truncated (deletion of 42-amino acid) human PR, the DNA-binding domain of yeast GAL4 protein, and the transcription activation domain of the p65 subunit of human nuclear factor kappa B transcription factor. It is inactive in its native state, because the modified ligand-binding domain lacks the ability to respond to progestins but responds to synthetic anti-progesterone mifepristone (RU486) (7). The target transgene is comprised of four placed in tandem 17-bp (CGGAGTACTGTCCTCCG) GAL4 upstream activating sequences (UAS) and TATA box positioned 5′ to a mutant cTnT-Q92 complementary deoxyribonucleic acid (cDNA). Expression of the target transgene could be turned on and off by adding or withdrawing mifepristone. The α-myosin heavy chain (MyHC) promoter affords cardiac-restricted expression of the regulator protein and consequently, that of the cTnT-Q92 (Fig. 1A).

The α-MyHC promoter (clone 26) was a kind gift from Dr. Jeffery Robbins (University of Cincinnati), and the PAP-CMV-GLp65-SV and p17x4-TATA-CAT plasmids were generous gifts from Dr. Sophia Y. Tsai (Baylor College of Medicine). To clone the regulator transgene, the GLp65 fragment containing the coding sequences for the tripartite regulator protein was excised from PAP-CMV-GLp65-SV at the unique *Asp718* and *BamH1* restriction sites, blunt ended and ligated to the blunt-ended *SalI* and *HindIII* cloning sites of clone 26, downstream to the α-MyHC promoter (Fig. 1A). To clone the target transgene, a previously described full-length (1.1 kb) human cTnT-Q92 cDNA (8) was released from the pSP73/cTnT-Q92 clone and inserted at *XhoI* and *KpnI* sites, replacing the CAT sequences in the p17x4-TATA-CAT clone. The correct orientations and the sequence of the transgenes were verified by direct sequencing. The regulator transgene (~7 kb) was released from the vector by digesting the clone at the encompassing *Not1* sites and the target transgene by digesting the clone at *HindIII* and *KpnI* sites (~2 kb). Transgenes were purified from the gel and injected into fertilized mouse zygotes (FVB/N), and the pups were screened by polymerase chain reaction (PCR) for the regulator and target transgenes and for the β casein, as an internal control.

Switch on and off protocol. Mifepristone (Biomol, Plymouth Meeting, Pennsylvania) was initially dissolved in dimethyl sulfoxide at a concentration of 40 mg/ml, and the working solution was prepared in sesame oil at a concentration of 1 mg/ml. To induce target gene expression, dose-titration and time-course experiments were performed and mifepristone was injected intraperitoneally (n = 5 to 12 adult mice per group) at three dosages of 300, 500, and 1,000 μg/kg/day for 2, 4, 8, or 16 days (short-term) or 70 days (long-term). To switch off expression of the transgene, mifepristone was discontinued for at least 16 days, which is longer than five half-lives of cTnT in the heart (9) and for 70 days in long-term experiments. Parallel experiments were performed in non-transgenic (NTG) littermates injected with mifepristone and in bigenic mice injected with a placebo (sesame oil). Expression of the target transgene was determined by reverse transcription-polymerase chain reaction (RT-PCR) and immunoblotting after switching on or off expression of the transgene at the above time points. Short-term (2, 4, 8, and 16 days) induction of expression of cTnT-Q92 transgene was achieved through daily intraperitoneal injections. To induce long-term expression of cTnT-Q92 transgene, mifepristone controlled-released pellets (Innovative Research of America, Sarasota, Florida) were prepared to deliver 1,000 μg/kg/day and implanted subcutaneously.

RT-PCR, real-time quantitative PCR, and immunoblotting. The RT-PCR and immunoblotting were performed using transgene-specific oligonucleotide primers and monoclonal anti-troponin T antibody 2D10 (Research Diagnostics Inc., Flanders, New Jersey), respectively, as described (8). Relative expression levels of the transgene and endogenous cTnT messenger ribonucleic acids (mRNAs) were determined by RT-PCR using primers (forward 5′TTCATGCCCAACTTGGTGCC and reverse 5′CTCTCTTCAGACAGGCGGTTC). A 260-bp of endogenous and transgene cTnT mRNAs was amplified and digested with 10 units of *BstU1* restriction enzyme, which digests the endogenous (mouse) but not the transgene (human) cTnT into two fragments of 184 and 76 bp.

Expression levels of molecular markers of cardiac hypertrophy (A-type natriuretic peptide [*NPPA*], B-type natri-

