Hypertrophic Cardiomyopathy in Cardiac Myosin Binding Protein-C Knockout Mice

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Abstract—Familial hypertrophic cardiomyopathy (FHC) is an inherited autosomal dominant disease caused by mutations in sarcomeric proteins. Among these, mutations that affect myosin binding protein-C (MyBP-C), an abundant component of the thick filaments, account for 20% to 30% of all mutations linked to FHC. However, the mechanisms by which MyBP-C mutations cause disease and the function of MyBP-C are not well understood. Therefore, to assess deficits due to elimination of MyBP-C, we used gene targeting to produce a knockout mouse that lacks MyBP-C in the heart. Knockout mice were produced by deletion of exons 3 to 10 from the endogenous cardiac (c) MyBP-C gene in murine embryonic stem (ES) cells and subsequent breeding of chimeric founder mice to obtain mice heterozygous (+/−) and homozygous (−/−) for the knockout allele. Wild-type (+/+) cMyBP-C+/+ and cMyBP-C−/− mice were born in accordance with Mendelian inheritance ratios, survived into adulthood, and were fertile. Western blot analyses confirmed that cMyBP-C was absent in hearts of homozygous knockout mice. Whereas cMyBP-C+/+ mice were indistinguishable from wild-type littermates, cMyBP-C−/− mice exhibited significant cardiac hypertrophy. Cardiac function, assessed using 2-dimensionally guided M-mode echocardiography, showed significantly depressed indices of diastolic and systolic function only in cMyBP-C−/− mice. Ca2+ sensitivity of tension, measured in single skinned myocytes, was reduced in cMyBP-C−/− but not cMyBP-C+/− mice. These results establish that cMyBP-C is not essential for cardiac development but that the absence of cMyBP-C results in profound cardiac hypertrophy and impaired contractile function. (Circ Res. 2002;90:594-601.)

Key Words: myosin binding protein-C heart myocardium gene knockout sarcomeric proteins

Myosin binding protein-C (MyBP-C), also known as C-protein,1 is a thick filament accessory protein that is present in nearly all vertebrate striated muscles but whose function is unknown. Nonetheless, there is compelling evidence to suggest that MyBP-C is a significant determinant of muscle contractile properties. In particular, cardiac MyBP-C (cMyBP-C) is a target for phosphorylation in response to various inotropic stimuli, including sympathetic stimulation that affect trisphosphorylation of cMyBP-C via cAMP-dependent protein kinase (PKA).2 In addition, mutations of the cMyBP-C gene are a leading cause of familial hypertrophic cardiomyopathy (FHC),3 an inherited disorder linked to mutations in sarcomeric proteins (for review, see Bonne et al4 and Seidman and Seidman5).

However, despite clues suggesting the importance of cMyBP-C to cardiac health, the function of cMyBP-C has remained enigmatic. For instance, although numerous studies have investigated effects of PKA on cardiac contractility (eg, Strang et al6 and Patel et al7), the role, if any, of cMyBP-C in mediating contractile responses to PKA has been difficult to discern.8–10 Similarly, the mechanisms by which cMyBP-C mutations affect cardiac function are not well understood. Unlike most missense FHC mutations, the majority of cMyBP-C mutations are predicted to encode truncated proteins that lack portions of the C-terminus.3 However, because truncated peptides have not been detected in FHC patients,11,12 it is unclear whether mutant proteins are incorporated into sarcomeres or whether they are degraded. Thus, it is unknown whether disease results from N-terminal cMyBP-C fragments that exert dominant-negative effects as “poison polypeptides”13 or from reduced expression of cMyBP-C due to a null allele (ie, haploinsufficiency).

The purpose of the current study was to investigate the role of cMyBP-C in normal cardiac function and investigate mechanisms by which cMyBP-C mutations cause disease. Targeted ablation of the cMyBP-C gene was used to produce mice lacking cMyBP-C in the heart so that structural and functional effects due to complete elimination of cMyBP-C...
C57/B66 blastocysts and implanted into pseudopregnant C57/B6 females (Jackson Laboratories, Bar Harbor, Maine).

