Neonatal Mouse-Derived Engineered Cardiac Tissue
A Novel Model System for Studying Genetic Heart Disease


Rationale: Cardiomyocytes cultured in a mechanically active 3-dimensional configuration can be used for studies that correlate contractile performance to cellular physiology. Current engineered cardiac tissue (ECT) models use cells derived from either rat or chick hearts. Development of a murine ECT would provide access to many existing models of cardiac disease and open the possibility of performing targeted genetic manipulation with the ability to directly assess contractile and molecular variables.

Objective: To generate, characterize, and validate mouse ECT with a physiologically relevant model of hypertrophic cardiomyopathy.

Methods and Results: We generated mechanically integrated ECT using isolated neonatal mouse cardiac cells derived from both wild-type and myosin-binding protein C (cMyBP-C)-null mouse hearts. The murine ECTs produced consistent contractile forces that followed the Frank-Starling law and accepted physiological pacing. cMyBP-C-null ECTs showed characteristic acceleration of contraction kinetics. Adenovirus-mediated expression of human cMyBP-C in murine cMyBP-C–null ECT restored contractile properties to levels indistinguishable from those of wild-type ECT. Importantly, the cardiomyocytes used to construct the cMyBP-C−/− ECT had yet to undergo the significant hypertrophic remodeling that occurs in vivo. Thus, this murine ECT model reveals a contractile phenotype that is specific to the genetic mutation rather than to secondary remodeling events.

Conclusions: Data presented here show mouse ECT to be an efficient and cost-effective platform to study the primary effects of genetic manipulation on cardiac contractile function. This model provides a previously unavailable tool to study specific sarcomeric protein mutations in an intact mammalian muscle system. (Circ Res. 2011;109:8-19.)

Key Words: engineered cardiac tissue ■ cardiomyopathy, hypertrophic ■ myosin-binding protein C
culture approaches. Clearly, establishing murine ECT would provide significant advantages by allowing study of the multiple existing genetic mouse models of cardiac disease. More importantly, murine ECT would permit the physiological characterization of mutations before the development of secondary changes (ie, hypertrophy, heart failure) that occur in older whole-animal and excised-tissue models.

One mouse model that has been studied extensively is that of cardiac myosin-binding protein C (cMyBP-C-ablation) (cMyBP-C−/−). cMyBP-C plays an important regulatory role in cardiac muscle contraction, and mutations in the cMyBP-C-encoding gene (MYBPC3) are prevalent causes of familial hypertrophic cardiomyopathy (HCM) in humans.17–19 cMyBP-C−/− mice share several of the known phenotypes of the human disease, including pronounced left ventricular and septal hypertrophy, systolic and diastolic dysfunction, and accelerated contractile kinetics.20–26

Here, we present data showing successful generation of ECT from cardiac cells derived from wild-type (WT) and cMyBP-C−/− neonatal mouse hearts. We demonstrate that ECT derived from cMyBP-C−/− cardiac cells recapitulates several contractile abnormalities previously observed in both intact hearts and isolated cardiac tissue from cMyBP-C−/− mice.20–26 Importantly, these abnormalities occur in the absence of detectable hypertrophic remodeling. Additionally, we show that transduction of cMyBP-C−/− mouse ECT with an adenovirus that encodes WT human cMyBP-C completely mitigates the contractile abnormalities in these ECTs. Thus, we demonstrate a robust and reproducible mouse ECT model that provides a novel tool that bridges the gap between isolated cell culture and the intact heart. We further demonstrate the ability to apply this tool to the study of genetic mouse models of important human cardiac diseases.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Animals
Ventricular tissue was harvested from 1-day-old homozygous cMyBP-C−/− mouse pups that were previously generated on the E129X1/SvJ background20 and from 1-day-old WT E129X1/SvJ mice (Taconic, Hudson, NY). Pups were anesthetized with inhaled isoflurane before ventricular tissue was harvested. This study was approved by the Animal Care and Use Committee of the School of Medicine and Public Health at the University of Wisconsin-Madison in accordance with the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health publication No. 85-23, revised 1985).

Isolation of Neonatal Mouse Ventricular Cardiac Cells
Ventricular cardiac cells were isolated by use of enzymatic dissociation based on methods used to isolate neonatal rat cardiomyocytes.2,3,27,28 Briefly, excised ventricular tissue was minced and suspended in 3 mg/mL collagenase type II (Gibco BRL, Invitrogen, Carlsbad, CA) in KG buffer (pH 7.4) and incubated at 37°C for 20 minutes with gentle agitation. KG buffer consisted of 127 mmol/L NaCl, 1.8 mmol/L L-glutamic acid potassium salt monohydrate (Sigma, St Louis, MO), 0.1335% (wt/vol) NaHCO3 (Gibco), 16.5 mmol/L D-glucose (Sigma), 0.42 mmol/L NaH2PO4 (Sigma), and 25 mmol/L HEPES (Sigma). Ventricular tissue was subsequently resuspended and agitated in 0.1% trypsin (Gibco) in KG buffer for 10-minute intervals until dispersed. Cells were pelleted at 200g and resuspended in mouse culture media that consisted of 60.3% high-glucose DMEM (Gibco), 20% F12 nutrient mix (Gibco) supplemented with 1 mg/mL gentamicin (Sigma), 8.75% fetal bovine serum (HyClone; Thermo Fisher Scientific, Waltham, MA), 6.25% horse serum (HyClone), 1% HEPES (Sigma), 1× nonessential amino acid cocktail (Gibco), 3 mmol/L sodium pyruvate (Gibco), 0.00384% (wt/vol) NaHCO3 (Gibco), and 1 μg/mL insulin (Sigma). Cell suspensions were subsequently pelleted into P100 cell culture dishes and incubated at 37°C for 45 minutes to allow preferential attachment of nonmyocyte cell populations and enrichment of the cardiomyocyte population. Cardiac cells remaining in suspension were collected, checked for viability by dye exclusion, counted, and prepared for subsequent ECT construction.