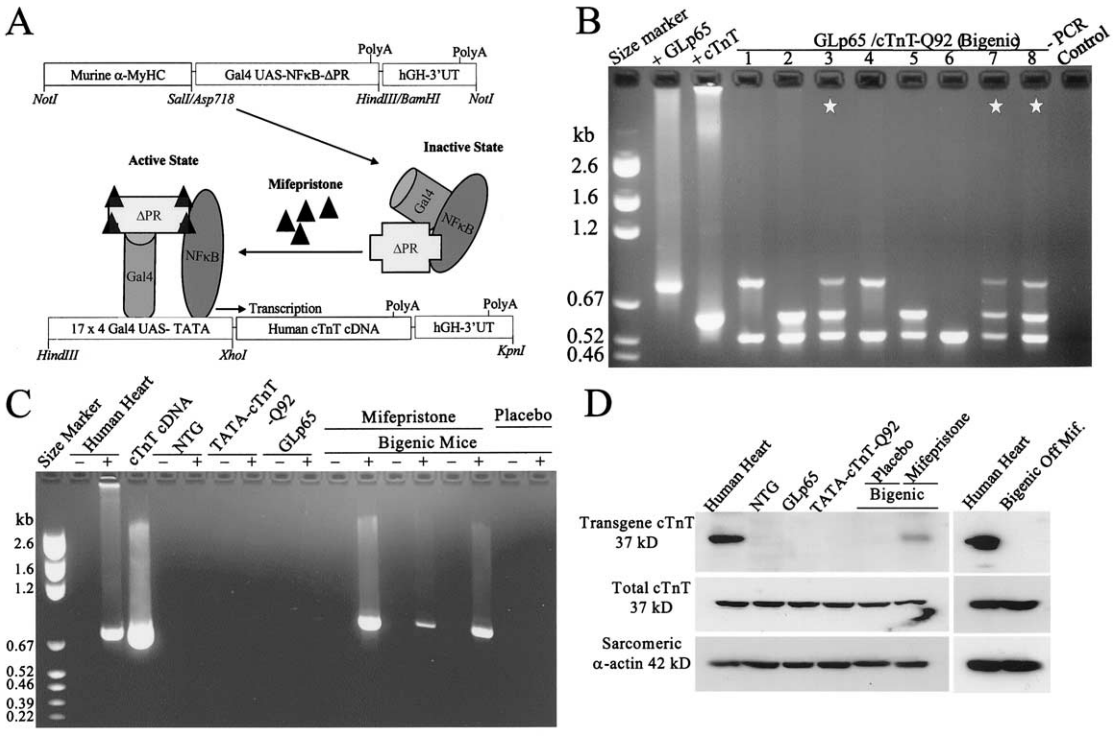

Figure 1. Schematic structure of the transgenes and documentation of transgene expression. **(A)** Schematic diagrams of the regulator and target transgenes. The regulator is an inactive tripartite protein that is expressed specifically in the heart. Mifepristone binds to the ligand-binding domain of the tripartite protein and induces conformational changes that expose the DNA binding domain of the protein. The latter domain binds to GAL4 sequences in the target transgene, juxtaposing the transcription activation domain of p65 to a minimal TATA box (TCTAGAGGGTATATAATGGATCCGGAT) and subsequent transcription of the downstream sequences (cTnT-Q92 transgene). **(B)** Polymerase chain reaction screening of bigenic mice: DNA size marker is pGEM. The + GLp65 and + cTnT denote positive controls for the regulator and the target transgenes, respectively. The remainder of the wells represent DNA from F2 mice screened for carrying the regulator (forward primer: 5′GGCTCCTCTGCCAGTGTTGATAT and reverse 5′AGAAGTTGC-CTCTCGCCTAGTTG) and target (forward 5′GAGCAGGAAGAAGCAGCTGTTGA and reverse 5′GGTCGAACTTCTCTGCCTCCAAG) transgenes. The lower band in each lane identifies an approximately 500-bp fragment of the mouse β casein gene used as an internal control for DNA quality (forward 5′ GATGTGCTCCAGGCTAAAGTT and reverse 5′AGAAACGCAATGTTGTGGAGT). Three bigenic mice carrying both the regulatory and the target transgenes are identified by *. **(C)** Detection of expression of inducible cTnT-Q92 mRNA in bigenic mice by RT-PCR. The cDNA for human cTnT and human heart mRNA are included as positive controls and cardiac mRNAs from non-transgenic (NTG) and monogenic TATA-cDNA as negative controls. The + and − denote RT-PCR with and without superscript reverse transcriptase in the reaction. Cardiac mRNA from one bigenic line treated with placebo, three bigenic lines treated with mifepristone (16 days), and one bigenic line 16 days after discontinuation of mifepristone are included. The presence of 760 bp PCR products (forward: 5′GGTTATCGTTGATCCTGATTGCC and reverse: 5′GGAGCAGGAAGAAGC-AGCTGTT) identifies expression of the induced cTnT-Q92 in three lines of bigenic mice. The absence of RT-PCR product in the target TATA-cTnT-92 monogenic mouse confirms the absence of background expression of cTnT-Q92. **(D)** Expression of induced cTnT-Q92 in the heart of bigenic mice. Cardiac protein extract from a human heart is included as positive control. Cardiac proteins from NTG, GLp65, and TATA-cTnT-Q92 monogenic mice and bigenic mouse treated with placebo (each 20 μg) are included as negative controls. The presence of a 37-kDa band identifies the expression of transgene protein in the heart of a bigenic mouse treated with mifepristone, which was detected using human specific anti-troponin T monoclonal antibody 2D10. The **mini-panels** to the right indicate switching off expression of cTnT-Q92 after discontinuation of mifepristone. The middle row shows expression of total cTnT (endogenous and transgene), detected using pan-specific anti-troponin T antibody JLT-12 (Serotec, Kidlington, Oxford). The **lower panel** shows expression of sarcomeric α-actin (Biomeda, Inc., Foster City, California), a marker for loading condition.

uretic peptide [*NPPB*], skeletal α-actin [*ACTA1*], and sarcoplasmic reticulum calcium adenosine triphosphatase (ATPase) 2a [SERCA2a or *ATP2A2*]), cardiac fibrosis [procollagens COL1(α1), COL1(α2), and COL3(α1) mRNAs (*COL1A1*, *COL1A2*, and *COL3A1*, respectively)], and glyceraldehyde-3-phosphate dehydrogenase were determined by real-time quantitative RT-PCR using specific Taqman probes and primers in a 7900HT SDS unit (Applied Biosystems, Inc., Foster City, California).

Expression levels of selected phosphorylated and total signaling kinases, implicated in affecting cardiac structure and function, were detected using 20 μg of cardiac protein extracts and phospho-specific and pan-specific antibodies, as described (10). Specific information about antibodies is available upon request.

M-mode, two-dimensional, and Doppler echocardiography. Echocardiography was performed before and after switching on and off expression of the transgene at each dose and time point. Mice were anesthetized with intraperitoneal injection of anesthetics (avertin 0.2 ml/10 g body weight, intraperitoneal injection of a 1.25% solution in the long-term experiments), and trans-thoracic echocardiography was performed using an Acuson Sequoia Cardiac System equipped with a 15-MHz linear transducer (Acuson Co., Mountain View, California) in accordance with conventional methods (11). Echocardiographic parameters

were measured in at least three consecutive cardiac cycles by an investigator totally unaware of the genotypes or the treatment groups.

Histologic studies. The extent of cardiac myocyte disarray and interstitial fibrosis were quantified as published (10,12). Percentage of myocardium showing disarray was determined in a semiquantitative manner in 12 fields per section and 10 sections per mouse. Similarly, collagen volume fraction (CVF) was calculated using quantitative automated planimetry in 12 fields per section and 10 sections per mouse. Myocyte cross-sectional area was determined by quantitative indirect immunofluorescence in approximately 2,500 myocytes per group, as published (10,12). All histologic quantifications were performed by an examiner who had no knowledge of group assignment.