Targeted clones from both ES cell lines gave rise to chimeric founder mice. Founders (designated 2G12 from the R1 line and 9F8 from the AB2.2 line) were bred to C57/B6 and 129/Sv females for transmission of the mutant cMyBP-C allele. Progeny of the 2 founder lines were maintained as separate colonies and offspring were genotyped by Southern blot and polymerase chain reaction (PCR). Appropriate breeding generated mice homozygous for the cMyBP-C knockout mutation. No phenotypic differences between offspring of the 2 founder lines were noted, and progeny from both lines were used for data analyses. Unless noted otherwise, all measurements were performed using adult (8- to 36-week-old) littermate mice of both sexes on mixed (ie, C57/B6) or pure 129/Sv genetic backgrounds.

Care and handling of all mice was carried out according to institutional guidelines approved by the Association for Assessment and Accreditation of Laboratory Care International (AAALAC). For euthanasia, mice were injected with heparin (intraperitoneal), anesthetized with isofluran, and hearts rapidly excised.

RNA Analyses
Total RNA was isolated from whole hearts using Trizol (GIBCO BRL Life Technologies). cDNA was synthesized for reverse transcription (RT-PCR using oligo-d(T) primers and the Thermoscript RT-PCR system (GIBCO BRL Life Technologies). Primers to exons 1 to 2, 3 to 5, and 25 to 34 are listed in the online data supplement available at http://www.circresaha.org.

Northern and dot blot analyses were performed as described.15 Template DNA for cMyBP-C probes was obtained by PCR of a mouse cMyBP-C cDNA (Accession No. AF097333) using primers to exons 1 to 2 and 25 to 34. DNA was 32P-labeled by random priming, hybridized at 45°C in ULTRAlabel by random priming, hybridized at 45°C in ULTRAhyb (Ambion, Inc), and washed at 55°C in 0.1X SSC, 0.1% SDS. Transcript-specific oligonucleotides (sequences in online data supplement) to atrial natriuretic factor (ANF), brain natriuretic factor (BNP), a-skeletal actin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were 32P end-labeled and hybridized with RNA dot blots at 42°C in ULTRAhyb-oligo buffer.

Protein Analyses
Total heart proteins were prepared by homogenization in (mmol/L) 50 KCl, 5 Tris-Cl pH 7.5, 5 EGTA, 2 NaCl, 1 DTT, 1% Triton X-100, leupeptin (20 μg/mL), pepstatin (20 μg/mL), 0.2 PMSF, and pepstatin (0.2 mg/mL). Myofibrils were pelleted from homogenates, washed by repeated suspension/homogenization in fresh buffer, and resuspended in buffer lacking Triton X-100. SDS-PAGE analysis of proteins was performed as described.16

Antibodies
A polyclonal antibody against cMyBP-C was made by immunizing rabbits against rat cMyBP-C purified as described.17 cMyBP-C was detected on immunoblots by incubation with antibody (1:10 000) and colorimetric detection (Bio-Rad) using a secondary antibody conjugated to alkaline phosphatase. Polyclonal antibodies against rabbit skeletal MyBP-C18 and mouse cardiac C0-C1 19 were diluted 1:3000. A herpes virus thymidine kinase (TK) gene permitted negative selection with ganciclovir.

The targeting vector (SacII-digested) was electroporated into 2 separate 129/Sv ES cell lines, R114 and AB2.2 (Stratagene, La Jolla, Calif). Recombinant cells were identified by geneticin (G418 sulfate) and ganciclovir resistance. Southern blots using 5′ and 3′ probes outside the homology units confirmed proper targeting due to homologous recombination between vector and the endogenous cMyBP-C locus (Figure 1B). Selected clones from R1 and AB2.2 lines were karyotyped and 1 clone from each line was injected into could be assessed. To investigate potential effects due to haploinsufficiency, mice heterozygous for the knockout (null) allele were also studied.

Materials and Methods

Gene Targeting
A P1 clone containing the entire murine cardiac MyBP-C sequence was obtained from an embryonic stem (ES) cell library (strain 129/SvJ, Genome Systems, St Louis, Mo). A 10.5-kb EcoRI fragment containing the 5′ end of the MyBP-C gene was subcloned and mapped. A 1.8-kb KpnI/EcoRV fragment upstream of exon 3 and a 3.8-kb BamHI fragment downstream of exon 10 were used as 5′ and 3′ gene homology units in the targeting vector and exons 3 to 10 were replaced with a neomycin (neo) resistance gene (Figure 1A). A herpes virus thymidine kinase (TK) gene permitted negative selection with ganciclovir.