Generation and Characterization of Human MYBPC3-Expressing Adenovirus
An adenovirus encoding N-terminally Myc-tagged full-length human cMyBP-C (adWT) was generated with the ViraPower Adenoviral Expression System (Invitrogen) according to the manufacturer’s protocol. The transduction efficiency of adWT was assessed in neonatal murine cardiac cells in 2-dimensional culture. Control adenoviruses expressing the LacZ reporter gene (adLacZ) and lacking any significant reading frame (adC−) were similarly prepared (see the Online Data Supplement for detailed methodology).

ECT Construction
WT and cMyBP-C−/− neonatal mouse ventricular cells suspended in mouse media were rotated at 50 rpm on a gyratory shaker (195 mm diameter) for 6 to 8 hours at 37°C and 5% CO2 to allow aggregation of small, uniform clusters of viable cells. For production of cMyBP-C−/− adWT ECT, cMyBP-C−/− cardiac cells were transduced with adWT at a multiplicity of infection (MOI) of 20 during the rotational culture period. After rotational culture, approximately 8.0×108 cardiomyocytes were suspended in 83.3 μL of mouse media and added to 116.7 μL of an ECT matrix mixture that consisted of 66.7 μL of 2-mg/mL acid-soluble rat-tail collagen type 1 (pH 3.0; Sigma), 8.3 μL of 10× MEM (Gibco), 8.3 μL of reconstituted buffer (200 mmol/L NaHCO3 [Gibco], 200 mmol/L HEPES [Sigma], 100 mmol/L NaOH [Sigma]), and 33.3 μL of Matrigel (BD Biosciences, San Jose, CA). A total of 200 μL of the cell/matrix suspension was cast into 20-mm×3-mm cylinder constructs with a Flexcell Tissue Train silicone membrane culture plate (Flexcell International, Hillsborough, NC) and incubated under preprogrammed vacuum conditions for 120 minutes (37°C, 5% CO2) to form cylindrical ECT constructs that were attached at each end to fibrous tabs. On matrix polymerization, mouse medium was added.
to the ECT within the 6-well culture dish. ECT constructs were maintained in culture for 7 days, with media changes every other day.

**Results**

**Structure of Murine ECT**

Spontaneous contraction began in an uncoordinated manner on the second day after ECT construction, with gradual transition to coordinated rhythmic contraction by day 5. During the 7 days in culture, ECT diameter decreased from the mold width of 3 mm to approximately 1.1 mm as the construct formed and compacted (Figures 1A and 1B). Cells were distributed unevenly through the ECT, with elongated cardiomyocytes predominantly located near the surface, whereas noncardiomyocyte cell populations were distributed evenly throughout the ECT (Figures 1C–F). Cardiomyocytes-dense areas were often noted at the bottom of the ECT, presumably because of gravitational settling of the cardiomyocytes during matrix polymerization. Cardiomyocytes in this region were particularly well defined, elongated, and rich in mitochondria, with the sarcomeres aligned to the long axis of the ECT (Figures 1C–J). Gap junctions were frequently noted between adjacent cardiomyocytes in these areas (Figure 1J). Additionally, isolated round cardiomyocytes with disorganized sarcomeric orientation were often observed closer to the center of the structure. There were no genotype-dependent differences in the final diameter (WT 1.13±0.04 mm; cMyBP-C−/− 1.11±0.07 mm; cMyBP-C−/− adWT 1.05±0.03 mm; Figures 1A and B), gross structure, or cellular distribution of ECT (Online Figure V).

**Physiological Response of WT Murine ECT**

ECTs generated from rat and chick hearts recapitulate several aspects of the contractile phenotype of intact cardiac tissue. We therefore examined the contractile responses of murine ECT to standard physiological challenges. Maximal twitch force (FMax) of mouse ECT increased in response to increasing stretch applied to ECT in accordance with the Frank-Starling law (Figures 2A and 2B). Furthermore, ECT responded appropriately to an increase in perfusion temperature from 24°C to 37°C with a significant increase in FMax and the rate of contraction, assessed as the elapsed time between the electric pulse and peak developed twitch force (Ct100). Increasing temperature similarly increased the rate of relaxation, assessed from elapsed time between peak developed twitch force and 50% twitch force decay (Rt50; Figures 2C and 2D). FMax decreased as stimulation frequency was increased from 4 to 8 Hz in mouse ECT incubated at 37°C. This negative force-frequency relationship was less pronounced at slower pacing frequencies (4 to 6 Hz; Figures 2E and 2F). The magnitude of twitch force generated by, and physiological responsiveness of, murine ECT was similar to that of similar ECT produced from rat cardiomyocytes.

**Effect of cMyBP-C Ablation on Contractile Function**

cMyBP-C ablation increases the rates of contraction and relaxation in both intact hearts and isolated papillary muscle, with an increase in power output noted in skinned cardiac fibers. We therefore investigated the contractile properties of cMyBP-C−/− ECT. Contractile function was assessed in WT and cMyBP-C−/− ECTs that were electrically stimulated at murine physiological frequencies of 6, 7, and 8 Hz (Figure 3). FMax was significantly greater in cMyBP-C−/− ECT than in WT ECT at all frequencies (Figure 3B; Online Table I). Expression levels of several sarcomeric components were similar between WT and cMyBP-C−/− ECTs at both the transcript and protein levels (Figures 4 and 5; Online Figures VI, VII, and VIII), which indicates that increased force production by cMyBP-C−/− ECT is unlikely to be due to differences in cardiomyocyte number. Additionally, Ct100 and Rt50 were significantly shorter in cMyBP-C−/− than in WT ECT at all stimulation frequencies (Figures 3C and 3D;
Online Table I), which indicates accelerated kinetics of contraction and relaxation in cMyBP-C<sup>H11546</sup>/H11002 ECT. cMyBP-C<sup>H11546</sup>/H11546 ECT Represents “Prehypertrophic” HCM Much of the contractile data characterizing the effects of genetic manipulation on heart function are necessarily derived from adult mice, which typically have already undergone notable hypertrophic remodeling. A potential advantage of ECT in studies of genetically manipulated cardiomyocytes is the relative immaturity of the cardiomyocytes at the time of isolation for ECT construction. To evaluate the status of our source cardiomyocytes, we first measured the heart-to–body weight ratio in newborn (postnatal day 1) and juvenile pups.