Myocardial cyclic adenosine monophosphate (cAMP) levels. Because cAMP is a major mediator of myocardial contractile function, we measured total myocardial cAMP levels by enzyme immunoassay according to the instructions of the manufacturer (Amersham Biosciences, Piscataway, New Jersey). In brief, 100-mg aliquots of ventricular myocardium (n = 4 to 8 mice per group) were homogenized in Hanks' balanced salt solution with 5-mM ethylenediaminetetraacetic acid without calcium and magnesium and centrifuged at 1,000 *g* at 4°C for 15 min. The homogenates were subjected to ion-exchange chromatography using columns containing anion exchange silica sorbents followed by analyte elution with acidified methanol. The eluted samples were added to an assay buffer containing rabbit anti-cAMP antibody in 0.05-M acetate, 0.02% (w/v) bovine serum albumin and mixed thoroughly. Then, an equal volume of cAMP-horseradish peroxidase conjugate in 0.05-M acetate buffer, pH 5.8 was added to each reaction and washed with 0.01-M phosphate buffer pH 7.5 containing 0.05% (v/v) Tween 20. The mixtures were added to microplates coated with a donkey anti-rabbit immunoglobulin G antibody, incubated at 4°C for 60 min. After washing, the enzyme substrate was added to the wells, and the reactions were terminated after 1 h of incubation at room temperature with 1.0 M sulfuric acid. The optical density was determined immediately in a plate reader at 450 nm.

Statistical methods. Differences between the baseline and follow-up values in each group were compared by paired *t* test. Differences in variables among multiple groups, such as NTG, placebo, and mifepristone groups, were compared by analysis of variance (ANOVA). Similarly, differences in the phenotype at different experimental points, that is, before and after switching on and off expression of the transgene, in each group of mice were compared by repeated measures ANOVA. Homogeneity of the variances was tested by Bartlett's test. Variables with unequal standard deviation (LVEF by two-dimensional echocardiography, deceleration time of mitral inflow E velocities, and myocyte disarray) were compared by the non-parametric Kruskal-Wallis test. Minitab software (version 14) was used for the statistical analysis.

RESULTS

Bigenic mice. Three lines of regulator (α-MyHC/GLp65-GAL4-ΔPR) and seven lines of target (4xGAL4UAS-TATA-cTnT-Q92) transgenic mice were generated. The target transgenic lines were tested for background expression of the cTnT-Q92, after confirmation of the absence of background leak, mated to the regulator transgenic mice, and six lines of bigenic mice were established. Monogenic and bigenic mice were identified by PCR using transgene-specific oligonucleotide primers (Fig. 1B). Mice from three lines, backcrossed at least five times, were used in these experiments.

Switching on and off cTnT-Q92 mRNA and protein expression. Expression of the transgene mRNA and protein was detected consistently after daily administration of mifepristone at 1,000 μg/kg/day for 16 days. Shorter durations or lower doses of administration of mifepristone did not consistently induce expression of the transgene. The results of RT-PCR (in three lines, Fig. 1C) and immunoblotting (in one line, Fig. 1C) confirmed expression of the cTnT-Q92 mRNA and protein, respectively. No significant cTnT-Q92 expression was detected in bigenic mice injected with a placebo or in the target monogenic mice. The relative expression level of the induced transgene cTnT was low in all lines and comprised <10% of the total cTnT mRNA in the heart. Transgene protein and mRNA were not detected 16 days after discontinuation of mifepristone (Fig. 1D).

In the long-term study, 2 of 9 bigenic mice died 3 and 9 days after mifepristone injection, in contrast to 0 of 11 NTG mice injected with mifepristone or 0 of 9 bigenic mice injected with the placebo. Baseline echocardiographic indices were unremarkable in two mice that died after induced transgene expression. Because these mice were found dead, no histologic or molecular studies were performed.

Myocardial systolic and diastolic function. Echocardiographic indices of myocardial function were compared at the baseline before injection of mifepristone; 16 and 70 days after induction of expression of the cTnT-Q92 in the heart; and 16 days and 70 days after discontinuation of mifepristone administration. The results were remarkable for a smaller left ventricular (LV) end-systolic diameter, increased LV fractional shortening, LVEF, velocity of circumferential fiber shortening, and maximum aortic flow velocity in induced state at both time points (16 and 70 days) compared with the baseline values. There were no significant differences in the LV wall thickness, end-diastolic diameter, or mitral inflow early (E), late (A), and E/A. However, deceleration time of E velocity was prolonged in the bigenic mice injected with mifepristone. Table 1 shows the summary of echocardiographic data at the baseline, 16 days after induced gene expression, and 16 days after discontinuation of mifepristone injection (data for 70 days induced expression were largely similar, except the mean heart rate was 462 ± 71 beats/min, and are not shown). Discontinuation of mifepristone led to reversal of

Table 1. Echocardiographic Phenotype in Bigenic Mice After Switching On and Off Expression of cTnT-Q92