The targeting vector (SacII-digested) was electroporated into 2 separate 129/Sv ES cell lines, R114 and AB2.2 (Stratagene, La Jolla, Calif). Recombinant cells were identified by geneticin (G418 sulfate) and ganciclovir resistance. Southern blots using 5′ and 3′ probes outside the homology units confirmed proper targeting due to homologous recombination between vector and the endogenous cMyBP-C locus (Figure 1B). Selected clones from R1 and AB2.2 lines were karyotyped and 1 clone from each line was injected into
LV mass as \(1.05 \times \left[\frac{1}{2}(PWd + AWd + LVDd) - (LVDd)^2\right]\), and relative wall thickness (RWHt) as \(\frac{1}{2}(PWd + LVDd)\).

**Histology and Ultrastructure**

For light microscopy, hearts were dissected and fixed in 10% formalin. Fixed tissue was paraffin embedded, sectioned (5 µm), and stained with Masson’s trichrome. For transmission electron microscopy, LV cubes (~1 to 2 mm³) were immersed in PBS containing 2% formaldehyde/2% glutaraldehyde. Samples were fixed (micro-wave facilitated) and postfixed in 2% osmium tetroxide. Propylene oxide was used as transition solvent, and a 1:1 mixture of Polybed 812:Spurr’s resin (Polysciences, Inc) was used for infiltration and embedding. Sections (70 nm) were cut on a Reichert-Jung Ultramicrotome, placed on Cu grids, and post-stained with uranyl acetate and lead citrate. Sections were viewed using a Philips CM120 STEM at 80 kV.

**Force Measurements**

Myocyte preparations were isolated from whole hearts as described and permeabilized (skinned) for 10 minutes in (mmol/L) 1 Mg²⁺, 100 KCl, 2 EGTA, 4 ATP, 10 imidazole, pH 7.0, 0.3% Triton X-100, and 0.65 mg/mL saponin. After skinning, myocytes were washed and stored on ice for use the same day. Force measurements were performed as described with relaxing and activating solutions as described.

**Statistical Analysis**

Unless otherwise noted, data were reduced to mean ± SD. Comparisons were performed by 1-way ANOVA and Post hoc comparisons were made using Tukey’s or Dunn’s tests.

**Results**

**Generation of cMyBP-C Knockout Mice**

Figure 1 shows the targeting strategy used to ablate the cMyBP-C gene in mouse ES cells by homologous recombination. The strategy was designed so that exons 3 to 10 of the endogenous cMyBP-C gene could be replaced with a gene encoding neo resistance. Accordingly, knockout of cMyBP-C was expected to result from combined effects of (1) inappropriate splicing of exons 2 and 11, leading to a reading frame shift (at position 98) and introduction of a premature stop codon (at 118), and (2) aberrant transcript expression due to the presence of the neo cassette.

Targeting was applied in 2 independent ES cell lines, RT1 and AB2.2; 288 and 192 G418-resistant clones were selected from the respective lines. Correct targeting was confirmed by Southern blot in at least 6 clones from each line, and clones from both lines gave rise to founders. Analysis of progeny derived from the 2 lines showed that mice heterozygous (+/−) and homozygous (−/−) knockout mice. Total RNA (≈3 μg/lane) was initially hybridized with a probe to exons 25 to 34 (3’ probe). The blot was stripped and re-hybridized with a probe to exons 1 to 2 (5’ probe).