Figure 2. Responsiveness of mouse ECT to physiological stimuli. A and B, Representative force trace from a mouse ECT that was electrically paced at 2 Hz showing increased twitch force as the length is increased, corresponding to the Frank-Starling relationship. C and D, Representative trace showing the effect of temperature on twitch force in a mouse ECT electrically paced at 4 Hz showing an increased twitch force, as well as accelerated kinetics of contraction and relaxation when perfusion temperature is increased from 24°C to 37°C. E and F, Representative trace showing the effect on force caused by increasing pacing frequency from 4 to 8 Hz in a mouse ECT at 37°C.
from WT and cMyBP-C−/− litters. Heart-to–body weight ratios were similar in neonatal WT and cMyBP-C−/− mouse pups (Online Figure IA). By postnatal day 10, heart-to–body weight ratios were significantly higher in the cMyBP-C−/− pups and remained elevated at postnatal day 35.

We used quantitative real-time polymerase chain reaction to examine expression of early (atrial natriuretic peptide [Nppa] and brain natriuretic peptide [Nppb]) and long-term (β-myosin heavy chain [Myh7] and α-myosin heavy chain [Myh6]) hypertrophic marker genes. In postnatal day 1 cMyBP-C−/− hearts, expression levels of Myh6 and Myh7 were not differentially regulated (Online Figures IB, C), whereas expression of Nppa and Nppb was already significantly upregulated (Online Figures ID, F). This suggests activation of early hypertrophic pathways in the “prehypertrophic” myocardium. The expression profiles of hypertrophic marker genes confirmed the presence of overt cardiac hypertrophy in postnatal day 10 and day 35 cMyBP-C−/− mice (Online Figure I).

Because ECTs were isolated from postnatal day 1 pups, the initial expression levels of early- and late- hypertrophic genes would presumably be the same between the newly formed ECT and the postnatal day 1 hearts of the respective genotype; however, over the 10 days in vitro, ECT might progress along the same pathway as the intact hearts, which in the cMyBP-C−/− pups showed significant activation of hypertrophic signaling. We therefore examined WT and cMyBP-C−/− ECT for hypertrophic signals by quantitative real-time polymerase chain reaction. All marker genes (early and late) were expressed at similar levels in WT and cMyBP-C−/− ECT (Figure 4), which indicates relative quiescence of hypertrophic signaling in cMyBP-C−/− ECT.

**Adenovirus-Mediated Expression of Human cMyBP-C Restores Contractile Function in cMyBP-C−/− ECT**

Two-dimensional cultures of WT ventricular myocytes were transduced with an adenovirus that expressed Myc-tagged WT human cMyBP-C (adWT). Quantitative real-time polymerase chain reaction was used to determine the expression of endogenous (murine) and exogenous (human) cMyBP-C over a range of MOIs. Expression of human and murine cMyBP-C was similar at an MOI of 5, whether using a standard curve method (Figure 5A) or expressed as a fraction of β-actin expression levels with the Δ-ΔCt method (data not
Furthermore, expression of the human MYBPC3 transgene did not have a significant effect on endogenous mouse Mybpc3 mRNA levels, irrespective of MOI (Figure 5A). Expression of human MYBPC3 was, however, significantly higher than that of endogenous mouse Mybpc3 at >5 MOI (Figure 5A).

We then examined protein expression by Western blot analysis using an anti-cMyBP-C antibody. Total cMyBP-C and myosin heavy chain protein levels were assessed in untransduced WT cardiac cells and cMyBP-C−/− cardiac cells transduced at MOIs of 0, 5, 10, 20, 50, and 100 in 2-dimensional culture. Human cMyBP-C protein levels were expressed as a fraction of myosin heavy chain protein level and compared with endogenous mouse Mybpc3 at ≥5 MOI (Figure 5A).

We chose to use an MOI of 20 for subsequent generation of ECT because even marginally lower cMyBP-C protein levels have been shown to cause HCM in humans. Transduction was performed during the 8-hour rotational culture. Quantitative Western blots confirmed the presence of equivalent cMyBP-C levels in untransduced WT cardiac cells and cMyBP-C−/− cells at MOIs of 5, 10, or 20 did not significantly differ from cMyBP-C levels in untransduced WT cells. Transduction at higher MOIs resulted in significantly higher cMyBP-C levels (Figure 5B). Transduction of cardiac cells with adWT adenovirus did not significantly affect cell survival, as assessed by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT assay), at any MOI used (Online Figure IIIB). Additionally, LacZ reporter-gene expression in mouse neonatal cardiac cells transduced with the adLacZ control virus was assessed by β-galactosidase assay 48 hours after transduction. Transduction efficiency approached 100% at an MOI of 5, whereas transduction at higher MOIs resulted in more intense staining, likely due to β-galactosidase buildup in the cytoplasm (Online Figure II). Transduction with adLacZ had a modestly adverse effect on cell survival, as assessed by an MTT assay, that was statistically different from untransduced cells at MOIs of 10 and 100 (Online Figure IIIA).

To confirm that exogenous human cMyBP-C normally inserted into the sarcomere, we used immunofluorescence microscopy to detect endogenous and human cMyBP-C. WT

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**Figure 4. Expression of hypertrophic response genes in WT and cMyBP-C−/− ECT.** mRNA expression levels of MYBPC3 (A), Nppa (B), Nppb (C), Myh7 (D) and Myh6 (E) in WT (open bars) and cMyBP-C−/− (solid bars) ECT. F, Myh7-Myh6 ratio. *P<0.05 vs WT (Student t test; WT n=5; cMyBP-C−/− n=5). Actb indicates mouse β-actin gene.
ECT showed a double cross-striated pattern, characteristic of C-zone–specific incorporation of cMyBP-C into the sarcomere (Figures 6B and 6C). The double cross-striated cMyBP-C staining pattern was restored after adWT transduction in cMyBP-C−/− ECT (Figures 6H and 6I). This labeling pattern was absent in untransduced cMyBP-C−/− ECT constructs incubated with the same anti-cMyBP-C antibody (Figures 6E and 6F). These data indicate that exogenous human cMyBP-C is appropriately incorporated into the mouse sarcomere in ECT constructs.