	NTG			Bigenic Placebo			Bigenic Mifepristone				
	Baseline (n = 7)	**On Mife (n = 5)**	**p**	**Baseline (n = 7)**	**On Placebo (n = 5)**	**p**	**Baseline (n = 14)**	**On Mife (n = 11)**	**Off Mife (n = 4)**	**p**	**p***
Gender (M/F)	5/2	3/2	—	4/3	2/3	—	7/7	5/6	2/2	—	—
Age (days)	132 ± 62	148 ± 61	—	121 ± 29	137 ± 28	—	112 ± 32	128 ± 36	156 ± 34	—	—
HR (beats/min)	380 ± 36	393 ± 24	0.504	384 ± 30	358 ± 32	0.756	369 ± 38	373 ± 43	382 ± 48	0.148	0.620
Body weight (g)	27 ± 5	24 ± 3	0.262	28 ± 5	29 ± 4	0.500	28 ± 4	29 ± 5	29 ± 2	0.637	1.0
ST (mm)	0.77 ± 0.09	0.70 ± 0.10	0.244	0.72 ± 0.05	0.77 ± 0.07	0.162	0.72 ± 0.05	0.73 ± 0.1	0.78 ± 0.05	0.369	0.396
PWT (mm)	0.77 ± 0.1	0.70 ± 0.01	0.244	0.71 ± 0.06	0.72 ± 0.04	0.699	0.73 ± 0.09	0.74 ± 0.07	0.78 ± 0.05	0.538	0.529
LVEDD (mm)	4.1 ± 0.3	4.1 ± 0.06	0.882	4.1 ± 0.3	4.4 ± 0.4	0.158	4.3 ± 0.4	4.2 ± 0.4	4.4 ± 0.4	0.746	0.624
LVESD (mm)	2.9 ± 0.3	2.8 ± 0.1	0.612	2.7 ± 0.3	3.0 ± 0.3	0.264	2.9 ± 0.4	2.5 ± 0.3	2.9 ± 0.2	**0.050**	**0.008**
FS (%)	28.8 ± 3.9	29.9 ± 2.2	0.452	35.4 ± 4.3	33.3 ± 2.0	0.466	31.1 ± 3.3	40.0 ± 3.7	33.9 ± 5.0	**<0.001**	**<0.001**
LVEF-M mode (%)	60.2 ± 7.2	63.7 ± 3.8	0.446	67.8 ± 3.7	68.6 ± 3.7	0.515	65.6 ± 5.3	77.3 ± 3.5	69.2 ± 5.8	**<0.001**	**<0.001**
LVEF-2D (%)	47.4 ± 4.5	46.0 ± 3.3	0.365	47.7 ± 6.1	51.4 ± 5.7	0.141	51.2 ± 6.9	62.8 ± 3.2	49.4 ± 3.8†	**<0.001**	**<0.001**
Vcf (c/s)	4.6 ± 0.83	5.0 ± 0.71	0.557	5.6 ± 0.66	5.7 ± 0.06	0.539	5.3 ± 1.0	6.3 ± 0.60	4.8 ± 0.40	**0.004**	**0.008**
Ao Vmax (m/s)	1.1 ± 0.09	1.1 ± 0.04	0.804	1.1 ± 0.16	1.0 ± 0.13	0.204	1.1 ± 0.13	1.2 ± 0.11	0.98 ± 0.07	**0.001**	**0.006**
E (m/s)	0.90 ± 0.08	0.97 ± 0.1	0.253	0.97 ± 0.08	1.0 ± 0.10	0.670	0.90 ± 0.10	0.98 ± 0.17	0.82 ± 0.09	0.124	0.812
E/A	2.4 ± 0.7	2.7 ± 0.8	0.705	2.2 ± 0.2	2.2 ± 0.6	0.639	2.3 ± 0.7	3.1 ± 1.9	2.3 ± 0.3	0.425	0.128
DT (ms)	35.1 ± 2.8	32.3 ± 2.1	0.169	32.7 ± 4.2	35.2 ± 4.1	0.234	33.9 ± 3.1	39.2 ± 1.6	33.0 ± 1.2†	**0.007**	**0.028**
LVM (mg)	114 ± 29	110 ± 5	0.374	113 ± 21	114 ± 11	0.951	114 ± 20	116 ± 19	130 ± 17	0.292	0.076
LVM/BW (mg/g)	4.1 ± 0.4	4.4 ± 0.09	0.237	4.0 ± 0.4	3.7 ± 0.3	0.138	4.0 ± 0.4	4.0 ± 0.9	4.6 ± 0.5	0.177	0.340
HW/BW (mg/g)	—	4.4 ± 0.5	—	—	4.7 ± 0.3	—	—	4.9 ± 0.3	4.5 ± 0.3	0.126	0.237

Bold face p values indicate statistically significant p values. The p values reflect comparisons between the baseline and follow-up data in each group (paired *t* test). The p* values reflect comparisons of values in bigenic-placebo and bigenic-mifepristone groups. †Unequal SD among the groups ($p < 0.05$) and analysis by Kruskal-Wallis test. The differences among the NTG (on mifepristone), Bigenic on Placebo, and Bigenic on Mifepristone groups were statistically significant (ANOVA) for LVESD ($F = 6.5$, $p = 0.008$), FS ($F = 20.8$, $p < 0.001$), LVEF ($F = 27.2$, $p < 0.001$), Vcf ($F = 9.5$, $p = 0.002$), Ao Vmax ($F = 5.6$, $p = 0.013$), and DT ($F = 14.4$, $p = 0.0002$).

Ao Vmax = aortic maximum velocity; c/s = circumference per second; DT = deceleration time; E = mitral valve inflow E velocity; E/A = mitral valve inflow E/A ratio; FS = fractional shortening; HR = heart rate; HW/BW = heart weight/body weight ratio; LVEDD = left ventricular end-diastolic diameter; LVEF = left ventricular ejection fraction; LVESD = left ventricular end-systolic diameter; LVM = left ventricular mass; LVM/BW = left ventricular mass/body weight ratio; Mife = mifepristone; NTG = non-transgenic; PWT = posterior wall thickness; ST = septal thickness; 2D = two-dimensional; Vcf = velocity of circumferential fiber shortening.