**cMyBP-C RNA and Protein Expression in Knockout Mice**

RT-PCR was performed using primers to 3 regions of cMyBP-C cDNA to assess cMyBP-C transcript expression in wild-type, cMyBP-C+/-, and cMyBP-C−/− knockout hearts (Figure 2). Primers to exons 3 to 5 amplified a 345-bp product from wild-type and cMyBP-C+− cDNA. However, as expected following deletion of exons 3 to 10, the product was not detected using cMyBP-C−/− cDNA. Transcription from the mutant allele was still evident because primers to exons 1 to 2 and 25 to 34 resulted in PCR products of expected size. Northern blots were performed using probes complementary to 5’ and 3’ regions of the cMyBP-C mRNA to evaluate cMyBP-C transcript size. Probes to exons 1 to 2 (5’ probe) and exons 25 to 34 (3’ probe) hybridized to a transcript at ≈4.5 kb in total RNA from wild-type and cMyBP-C+/- hearts (Figure 2B). However, the 4.5-kb transcript was not detected in cMyBP-C−/− RNA. Instead, the 5’ probe identified 2 to 3 shortened transcripts in cMyBP-C+/- and cMyBP-C−/− RNA, whereas no transcripts were detected in cMyBP-C−/− RNA using the 3’ probe. These data suggest that transcription from the mutant allele is initiated properly (because the 5’ probe recognized transcripts in all RNA), but that transcription...
and/or splicing are altered such that multiple transcripts are produced, and expression downstream of the deletion (eg, exons 25 to 34) is reduced.

To determine whether the truncated cMyBP-C mRNA transcripts produced stable proteins, western blots were performed using total heart homogenates and myofibril proteins from wild-type and cMyBP-C knockout mice. As shown in Figure 3, a polyclonal antibody against rat cMyBP-C (see Materials and Methods) recognized a band at ~150 kDa in rat and (wild-type) mouse cardiac myofibrils. The antibody was specific for cMyBP-C because little or no cross-reactivity with skeletal MyBP-C isoforms was observed. The antibody also detected full-length cMyBP-C in cMyBP-C−/− hearts. Densitometric analysis of cMyBP-C to α-actinin ratios on Coomassie-stained gels showed that the relative abundance of cMyBP-C in cMyBP-C−/− hearts was the same as in wild-type hearts (1.20±0.09, n=6, for +/+ and 1.15±0.11, n=6, for +/−). In contrast, the 150-kDa cMyBP-C band was not detected by Western blot in cMyBP-C−/− hearts, indicating that full-length cMyBP-C was absent in homozygous knockout mice. Although faint bands at lower molecular weight were occasionally seen on overloaded blots of total homogenates, consistent evidence for expression of truncated cMyBP-C peptides was not found. The latter result suggests that either the cMyBP-C transcripts are not translated into stable proteins23 or that they are not detectable by our assays. The former seems more likely because an antibody against the N-terminus (C0-C1) of mouse cMyBP-C19 also failed to detect proteins in cMyBP-C−/− mice (data not shown).

Western blots were also probed with an antibody against rabbit (fast) skeletal MyBP-C.18,22 Although a band at ~140 kDa was identified in mouse skeletal myofibrils, the antibody did not detect protein in cardiac myofibrils from cMyBP-C knockout mice (data not shown). This result agrees with studies showing that skeletal MyBP-C isoforms are not normally expressed in mammalian heart19,23 and suggests that even under conditions of hypertrophy transcomplementation of (fast) skeletal MyBP-C does not occur.

Cardiac Morphology and Ultrastructure in cMyBP-C Knockout Mice

As shown in Figure 4A, adult homozygous knockout mice exhibited significant cardiac hypertrophy evident as visible enlargement of cMyBP-C−/− hearts compared with either wild-type or cMyBP-C+/− littermates. Heart-to-body weight ratios were significantly increased in cMyBP-C−/− mice.
pertrophic cardiomyopathy, including myocyte disarray and hearts showed structural rearrangements consistent with hy-
upregulated in cMyBP-C protein expression of

<table>
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<th>Echocardiography Summary Data</th>
<th>+/+ (n=9)</th>
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<th>+/- (n=7)</th>
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Values are mean±SD. Significantly different vs age-matched *wild-type or †heterozygous mice, P<0.05 (1-way ANOVA).

(Figure 4B and Table), as were LV diameter and AW and PW thicknesses. Molecular markers of hypertrophy, including transcript expression of ANF, BNP, and α-skeletal actin and protein expression of β-myosin heavy chain, were also upregulated in cMyBP-C⁻/⁻ knockout mice but not cMyBP-C⁺/⁺ mice (Figures 4C and 4D). Hypertrophy was evident at early time points as shown by elevated LV-to-body weight ratios and LV diameter in mice as young as 3 weeks (Table).