One of the major difficulties with genetically engineered mice is determining whether the phenotype is entirely due to deficiency of the specific gene or to secondary remodeling events. We therefore sought to determine whether adenovirus-mediated transduction of human cMyBP-C could rescue the physiological deficits identified in the cMyBP-C−/− ECT. Fmax in cMyBP-C−/− adWT ECT was restored to levels similar to that of WT ECT at all stimulation frequencies and was significantly lower than that of cMyBP-C−/− ECT (Figure 7B; Online Table I). Furthermore, C100 and R50 in cMyBP-C−/− adWT ECT were similar to those of WT ECT and significantly longer than those of cMyBP-C−/− ECT at all 3 pacing frequencies (Figures 7C and 7D; Online Table I), whereas transduction of cMyBP-C ECT with the ad− control virus failed to alter these parameters (Online Figure IV). We found no genotype-dependent differences in total protein or RNA yields (Online Figure VI, A and B), whereas expression levels of total myosin heavy chain protein and Myh6 transcript levels were not influenced by ECT genotype (Online Figure VI, C and D), which indicates that functional differences are unlikely to be due to total cell or cardiomyocyte number. Additionally, expression levels of several genes that encode sarcomeric proteins or proteins involved in excitation-contraction coupling were similar in WT, cMyBP-C−/−, and cMyBP-C−/− adWT ECT (Online Figure VIII). These findings confirm that exogenous human cMyBP-C can incorporate into the mouse sarcomere and restore normal physiological function. Furthermore, these data reinforce the idea that the physiological abnormalities identified in the cMyBP-C−/− ECT are specifically due to the deficiency of cMyBP-C and not to compensatory mechanisms.

Discussion
The data presented herein demonstrate the feasibility of applying 3-dimensional culture methods in the study of both murine and human cardiac physiology and of validating a
novel method to obtain fundamental contractile data from “unremodeled” cardiomyocytes that carry mutations that lead to HCM. Furthermore, we have demonstrated the feasibility of introducing sarcomeric proteins into a multicellular cardiac muscle preparation with the potential to develop a robust screening system for genetic cardiac disease.

Generation of Murine ECT
To the best of our knowledge, this is the first report describing ECT generated from neonatal mouse cardiac cells. As described here, the murine ECT provides a powerful, high-throughput tool to test how specific alterations in cardiac genes contribute to abnormal cardiac physiology and produce disorders such as HCM and dilated cardiomyopathies. Although our approach was similar to those used previously for the generation of rat ECT,2,4 we found several adaptations that were essential for the generation of well-organized, functioning mouse ECT. First, viability and quality of the resulting ECT were improved when cells were harvested from mouse pups less than 24 hours old. In agreement with previous studies,3,4 we also observed that incubation of mouse cardiac cells with gentle gyration (rotational culture) to allow aggregation of the isolated cells was beneficial to establishing mechanically active ECT. However, whereas previous studies maintained cells in rotational culture for more than 12 hours,3,4 we found that a culture period of 6 to 8 hours yielded more uniform small clusters of cells and reduced excessive cell death. Third, seeding neonatal mouse cardiomyocytes at a density of $8 \times 10^5$ per ECT with a concentration of $4 \times 10^6$ cells/mL was necessary to establish robust muscle strips that extended the length of the construct. This optimal seeding density was similar to that used to produce fibrin-based rat ECT ($4.1 \times 10^6$ cells/mL)5 but higher than previously used for the generation of chick (1.3 $\times 10^6$ cells/mL)1 and rat (3.2 $\times 10^6$ cells/mL)2 ECT in collagen matrices. Generally, cardiac cells from 4 to 5 neonatal hearts were required for each ECT generated. Finally, we found that addition of F12 nutrient supplement mixture to the culture media during the extended 7- to 10-day culture period was essential for optimal cell viability.

Mouse ECT produced measurable force, accepted electric pacing, and responded appropriately to physiological stimuli, including increases in passive tension and temperature. This is not surprising given that cardiomyocytes in mouse ECT align with the long axis of the construct and form myofibrils that span the length of the ECT, with preserved sarcomeres, abundant mitochondria, and numerous gap and tight junctions. This level of organization observed in mouse ECT is distinctly absent from the stellate cardiomyocytes commonly observed in monolayer cultures. As in other species,1–3,29,31,32 mouse cardiomyocytes in the ECT responded appropriately to increasing stretch in accordance with the Frank-Starling law. Similar to observations in intact myocardial tissue,34–36 increasing the perfusion temperature to 37°C significantly accelerated the rates of contraction and relaxation in mouse ECT. The ability to acquire measurements under physiological conditions is crucial to drawing physiologically relevant conclusions about contractile kinetics.

Figure 6. Incorporation of human cMyBP-C into the mouse sarcomere. Immunofluorescence in thin sections through a WT (A–C), cMyBP-C–/– (D–F), and cMyBP-C–/– adWT (G–I) ECT. Desmin was detected by a mouse anti-desmin primary antibody and AlexaFluor 568 goat anti-mouse secondary antibody (red fluorescence; A, D, G), cMyBP-C was detected by rabbit anti-cMyBP-C primary antibody and AlexaFluor 488 goat anti-rabbit secondary antibody (green fluorescence; B, E, H). Panels C, F, and I represent desmin and cMyBP-C immunofluorescence overlay. DAPI was used as a nuclear counterstain in all panels (blue fluorescence). Scale bars, 10 μm.
Unlike intact neonatal and adult mouse cardiac tissue, mouse ECT did not display a positive force-frequency response. Indeed, the force frequency tended to be negative in mouse ECT, a phenomenon that was more striking at higher pacing frequencies. Similar negative force-frequency relationships were noted previously in rat ECT. This negative force-frequency relationship may be a result of the dimensions of ECT constructs. Previously, Janssen et al showed a positive force-frequency response in ultrathin rat trabeculae but a flat or negative relationship in trabeculae with a diameter of \( \frac{1}{100} \) mm, specifically at higher pacing frequencies. They speculated that diffusion of oxygen and nutrients, as well as removal of toxic metabolites, may become limiting at higher pacing frequencies when diffusion distances exceed \( \frac{1}{50} \) to \( \frac{1}{75} \) mm, resulting in a negative force-frequency relationship. Because mouse ECT are generally approximately 1 mm in diameter, and well-organized cardiomyocyte-rich cell layers often exceed 100 \( \mu \)m, diffusion distance may account for the negative force-frequency response in ECT derived from mice and from other species.