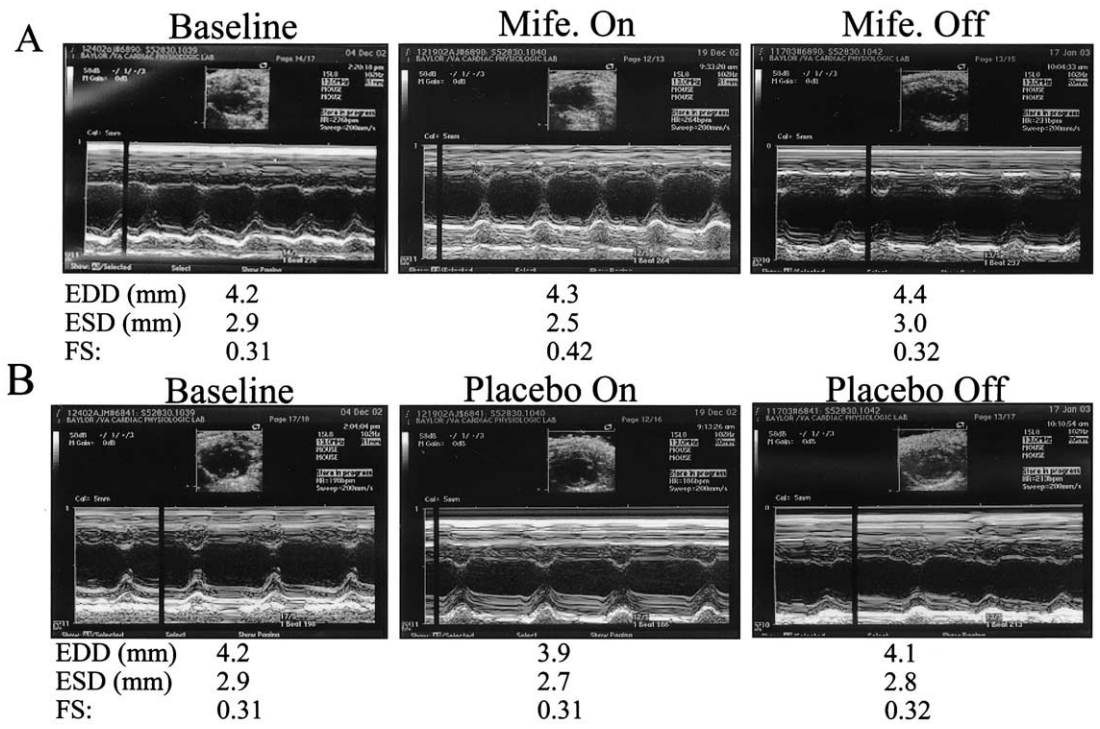

Figure 2. Representative M-mode echocardiographic views of the left ventricle in the treatment **(A)** and placebo **(B)** groups. In each panel, M-mode views are shown before and after switching on and off expression of the cTnT-Q92 transgene. Left ventricular end-diastolic and end-systolic diameters and fractional shortening (EDD, ESD, and FS, respectively) are depicted under each panel.

the echocardiographic phenotypes. Representative M-mode echocardiographic views of the left ventricle in bigenic mice before and after switching on and off expression of the cTnT-Q92 transgene are shown in Figure 2. There were no discernible differences in induced cardiac phenotypes among the three lines used in these experiments. There were no significant changes in other echocardiographic indices. In addition, echocardiographic indices of cardiac systolic function were not significantly different among NTG, monogenic target (TATA-cTnT-Q92), and monogenic regulator (GLp65) mice. The LV fractional shortening, LVEF, and velocity of circumferential fiber shortening values were 28.8 ± 3.9, 60.2 ± 7.2, and 4.6 ± 0.83 in NTG mice (Table 1); 27.6 ± 2.01, 58.3 ± 8.2, and 4.3 ± 0.74 in monogenic target mice; and 29.3 ± 2.45, 61.5 ± 6.7, and 4.7 ± 0.54 in monogenic regulator mice. Injection of mifepristone, at 1,000 μg/kg/day, to NTG mice alone had no significant effects on LV systolic function or other echocardiographic indices (Table 1).

Myocardial cAMP levels. To determine whether increased cAMP levels were responsible for the enhanced myocardial systolic function, we measured total myocardial cAMP levels in the experimental groups. There were no significant differences in myocardial cAMP levels among the experimental groups (NTG: 15.9 ± 3.9 fmol/g; target transgenic: 17.5 ± 3.0 fmol/g; regulator transgenic: 17.0 ± 3.3 fmol/g; bigenic placebo: 16.3 ± 3.4 fmol/g; bigenic mifepristone on: 13.5 ± 4.8 fmol/g; bigenic mifepristone off: 13.1 ± 1.5 fmol/g, $F = 1.70$, $p = 0.162$).

Levels of activated signaling kinases. To determine whether induced expression of cTnT-Q92 also led to changes in expression of activated signaling molecules, we assayed for levels of selected signaling kinases, implicated in cardiac function, by immunoblotting using phospho-specific antibodies. The results, partially shown in Figure 3, were notable for a significant reduction in the expression level of PTEN and to a lesser extent, in levels of p-protein kinase C (PKC)-Zλ-Threonine 538 and p-protein kinase D (PKD)-Serine 744-748, when transgene was turned on for 16 days. There were no significant changes in levels of PI3K-p110-α, β, δ; and p85 or p-PKC-δ-Serine 643, p-protein kinase A (PKA), p42/p44 mitogen-activated protein kinases, glycogen synthase kinase 3β, calcineurin, or phosphorylated phospholamban. Discontinuation of mifepristone led to normalization of the activated signaling molecules. There were no statistically significant changes in the expression levels of selected signaling kinases after shorter (2, 4, and 8 days) or longer (70 days) duration of induction of expression of cTnT-Q92 in the heart. To determine whether changes in the expression levels of signaling kinases were independent of administration of mifepristone, NTG mice were injected with 1,000 μg/kg/day of mifepristone ($n = 4$) and immunoblotting was repeated. Mifepristone injection alone did not induce sig-