Histological examination of the cMyBP-C⁻/⁻ knockout hearts showed structural rearrangements consistent with hypertrophic cardiomyopathy, including myocyte disarray and fibrosis. In cMyBP-C⁻/⁻ hearts, the disarray was evident as an overall decrease in apparent tissue organization such that it was more difficult to find regions of well-organized myocytes in either longitudinal or cross-sectional planes. Foci of interstitial fibrosis were also noted in 3 of 5 cMyBP-C⁻/⁻ hearts. However, misshapen or distorted myocytes (eg, stellar shapes) were not typically observed. Although somewhat less distinct in cMyBP-C⁻/⁻ hearts, sarcomere striation patterns were seen in nearly all sections examined (Figures 5A through 5F). Ultrastructural examination of sections by transmission electron microscopy also showed sarcomeres with

Figure 5. Cardiac histology and ultrastructure in cMyBP-C knockout mice. Light micrographs (100× oil) of sections stained with Masson’s trichrome from wild-type (a), heterozygous (b), and homozygous (c) cMyBP-C littermate knockout mice (14 weeks). Note the presence of cross-striations in all fields. d, e, and f, Higher magnification of sarcomeres in a, b, and c, respectively. Scale bars=10 μm. Transmission electron micrographs showing sarcomere ultrastructure in wild-type (g), heterozygous (h), and homozygous (i) littermate knockout mice (16 weeks). Scale bar=2 μm. j through l, Higher magnification of sarcomeres in g through i. Scale bar=1 μm.
prominent Z lines, M lines, and A bands in cMyBP-C−/− and cMyBP-C+/− hearts (Figures 5G through 5L). However, the close lateral alignment of myofibrils was often not maintained in cMyBP-C−/− hearts such that Z lines of adjacent myofibrils were frequently out of register. Mitochondrial abnormalities, including an increase in number and decrease in size, were also common in cMyBP-C−/− hearts.

Cardiac Function in cMyBP-C Knockout Mice

Transthoracic 2-dimensionally directed M-mode echocardiography was used to assess LV function in young (∼3 weeks) and adult (>8 weeks) cMyBP-C knockout mice. Representative M-mode and Doppler images from adult mice are shown in Figure 6. Summary data for all mice are shown in the Table. Body weight and heart rates did not differ with genotype, but LV fractional shortening (FS%), an index of systolic function, was significantly reduced in both young and adult cMyBP-C−/− mice compared with age-matched wild-type and cMyBP-C+/+ mice. FS% values for wild-type and cMyBP-C+/+ mice, although lower than those reported for another cMyBP-C mouse model,24 overlapped with respect to standard deviation and were similar to other published values.25,26 Differences in anesthetic, age, sex, strain, and interpretive variability in wall thickness measurements may all affect calculated FS% values. Isovolumic relaxation time (IVRT), ie, the time between closure of the aortic valve and opening of the mitral valve, was increased in young and adult cMyBP-C−/− mice, indicating that relaxation was significantly impaired in cMyBP-C−/− mice.

Myofilament Ca2+ Sensitivity of Tension

Force was measured in permeabilized myocytes to assess contractile deficits at the cellular level. As shown in Figure 7, plots of normalized tension versus pCa for cMyBP-C−/− myocytes were right-shifted relative to those of wild-type littermates, indicating reduced myofilament Ca2+ sensitivity of tension. Summary data showed a small but significant reduction in the midpoint of the tension-pCa curve (ie, the pCa50) in cMyBP-C−/− myocytes compared with wild-type (5.66±0.03, n=10, versus 5.73±0.05, n=7), whereas the pCa50 was unchanged in cMyBP-C+/− myocytes (5.71±0.06, n=9). Slopes (ie, Hill coefficients) of tension-pCa relationships were not different between wild-type and cMyBP-C+/− or cMyBP-C−/− myocytes.

Discussion

In the present study, we used gene targeting to produce knockout mice that lack cMyBP-C in heart. The results show that cMyBP-C is not essential for sarcomere assembly or cardiac development but that the absence of cMyBP-C is sufficient to trigger profound cardiac hypertrophy, impaired systolic and diastolic function, and depressed myocyte contractile properties.

cMyBP-C Is Not Essential for Sarcomere Assembly

Our findings that cMyBP-C knockout mice are viable and display well-developed sarcomeres conflict with previous suggestions that cMyBP-C performs essential structural roles during sarcomere assembly and myofibrillogenesis. The proposals were based in part on observations that MyBP-C affects thick filament assembly in vitro27,28 and that MyBP-C expression coincides with myosin and myofibril assembly during development.19,29 However, the occurrence of sarcomeres with an overtly normal appearance in cMyBP-C−/− mice and striation patterns visible at the light microscope

Figure 6. Echocardiographic recordings of hearts from cMyBP-C knockout mice. Left, M-mode LV images from representative wild-type (+/+), heterozygous (+/−), and homozygous (−/−) knockout mice. Right, Doppler images of aortic flow (downward deflections) and mitral flow (upward deflections) in wild-type (+/+), heterozygous (+/−), and homozygous (−/−) knockout mice.