Further refinement of the mouse ECT model is desirable to achieve a more defined growth environment. Current methods of culturing rat ECT have successfully reduced or eliminated serum from the growth media by substituting a complex cocktail of supplements. Attempts to replace serum in the mouse cultures have not been successful thus far. Additionally, alternatives to the collagen matrix and Matrigel have been explored in rat ECT. Most promising is the use of fibrin, which supports better cell survival and more uniform final tissue configuration. Fibrin also appears to undergo more extensive remodeling by the cardiomyocytes and nonmyocytes within the ECT, which likely results in a more physiological extracellular matrix than achieved with collagen. The distribution of cardiomyocytes around the outer margin of the ECT is commonly observed in collagen-based ECT. This likely reflects both the gradient of nutrients and oxygen, which diminish 50 to 100 \( \mu \)m from the outer surface of the construct, as well as a settling of cells as the collagen matrix slowly polymerizes. Development of a fibrin-based matrix might reduce this phenomenon, because the fibrin polymerizes more quickly, maintaining a better distribution of cardiomyocytes throughout the full cross section of the ECT. Finally, the murine ECTs are typically

![Figure 7. Effect of expression of human cMyBP-C on ECT contractile function. A, Representative twitches of WT, cMyBP-C/−/−, and cMyBP-C/−/+ adWT ECT paced at 6 Hz. B, Maximal twitch force (FMax) in WT, cMyBP-C/−/−, and cMyBP-C/−/+ adWT ECT paced at 6, 7, and 8 Hz. C, Time to peak developed twitch force in WT, cMyBP-C/−/−, and cMyBP-C/−/+ adWT ECT paced at 6, 7, and 8 Hz. D, Time from peak developed twitch force to 50% force decay in WT, cMyBP-C/−/−, and cMyBP-C/−/+ adWT ECT paced at 6, 7, and 8 Hz. Open bars, WT ECT; black bars, cMyBP-C/−/− ECT; gray bars, cMyBP-C/−/+ adWT ECT. *P<0.05 vs WT and cMyBP-C/−/+ adWT (1-way ANOVA, Bonferroni post hoc analysis; WT n=7, cMyBP-C/−/− n=6; cMyBP-C/−/+ adWT n=6). KO indicates knockout.](http://circres.ahajournals.org/doi/abs/10.1161/CIRCRESAHA.110.242570)
avascular, although cocultured endothelial cells have been observed to self-assemble into rudimentary capillary structures.\textsuperscript{14} Recent advances in promoting vascularization of 3-dimensional tissue constructs\textsuperscript{42–44} may facilitate greater cardiomyocyte densities in collagen-based murine ECT.

**Specific Physiological Functions of cMyBP-C**

Mutations in \textit{MYBPC3} are a common cause of human familial HCM, with more than 150 distinct mutations implicated thus far.\textsuperscript{17–19} The impact of most of these mutations on cardiac function remains unknown, mainly because of the prohibitive cost and time required to generate mouse models that express specific gene mutations. The murine ECT cMyBP-C\textsuperscript{−/−} model, in which human mutations can be introduced and characterized, therefore provides a readily accessible tool to screen disease-causing mutations. The cost is comparatively low, and the time from design to physiological testing is approximately 3 months.

Our initial application of the murine ECT to study human disease has focused on the cMyBP-C\textsuperscript{−/−} mouse precisely because it is a well-characterized mouse model of cardiomyopathy. Overall, cMyBP-C ablation does not affect birth frequency or long-term survival of cMyBP-C\textsuperscript{−/−} mice.\textsuperscript{20} However, at the whole-organ level, cMyBP-C ablation causes significant cardiac hypertrophy, myocyte disarray, fibrosis, and impaired left ventricular fractional shortening and ejection fractions.\textsuperscript{20,26,45} The accelerated rates of contraction and relaxation we have identified in cMyBP-C\textsuperscript{−/−} ECT are similar to contraction kinetics observed in isolated skinned myocardium from cMyBP-C\textsuperscript{−/−} hearts.\textsuperscript{20–26}

The increased F_{\text{Max}} in cMyBP-C\textsuperscript{−/−} ECT contrasts with the impaired left ventricular fractional shortening and ejection fractions in the intact cMyBP-C\textsuperscript{−/−} heart and the F_{\text{Max}} observed in isolated skinned fibers, which was similar between the WT and MyBP-C\textsuperscript{−/−} preparations\textsuperscript{20,22,26,45}, however, in those studies, functional measurements were generally performed in tissue derived from older hearts that had already undergone hypertrophic remodeling, fibrosis, and changes in extracellular matrix elasticity. The present data are, however, consistent with findings by Korte et al\textsuperscript{21} who showed an increase in power output in skinned cMyBP-C\textsuperscript{−/−} fibers. This is compatible with a model in which cMyBP-C ablation increases the probability of cross-bridge formation, as was previously proposed,\textsuperscript{24–26} and supports the assertion that ECT may accurately reflect the inherent contractile properties of cardiomyocytes before remodeling and not the consequences of remodeling and other secondary events. Our findings also suggest that the ECT model may be well suited to examining the chain of events that lead from the initial genetic mutation and contractile phenotype to the manifestation of hypertrophy and heart failure. Future experiments will focus on further evaluation of this model of early MYBP-C deficiency, with careful assessment of other factors that may contribute to contractile performance, such as the phosphorylation status of sarcomeric proteins. Although we would not expect there to be differences between overall cMyBP-C\textsuperscript{−/−} and WT ECT protein phosphorylation, this remains to be fully explored, because potential differences could have important effects on both F_{\text{Max}} and contractile kinetics.