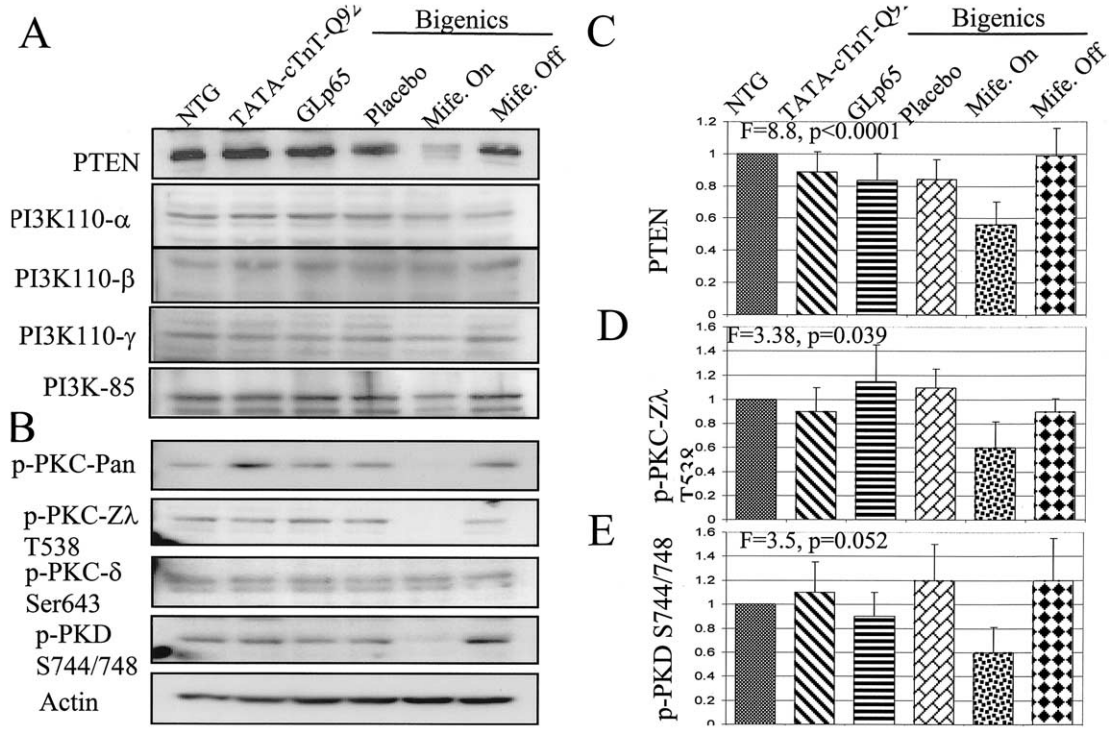

Figure 3. Expression of selected signaling molecules after short-term induced expression of cTnT-Q92 in bigenic mice. **(A)** Immunoblots showing levels of the major components of PI3K pathway: each **lane** represents one experimental group, as labeled. Bigenic mice were injected with either a placebo or mifepristone (Mife On) at 1,000 μg/kg/day for 16 days. Mife Off indicates bigenic mice after withdrawal of mifepristone for 16 days. **(B)** Immunoblots showing expression levels of selected phosphorylated PKC isoforms; the **bottom panel** shows expression levels of sarcomeric actin, used as a control for loading condition. Groups are as indicated for **panel A**. **(C, D, and E)** Quantitative levels of PTEN, p-PKC-Zλ-T538, and p-PKD-S744-748. The F and p values are shown for the entire groups. All p values were <0.05 when levels of PTEN, p-PKC-Zλ-T538, and p-PKD-S744-748 were compared with their corresponding levels in other experimental groups (ANOVA).

nificant changes in levels of p-PKC-Zλ-Threonine 538 and p-PKD Serine 744-748 or PTEN in the heart.

Myocardial levels of molecular markers of hypertrophy and fibrosis. Expression levels of *NPPA*, *NPPB*, *ACTA1*, *ATP2A2*, *COL1A1*, *COL1A2*, and *COL3A1* were determined using real-time quantitative RT-PCR, 16 and 70 days after induction of expression of cTnT-Q92 and after switching off expression of the transgene. After 16 days of induced expression, myocardial levels of molecular markers of hypertrophy and fibrosis were largely unchanged with the exception of a modest decrease in ATP2A2 levels (data not shown). However, long-term (70 days) induced expression of cTnT-Q92 led to significant reductions in expression levels of *NPPA* and *NPPB* without a significant change in *ATP2A2* levels (Fig. 4A). Furthermore, expression levels of *COL1A1* and *COL1A3*, encoding the predominant cardiac collagen, were increased significantly, whereas expression level of *COL1A2* was largely unchanged (Fig. 4A).

Histologic phenotype. To detect whether induction of expression of cTnT-Q92 was associated with morphologic or histologic phenotypes characteristics of human HCM, we determined heart weight, heart weight/body weight ratio, LV weight/body weight ratio, myocyte cross-sectional area, number of myocytes per microscopic fields, % CVF, and the extent of myocyte disarray. Short-term induced expression of the cTnT-Q92 did not lead to discernible changes in histologic or morphologic phenotypes, as no significant differences in morphometric histologic indices were detected among NTG alone, NTG injected with mifepristone, target and regulator monogenic mice, and bigenic mice in the placebo and mifepristone groups. However, as shown in Figure 4B, induced expression of cTnT-Q92 for 70 days led to a 1.45-fold increase in CVF, in accord with the observed increased expression of the procollagen mRNAs. Turning off expression of cTnT-Q92 led to normalization of CVF (bigenic placebo: 2.9 ± 0.65; switch on: 4.2 ± 1.0; switch off: 2.0 ± 0.24; $F = 12.4$, $p = 0.001$; $p < 0.05$ between placebo and switch on). Long-term treatment of NTG mice with mifepristone had no significant effect on CVF (2.5 ± 0.66 vs. 2.8 ± 0.73, $n = 5$, $p = 0.538$). With regard to indices of hypertrophy, neither heart weight/body weight ratio, echocardiographic indices, nor myocyte cross-sectional area was significantly different among the experimental groups. Myocyte disarray comprised 6.3 ± 3.6% of the myocardium in the bigenic mice induced with mifepristone for 70 days, whereas it was 2.8 ± 2.6% in NTG mice ($T = 2.02$, $p = 0.08$) and 5.0 ± 4.7% in bigenic mice after turning off expression of the transgene (Kruskal-Wallis test: $H = 2.92$, degrees of freedom = 2, $p = 0.232$).