Figure 7. Ca2+ sensitivity of tension in skinned myocytes from cMyBP-C knockout mice. Normalized tension-pCa relationships from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) knockout myocytes were plotted by expressing Ca2+-activated tension as a fraction of maximal tension in a pCa 4.5 solution. Error bars represent SEM of 7 to 10 myocytes from 3 to 5 age-matched mice (12 to 15 weeks).
Hypertrophic Cardiomyopathy in Homozygous cMyBP-C Knockout Mice

On the other hand, genetic ablation of cMyBP-C leads to significant hypertrophy and contractile deficits evident at both the whole heart and single cell levels. These results indicate that loss of cMyBP-C, distinct from expression of mutant cMyBP-C proteins, is sufficient to induce hypertrophic remodeling. Thus, whereas a similar phenotype was reported for homozygous knock-in mice expressing a cMyBP-C exon 30 deletion,24 it is likely that different stimuli initiated remodeling, because unlike mice bearing the cMyBP-C<sup>−/−</sup> knockout mutation, mice carrying the exon 30 deletion express detectable quantities of mutant protein. The ability of mutant peptides to exert dominant-negative effects was demonstrated by transgenic expression of a comparable cMyBP-C deletion mutation.30 The latter mutation also led to hypertrophy and functional defects even though expression of the mutant protein was modest and expression of endogenous cMyBP-C was unchanged. Thus, it appears that either loss of cMyBP-C or expression of cMyBP-C mutant peptides are distinct and adequate signals for hypertrophic remodeling.

Although a reduction of cMyBP-C protein has been proposed in disease etiology of FHC patients bearing cMyBP-C mutations, the complete absence of cMyBP-C in cMyBP-C<sup>−/−</sup> knockout mice differs from cMyBP-C expression in FHC patients. Because FHC is inherited as an autosomal dominant trait, affected individuals carry 1 mutant and 1 normal allele and consequently express some level of the wild-type protein.11,12 cMyBP-C expression therefore more closely parallels that encountered in heterozygous cMyBP-C<sup>+/−</sup> mice, which carry 1 wild-type and 1 mutant (null) allele.

In humans, an unresolved question is whether disease arises from reduced expression of normal cMyBP-C or from expression of cMyBP-C mutant proteins that act in a dominant-negative fashion to disrupt sarcomeric function. Our observations that cMyBP-C protein expression was not reduced in cMyBP-C<sup>−/−</sup> mice and that the mice did not develop hypertrophy suggest that a null allele alone is not sufficient to trigger disease. Instead, posttranscriptional mechanisms can apparently compensate for the loss of a functional allele. Consistent with this, transgenic overexpression of wild-type cMyBP-C led to increased transcript expression while cMyBP-C protein abundance remained constant.31 Results in cMyBP-C<sup>−/−</sup> mice therefore provide indirect support for hypotheses implicating poison polypeptides in cMyBP-C disease etiology. Nonetheless, it is still possible that deleterious effects of a null allele develop slowly over time or become evident only after stress. Longitudinal studies utilizing the cMyBP-C<sup>−/−</sup> mice should prove helpful in resolving these points and determining whether, as in humans with cMyBP-C mutations, disease penetrance is age-dependent, remaining low until late in life.32,33

Depressed Contractile Function in cMyBP-C Knockout Mice

The finding that myocytes from cMyBP-C<sup>−/−</sup> knockout mice exhibited reduced contractile properties is consistent with the overall reduction in diastolic and systolic function observed at the whole heart level. However, the finding that Ca<sup>2+</sup> sensitivity of tension was reduced in cMyBP-C<sup>−/−</sup> mice contrasts with enhanced Ca<sup>2+</sup> sensitivity reported after extraction of ∼60% of cMyBP-C from rat myocytes using biochemical techniques.34,35 A potential explanation to account for the different results is that the biochemical extractions occurred in vitro over short time periods, thus precluding adaptive responses, whereas compensatory effects (eg, protein phosphorylations or structural changes during hypertrophy) after genetic elimination of cMyBP-C may be additional factors in the present study.