Rescue of normal physiology by adenovirus-mediated expression of WT human protein confirms that our findings are due specifically to absence of cMyBP-C. Importantly, virally expressed protein incorporated normally into the sarcomere. By transducing cells during the rotational cultures, we ensured that exogenous human cMyBP-C in cMyBP-C\textsuperscript{−/−} adWT ECT was expressed at levels that were nearly identical to those of the endogenous protein in WT ECT without causing a significant reduction in cell survival. Immunohistochemical analysis of cMyBP-C\textsuperscript{−/−} adWT ECT showed a uniform transduction efficiency of most of the cells throughout the construct, with exogenous human cMyBP-C appropriately incorporated into the sarcomeric C-zones. A similar approach will be used in future studies to assess the contractile effects of expressing human HCM mutant cMyBP-C on the cMyBP-C\textsuperscript{−/−} background. On the basis of these data, we suggest that other sarcomeric proteins causative of human HCM can be similarly expressed and appropriately incorporated into murine ECT models derived from corresponding knockout animals.

In conclusion, we have developed a mouse ECT model for investigations of cardiomyocyte physiology and pathophysiology. We have shown that cMyBP-C\textsuperscript{−/−} ECTs recapitulate the accelerated contractile kinetics observed in isolated muscle from cMyPC\textsuperscript{−/−} mice in the absence of hypertrophic remodeling, thus confirming the fundamental contractile phenotype of cMyBP-C ablation. Furthermore, we have demonstrated the ability to introduce human sarcomeric proteins onto a knockout murine background. Murine ECT represents a new tool that bridges the gap between traditional isolated 2-dimensional cardiomyocyte cell culture and intact animal models. This technique will allow generation and characterization of novel genetic models more efficiently and at significantly less cost than with traditional animal models. This model could also provide an opportunity to study mutations that result in embryonic lethality in traditional animal models. More importantly, this model permits determination of the basic contractile phenotype in the absence of significant remodeling. Collectively, the strengths of this technique have the potential to increase our understanding of integrated cardiac physiology in health and disease.

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Novelty and Significance

What Is Known

- Three-dimensional engineered cardiac tissue constructs (ECTs) replicate important aspects of myocardial physiology in vitro.
- ECT with murine cardiomyocytes has not been available previously.
- Development of multicellular murine cardiac preparations would extend the utility of existing mouse models of genetic cardiac disease and facilitate the study of rare mutations.

What New Information Does This Article Contribute?

- It is feasible to create functionally intact murine ECT in a robust, reproducible, and cost-effective manner.
- The ability exists to appropriately express a human sarcomeric protein in a mouse cardiomyocyte and derive contractile function data.
- The model is a novel tool for studying the effects of sarcomeric protein mutations in an intact mammalian muscle system.

Hypertrophic cardiomyopathy, which affects nearly 1 of every 500 people, is highly heterogeneous. Mutations in several different proteins have been linked to the development of hypertrophy. Currently, many of the identified mutations that are linked to human hypertrophic cardiomyopathy are not well studied in any detail. Most of our understanding of the functional impact of specific mutations comes from mouse models in which the gene of interest has been knocked out or overexpressed. The development of these models is costly and time consuming.

We developed a method to culture mouse cardiomyocytes in a 3-dimensional tissue construct to form a functional strip of cardiac muscle tissue. This model allowed us to study contractile function and molecular events in existing mouse models of cardiac disease before the development of significant remodeling events. Furthermore, we can rapidly and efficiently express specific mutant proteins in an intact cardiac tissue system and identify the impact of the mutation on contractile function and the development of the hypertrophic phenotype. This technology will identify important genotype-phenotype relationships and identify potential pathways to target for therapeutic intervention.
Neonatal Mouse–Derived Engineered Cardiac Tissue: A Novel Model System for Studying Genetic Heart Disease


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An erratum has been published regarding this article. Please see the attached page for:
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Correction

Neonatal Mouse–Derived Engineered Cardiac Tissue: A Novel Model System for Studying Genetic Heart Disease: Correction

In the article that appears on page 8 of the June 24, 2011 issue, the Sources of Funding were missing from the article. The article was supported by NIH K08 HL074224-01 (J.C.R.); American Heart Association BGIA 0765303U (J.C.R.), NIH R37 HL089200 (R.L.M.), and the Children’s Cardiomyopathy Foundation, CCF 133-PRJ32ZU (J.C.R.).

The error has been noted and corrected in the online version of the article, which is available at http://circres.ahajournals.org/content/109/1/8.full.
Supplemental Material

Generation of Human MYBPC3 Expressing Adenovirus (adWT):

Full length human MYBPC3 cDNA in pCMV-SPORT6 (pCMV-MYBPC3WT) was obtained from Open Biosystems™. The full-length cMyBP-C open reading frame was subsequently PCR-amplified using PfuUltra (Agilent Technologies) according to the manufacturer’s protocol using the following primer sequences: hMYBPC3 C0 Myc F CACCATGGAACAAAACTTTATTTCTGAAGAAGATCTGATGCCTGAGCCGGGGAAG and hMYBPC3 C10R – TCACTGAGGCACCTCGCACCTCCAGG. During amplification, a Myc epitope tag was added inframe with the MYBPC3 coding region, allowing for subsequent production of N-terminally Myc-tagged wild type cMyBP-C. The 3,855 bp PCR product thus produced was subsequently cloned into the pENTR™/D-TOPO entry vector (Invitrogen) according to the manufacturer’s protocol. Following characterization by DNA sequencing, the Myc-tagged human cMyBP-C encoding inserts were subcloned into the pAd/CMV/V5-DEST adenoviral shuttle vector (component of the ViraPower Adenoviral Expression System, Invitrogen), using the Gateway LR Clonase recombination system (Invitrogen) to form the pAdWT plasmid. adWT adenoviral particles were produced using the ViraPower™ Adenoviral Expression System (Invitrogen), according to the manufacturer’s protocol. Briefly, the pAdWT plasmid was digested with PacI (New England BioLabs) to expose the left and right viral inverted terminal repeat sequences prior to transfection into the 293A cells using Lipofectamine™2000 (Invitrogen) according to the manufacturers’ protocol. Viral particles were harvested from 293A cells by repeated rapid freeze-thaw, 18 days post-transfection. The crude, low titer adWT-containing lysates were subsequently used to re-infect fresh 293A cells, from which adWT was harvested, purified and titred according to the manufacturer’s protocol (Invitrogen).