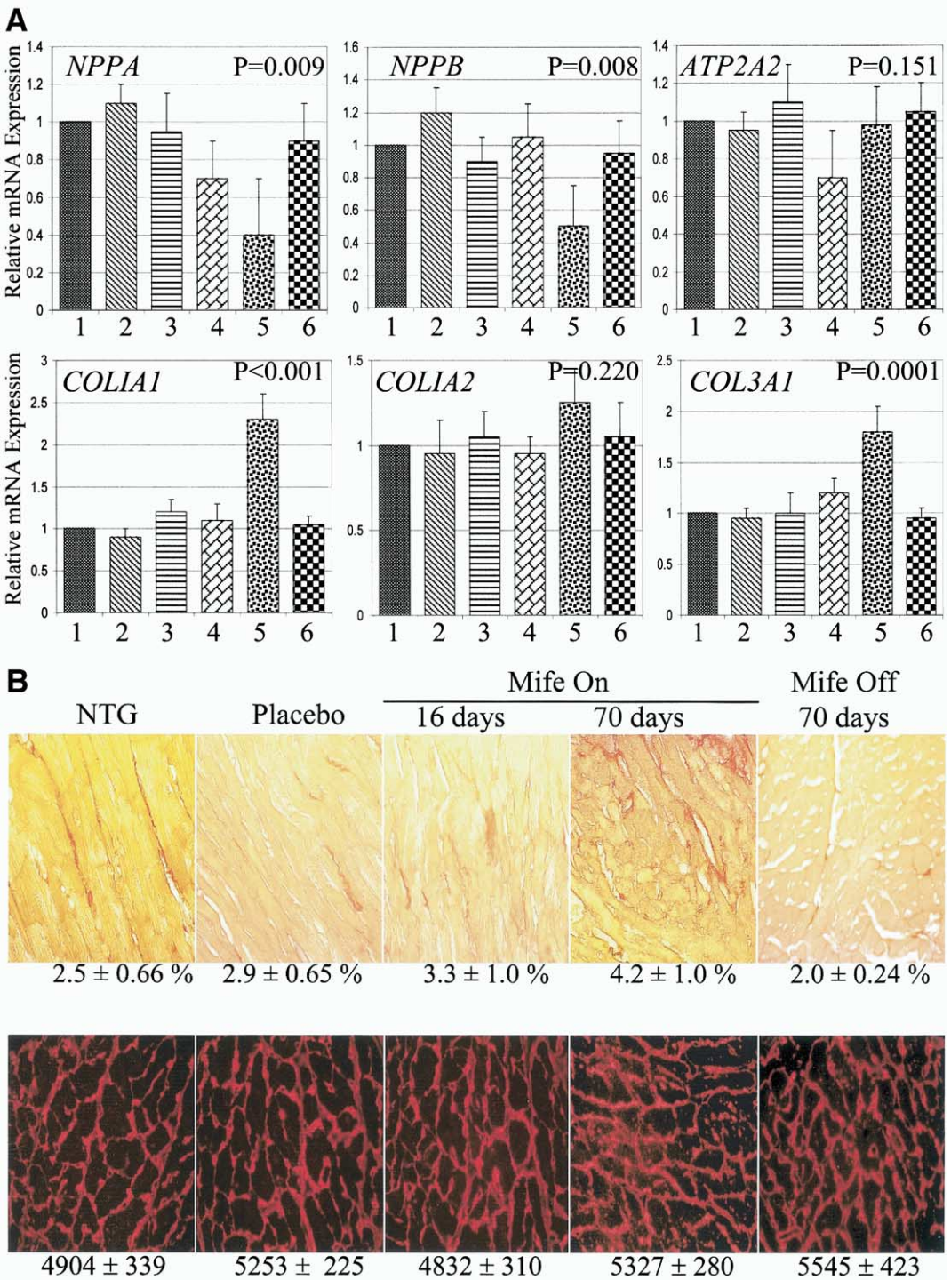

Figure 4. Induction of expression of markers of hypertrophy and fibrosis and histologic phenotypes. **(A)** Relative expression levels of marker of hypertrophy (cardiac load) and fibrosis. The **upper panels** show relative expression levels of A-type natriuretic peptide (*NPPA*), B-type natriuretic peptide (*NPPB*), and sarcoplasmic reticulum calcium ATPase 2a (SERCA2a or *ATP2A2*) in the heart in the experimental groups, determined by real-time quantitative RT-PCR. All values were corrected for levels of mRNA in the NTG control group. Labels are as follows: 1: non-transgenic littermates (NTG); 2: target transgene only (TATA-cTnT-Q92); 3: regulator transgene only (GLp65); 4: bigenic in the placebo group; 5: bigenic mice on mifepristone group (16 days); and 6: bigenic mice off mifepristone for 16 days. All p values were <0.05 for comparisons of *NPPA* and *NPPA* levels between bigenic mice on mifepristone versus each of the other groups (*t* test) and among all groups (ANOVA). The **lower panels** show relative expression levels of cardiac collagens, namely *COL1A1*, *COL1A1*, and *COL3A1* in the experimental groups. Labels are as above. All p values are <0.001 and <0.01 in comparing *COL1A1* and *COL3A1* levels, respectively, between bigenic mice on mifepristone versus each of the other groups (*t* test) and among all groups (ANOVA). **(B)** Histologic phenotypes in the NTG and bigenic mice in the placebo and mifepristone groups. The **upper panels** represent Sirius Red staining of thin myocardial sections used to determine CVF. The values under each micrograph indicate mean ± SD of CVF in the corresponding group. The lower panels show immunofluorescence micrographs stained with anti-laminin B monoclonal antibody and a rhodamine-conjugate secondary antibody to delineate the myocyte boundaries. Values under each panel indicate mean ± SD pixels per myocyte in each group.

DISCUSSION

We describe a switch on–switch off bigenic mouse model in which expression of the mutant sarcomeric protein cTnT-Q92, responsible for human HCM (4), was turned on and off by administering and withdrawing mifepristone, the activating ligand for the otherwise inactive regulatory protein. Short-term induced expression of the mutant cTnT-Q92 enhanced myocardial systolic function, a characteristic phenotype of HCM, independent of myocardial cAMP levels and in the absence of histologic or molecular phenotypes of hypertrophy, disarray, or fibrosis. Enhanced myocardial contractile performance was associated with reduced levels of selected active signaling kinases, implicated in regulating cardiac function. Long-term induced expression of cTnT-Q92 led to increased expression of procollagen genes and interstitial fibrosis, whereas levels of the molecular markers of hypertrophy were reduced. The induced phenotypes, namely, interstitial fibrosis, increased expression of procollagen genes and signaling kinases, and enhanced cardiac systolic function were completely reversed upon switching off expression of cTnT-Q92. These results, in a genetic animal model of human HCM mutation, establish the reversibility of HCM phenotypes, a prerequisite in designing future therapies targeted at correcting the underlying genetic mutations. The results also identify the primary defect conferred by cTnT-Q92 as enhanced myocardial systolic function, independent of myocardial cAMP levels. Thus, the potential utility of the switch on–switch off bigenic mouse model is at least twofold: first, to establish the reversibility of the cardiac phenotypes induced by HCM mutations; and second, to identify the initial defects conferred by the mutations through delineation of the sequence and pathogenesis of ensuing functional, molecular, histologic, and morphologic phenotypes in HCM.