Alternatively, differences in the extent of cMyBP-C removal might also account for differing results because extraction of cMyBP-C was incomplete in previous experiments, whereas cMyBP-C was totally eliminated in cMyBP-C<sup>−/−</sup> mice. If so, then the results might imply the existence of at least 2 functional pools of cMyBP-C. Differences in either spatial distribution or phosphorylation state of cMyBP-C could plausibly underlie nonequivalent extraction of different pools and account for disparate effects following partial or complete removal of cMyBP-C.

Our expectation that knockout of cMyBP-C should enhance contractile responses was also predicated on observations that partial extraction of MyBP-C from skeletal fibers sped shortening rates35 and on more recent experiments suggesting that cMyBP-C directly affects myosin contractile properties.36,37 Hofmann and colleagues35 interpreted their results in terms of a model where MyBP-C acts as an internal tether36 could affect directly37 or in combination with binding to the light meromyosin segment of myosin (ie, as a tether)36 could affect myosin head flexibility or position36 and influence myosin contractile properties.31,42 Although data from the present study do not address direct or indirect effects of MyBP-C motif binding to S2, the cMyBP-C<sup>−/−</sup> knockout mice provide an ideal null background on which to test the different hypotheses because competitive effects of endogenous cMyBP-C can be excluded. Future studies utilizing the cMyBP-C knockout mice should provide valuable insights into effects of cMyBP-C binding to S2, cMyBP-C phosphorylation, and mechanisms by which cMyBP-C mutations cause disease.

Acknowledgments

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HYPERTROPHIC CARDIOMYOPATHY IN CARDIAC MYOSIN BINDING PROTEIN-C (cMyBP-C) KNOCKOUT MICE

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Table 1: PCR Amplification Primers

<table>
<thead>
<tr>
<th>Exons</th>
<th>Forward Primer (5’→3’)</th>
<th>Reverse Primer (5’→3’)</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>F: ATGCCGGAGCCAGGGAAGAAACCAG</td>
<td>R: AGCCTGCAATGACCGCGTAGGAACC</td>
<td>253</td>
</tr>
<tr>
<td>2-12</td>
<td>F: CGACATCAACCGCAATGACAAATGATGG</td>
<td>R: TTGAGCCTCTTTCAACATGACCGTCAGG</td>
<td>905</td>
</tr>
<tr>
<td>3-5</td>
<td>F: GAGAAGGCAAACTGGAAGTGTGCTCC</td>
<td>R: CTGGCTCTGTCTAGCTGGGTCATGCA</td>
<td>345</td>
</tr>
<tr>
<td>25-34</td>
<td>F: GCCCTGCCTTCAGCCCTTTGTCATGCC</td>
<td>R: CCCGGATCTGGTCAGGGAACCTC</td>
<td>1264</td>
</tr>
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</table>

Table 2: Transcript-specific Oligonucleotides

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Oligonucleotide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrial Natriuretic Factor (ANF)</td>
<td>5’AATGTGACCAAGCTGACACACACACCAA</td>
</tr>
<tr>
<td></td>
<td>GGGCTTAGGATCTTTTTCGATCTGCTCAAG 3’</td>
</tr>
<tr>
<td>Brain Natriuretic Peptide (BNP)</td>
<td>5’CAGCTTGAGATATGTCACCATGGGAATTT</td>
</tr>
<tr>
<td></td>
<td>TGAGGTCTTCTGCTGACGGCAGGGGTTGCTG 3’</td>
</tr>
<tr>
<td>α Skeletal Actin</td>
<td>5’TGGAGCAAAAACAGAATGGCTGGCTTTAATGCTT</td>
</tr>
<tr>
<td></td>
<td>CAAGTTTTCCATTTCTTTCCACAGGG3’</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)</td>
<td>5’GGAACATGAGACCATGAGTTGAGGTCATGGAAG3’</td>
</tr>
</tbody>
</table>