Generation of adC Adenovirus

A control adenovirus (adC) was generated from a pENTR™/D-TOPO entry vector (Invitrogen) into which the full length cMyBP-C open reading frame was inadvertently cloned in the opposite orientation. adC is predicted to lack any significant open reading frame and was generated and titered using protocols identical to that used to generated adWT. The effect of adenoviral transduction on contractile kinetics was subsequently assessed in ECT generated from cMyBP-C−/− cells transduced with adC at an MOI of 20.

Adenoviral Transduction of Cardiac Cells in Two-Dimensional (2D) Primary Culture:

In order to assess adenoviral transduction efficiency, WT and cMyBP-C−/− neonatal mouse ventricular cardiac cells suspended in mouse media (60.3% high glucose DMEM (Gibco); 20% F12 nutrient mix (Gibco) supplemented with 1 mg/mL gentamicin (Sigma), 8.75% fetal bovine serum (HyClone®), 6.25% horse serum (HyClone®), 1% HEPES (Sigma), 1x non-essential amino acid cocktail (Gibco), 3 mmol/L sodium pyruvate (Gibco), 0.00384% (w/v) NaHCO₃ (Gibco), 1 µg/mL insulin (Sigma)) were plated in 12-well flat bottom tissue culture plates (Falcon) at a density of 2 x 10⁵ CMs per well. CMs were allowed to adhere to the surface of the dish for 48 hours prior to transduction with adWT at a multiplicity of infection (MOI) of 0, 5, 10, 20, 50 and 100. The adenovirus-containing media was removed and replaced with mouse
culture media 24 hours post-transduction and cells cultured for an additional 48 hours prior to harvesting of RNA or protein.

**RNA Extraction and qRT-PCR:**

Total RNA was extracted from WT and cMyBP-C hearts, WT, cMyBP-C and cMyBP-C adWT ECT, as well as WT CM transduced with adWT at MOI of 0, 5, 10, 20, 50 and 100 in 2D culture using standard protocols. Briefly, tissue/cells were homogenized in TriZol reagent (Invitrogen) according to the manufacturer’s protocol. Following addition of an appropriate amount of chloroform (Sigma), mixing, incubation and centrifugation according to the TriZol reagent protocol, the RNA containing aqueous phase was collected and treated with DNaseI (RNase-free DNase set; Qiagen) for one hour. RNA was subsequently purified using the RNeasy® mini kit (Qiagen), according to the manufacturer’s protocol. First strand cDNA synthesis was performed using the SuperScript III cDNA synthesis kit (Invitrogen) with minor modifications to the manufacturer’s protocol. In order to facilitate full length first strand cDNA synthesis of most transcripts, samples were incubated for 90 minutes at 50°C prior to heat inactivation of the reverse transcriptase at 70°C for 15 minutes, oligo dT priming and 200 to 500 ng of total RNA as template.

Expression levels of human MYBPC3, mouse Mybpc3, mouse Nppa, mouse Nppb, mouse Myh6, mouse Myh7, mouse Atp2a2, and mouse Actb were subsequently assessed using TaqMan® gene expression assays (Hs00165232_m1 detecting human MYBPC3; Mm00435104_m1 detecting mouse Mybpc3; Mm 01255748_g1 detecting mouse Nppa; Mm01255770_g1 detecting mouse Nppb; Mm00440359_m1 detecting mouse Myh6; Mm00600555_m1 detecting mouse Myh7; Mm01201431_m1 detecting mouse Atp2a2; Mm00465917_m1 detecting mouse Ryr2; Mm00441524_m1 detecting mouse Slc8a1; Mm00502426_m1 detecting mouse Tnni1; Mm00437164_m1 detecting mouse Tnni3; Mm01290252_g1 detecting mouse Tnnt2; Mm00600378_m1 detecting mouse Tpm1; Mm01333821_m1 detecting mouse Actc1; Mm00473657_m1 detecting mouse Actn2 and 4352933E detecting mouse Actb; all obtained from Applied Biosystems™). The equivalent of 10 ng reverse transcribed RNA and 2x TaqMan® Gene Expression Master Mix (Applied Biosystems™) was used as template in each TaqMan® qPCR reaction. Thermal cycling and fluorescence measurement was performed in an Mx3005P qPCR system (Stratagene). Data was analyzed using MxPro software (Stratagene) and expression levels calculated using the ΔCT-method and expressed as a percentage of the Actb expression level. Additionally, qPCR was performed on appropriate molar amounts of pCMV-MYBPC3WT (encoding full length human cMyBP-C) and pET-Mybpc3WT (encoding full length mouse cMyBP-C), allowing quantification and comparison of human MYBPC3 and mouse Mybpc3 transcript levels using the standard curve method.

**Protein Analysis:**

Total protein lysates were prepared from WT and cMyBP-C cardiac cells transduced with adWT at MOI of 0, 5, 10, 20, 50 and 100 in 2D culture, as well as WT, cMyBP-C and cMyBP-C adWT ECT. Protein lysates were electrophoresed using pre-cast 10% Criterion Gels (Bio-Rad) (10 μg for lysates from cells in 2D culture and 35 μg for lysates from ECT). Following electrophoresis, proteins were transferred to 0.2 μm nitrocellulose membranes (Bio-Rad) prior to being washed and equilibrated using standard techniques. Membranes were subsequently
incubated in blocking buffer for 1 hour (Odyssey® Infrared Imaging Systems), prior to overnight hybridization with primary specific antibodies. Primary antibodies used were, a goat anti-cMyBP-C polyclonal antibody diluted at 1:200 (Santa Cruz Biotechnology; sc-50115) that was able to detect both human and mouse cMyBP-C, and a rabbit anti-myosin heavy chain polyclonal antibody diluted at 1:500 (Sigma/Atlas HPA001239), a mouse anti-beta myosin heavy chain monoclonal antibody diluted at 1:200 (Millipore; MAB1552), a mouse anti-alpha cardiac actinin monoclonal antibody diluted at 1:5000 (Sigma; A7811), a mouse anti-cardiac troponin T monoclonal antibody diluted at 1:100 (University of Iowa Developmental Studies Hybridoma Bank; CT3) and a mouse anti-alpha tropomyosin monoclonal antibody diluted at 1:1000 (University of Iowa Developmental Studies Hybridoma Bank; CH1). Primary antibodies were subsequently visualized using secondary donkey anti-goat (Li-Cor, IRDye 800CW), goat anti-mouse (Li-Cor, IRDye 800CW) and goat anti-rabbit (Li-Cor, IRDye 680LT) immunofluorescent secondary antibodies diluted at 1:10,000 using an Odyssey imaging system (Li-Cor Biosciences) according to the manufacturer's protocols.