The finding of reversal and normalization of interstitial fibrosis after switching off expression of the mutant troponin T was confirmed by two complementary methods of real-time RT-PCR analysis of expression of major procollagen genes in the heart as well as by quantitative morphometric analysis of collagen protein in myocardial sections stained with collagen-specific dye. Reversal and normalization of interstitial fibrosis in the heart has potential clinical implications, because interstitial fibrosis is a potential risk factor for sudden cardiac death in humans with HCM (13). In contrast to interstitial fibrosis, however, cardiac hypertrophy was absent in the bigenic cTnT-Q92 mice, as was also the case in the conventional transgenic cTnT-Q92 mice (8,14). Notably, humans with HCM caused by cTnT-Q92 usually exhibit mild or minimal hypertrophy (15). The absence of discernible hypertrophy in the bigenic mice or even the presence of smaller myocyte and heart size in the conventional cTnT-Q92 mice (11,14) could simply reflect the enhanced cardiac performance, which affords the heart the ability to handle the normal load at a smaller cardiac mass. In accord with the foregoing, expression levels of markers of cardiac mass, namely *NPPA* and *NPPB*, were reduced significantly after long-term induced expression of cTnT-Q92. Regarding myocyte disarray, it was increased modestly in the bigenic mice following long-term induced expression. However, changes were of borderline statistical significance. Therefore, it remains to be determined whether switching off expression of the mutant protein could also lead to reversal of hypertrophy and disarray, as we have shown previously through pharmacologic interventions in genetically engineered animal models (10,12).

The molecular basis of enhanced myocardial contractile performance after induction of expression of cTnT-Q92 was not discerned, suffice that it was independent of myocardial cAMP levels or phosphorylation of phospholamban, or injection of mifepristone. We have shown previously that myofibrils isolated from the hearts of the conventional cTnT-Q92 transgenic mice exhibited enhanced calcium sensitivity of tension generation, evidenced by a higher values of half-maximally activating −log of the molar free Ca^{+2} concentration (pCa_{50}) (16). The latter, in conjunction with the results of studies in skinned fibers isolated from rabbit and pig hearts showing enhanced Ca^{+2} sensitivity (17,18), are likely to explain the observed enhanced cardiac systolic function in our bigenic mice in the induced state. In addition to enhanced Ca^{+2} sensitivity, other mechanisms, including changes in signaling molecules, as observed after induced expression of cTnT-Q92, could also affect cardiac function. Regarding the potential impact of signaling molecules, our study was limited to analysis of a limited number of signaling molecules, implicated in regulating cardiac structure and function, and was not comprehensive. Nonetheless, a significant change in the expression level of tumor suppressor PTEN, implicated in regulating cardiac function (19), was detected. The PTEN, in addition to affecting cardiac function, also plays an essential role in proper cell adhesion, alignment, and migration (20,21). Thus, given the essential role of PTEN in cellular attachment and alignment, reduced expression of PTEN could be involved in the subsequent evolution of myocyte disarray in HCM. The molecular mechanisms that regulate expression and activation of PTEN, p-PKC-Zλ-Threonine 538, and p-PKD-Serine 744–748 in the heart remain unknown. We showed that reduction in the expression levels of selected signaling molecules was independent of mifepristone administration. The role of changes in the activation state of selected signaling kinases, noted earlier, in the pathogenesis of cardiac phenotypes in HCM merits additional investigations.

The findings of the present study are restricted to the cTnT-Q92 mutation. We note that the initial impetus and the ensuing early and intermediary phenotypes, imparted by the mutant sarcomeric proteins are likely to be diverse and vary according to the topography of the causal mutations and genes. The bulk of experimental data implicates enhanced myofibrillar Ca^{+2} sensitivity as the initial defect imparted by mutations in the cTnT (16–18). However,

with regard to mutations in the β-MyHC and other genes, reduced myofibrillar and myocardial contractile performance and/or reduced ATPase activity have emerged as the prime candidates as the initial defects (reviewed in reference 2). The complexity of the pathogenesis of HCM phenotypes is further elucidated by the divergence of phenotypic expression of mutations in the same causal gene, which lead to morphologically contrasting phenotypes of hypertrophic, dilated, and restrictive cardiomyopathies (22,23). Therefore, a single animal model is insufficient to elucidate the pathogenesis of HCM caused by a diverse array of mutations.

Expression of transgene (human) cTnT in the background of endogenous cTnT (mouse) could confound the effect of the mutant transgene protein. However, we have previously shown that expression of wild-type transgene cTnT in the mouse heart did not induce discernible histologic or echocardiographic phenotypes (8). With regard to the potential background effects of the regulator protein, we included control groups composed of transgenic mice expressing only the regulator protein (GLp65) and bigenic mice treated with a placebo only. We detected no discernible phenotype in any of the control groups. Moreover, previous studies have shown that the tripartite regulator protein is inactive when not bound to mifepristone (6,24,25). Finally, with regard to the potential confounding effects of the administration of mifepristone, we injected mifepristone in NTG mice and detected no significant effect on cardiac structure or function. These precautions further strengthen our conclusions that the observed functional, molecular, and histologic changes reflect the direct effects of induced expression of the mutant cTnT-Q92 transgene protein in the heart.

In conclusion, we describe a switch on–switch off bigenic mouse model in which expression of cTnT-Q92, responsible for human HCM, could be turned on and off as needed. The results show that the established cardiac phenotypes in HCM are not permanent and could be reversed upon turning off expression of the mutant protein. The findings also indicate that the initial phenotype induced by the cTnT-Q92 is enhanced myocardial systolic function followed by changes in activation of signaling molecules implicated in cell organization and myocyte function, followed by interstitial fibrosis. These findings provoke pursuing specific therapies directed at correcting the underlying the genetic defect in HCM.

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