**Beta Galactosidase Staining of CM**

In order to establish transduction efficiency, mouse cardiac cells in 2D culture were transduced with the adLacZ control virus (in which expression of the LacZ reporter gene is driven by the same CMV promoter as is used to drive expression of human cMyBP-C in cells/ECT transduced with adWT) at MOI of 0, 5, 10, 20, 50 and 100. The virus was removed and the media changed after 24 hours and beta-galactosidase staining performed 48 hours post transduction. Fixing and beta-galactosidase staining of CMs were performed using the β-Gal staining kit (Invitrogen) according to the manufacturer's protocol. CMs were visualized and photographed on a Zeiss Stemi 2000 Stereomicroscope.

**MTT Assay**

In order to establish whether transduction of mouse cardiac cells adversely affected cell survival, WT and cMyBP-C−/− were transduced with adWT and the adLacZ control virus in 2D culture at MOI of 0, 5, 10, 20, 50 and 100. The virus was removed and the media changed after 24 hours. Following an additional 48 hours in culture, the media was changed and an equal volume of 2 μg/mL 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) diluted in 1x PBS was added to cells. Cells were subsequently incubated for 30 minutes at 37°C, where after an equal volume of cell lysis buffer (20% (w/v) SDS (Sigma); 50% (v/v) dimethyl formamide (Fischer Scientific); pH 4.7) was added. Cell lysates were incubated at room temperature for 3 - 4 hours where after optical densities at 550 nm were measured.

**Routine Histology and Light Microscopy**

Murine ECT were fixed with 4% paraformaldehyde (Sigma) in 1x PBS (pH = 7.2) (Mediatech) at 4°C overnight. The ECT were subsequently dehydrated and embedded with paraffin using standard protocols in an automated tissue vacuum infiltration processor (Tissue-Tek V.I P; Sakura). All tissue blocks are embedded in paraffin using a Sakura embedding center, prior to sectioning at a thickness of 5 – 6 μm using a microtome (Leica). Sections used for routine light microscopy were placed on non-charged glass microscope slides and incubated at 60°C for 15 minutes prior to hematoxylin and eosin (H&E) staining in a Tissue-Tek DRS automatic slide
stainer (Sakura). Following staining, sections were covered by a drop of Permount slide mounting fluid (The Science Company) prior to coverslipping. Sections were visualized and photographed on a Zeiss model T635b photomicroscope.

**Immunohistochemistry**

For immunohistochemistry, ECT constructs were rinsed 1 x 1 min in 0.1 mol/L KCl to relax the sarcomeres and then 1 x 1 min in 1x PBS (pH 7.0) before being fixed in Dent’s fixative (80% Methanol:20% DMSO) for 2 hours at 4 °C. Constructs were then dehydrated in methanol series and stored at -20 °C for 24 hours. For sectioning, fixed constructs were transferred directly to xylenes and allowed to equilibrate for 5 minutes prior to being placed in paraffin and maintained at 60 °C for 90 minutes. They were subsequently embedded in fresh paraffin in disposable plastic moulds and allowed to cool before being sectioned at 8 μm on a Leica RM2165 microtome, mounted on glass sides, and dried overnight on a flattening plate at 37 °C. After drying, slides were heated to 60 °C, placed in xylenes for 5 minutes, rehydrated through ethanol series to water and rinsed twice in 1x PBS. Blocking was performed in 2 steps to minimize nonspecific binding of mouse- and goat-derived antibodies. First, slides were blocked for 1 hour in 1x PBS containing 5 % [vol/vol] sheep serum, 2 mg/ml bovine serum albumin and 0.1 % [vol/vol] Tween20, then blocked secondarily with the Vectastain Mouse-on-Mouse (M.O.M) basic kit, following manufacturer’s instructions (Vector Laboratories, Burlingame, CA). Sections were incubated with 1:50 anti-Desmin monoclonal antibody (NCL-L-DES-DERII from Novocastra Laboratories, Buffalo Grove, NV) and 1:100 anti-cMyBPC rabbit polyclonal antibody for 1 h at room temperature in M.O.M protein solution (Vector Laboratories) in a humidity chamber. After rinsing 2 x 2 min in PBS sections were incubated for 1 hour at room temperature in the dark with 1:200 AlexaFluor 568 goat anti-mouse IgG1, 1:200 AlexaFluor 488 goat anti-rabbit IgG(H+L) and/or 1:250 AlexaFluor 647 goat anti-rabbit IgG(H+L) secondary antibodies (Molecular Probes, Eugene, OR) in M.O.M protein solution as before. After rinsing 2 x 2 min in PBS, sections were coverslipped using warmed ProLong Gold Antifade Reagent with 4′,6-diamidino-2-phenylindole (DAPI). Imaging was performed using the Nikon A1R inverted confocal microscope using a 60x oil-immersion objective and 405, 488, 568 and 647 nm to excite DAPI, AlexaFluor 488 and AlexaFluor 568 AlexaFluor 647, respectively. Images were acquired using a built-in automated tile scan routine and NIS-Elements software suite.

**Transmission Electron Microscopy**

Tissues were immersion fixed in a solution 2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.4) overnight at 4°C. The tissue was then post fixed in 1% OsO₄ in the same buffer for 2 hours at room temperature. Following OsO₄ post-fixation, the samples were dehydrated in a graded ethanol series, then further dehydrated in propylene oxide and embedded in Epon epoxy resin. Samples were sectioned for transmission electron microscopy using an ultramicrotome (Reichert-Jung Ultracut-E) and contrasted with Reynolds lead citrate and 8% uranyl acetate in 50% ethanol. Ultrathin sections were observed with a Philips CM120 electron microscope and images were captured with a MegaView III side mounted digital camera.

**Mechanical Testing of ECT:**
Isometric force generated by ECT was measured using protocols similar to those described by Tobita et al. (2006). In brief, each ECT construct was transferred from the culture dish (figure 1A) to a model 801B small intact fiber test apparatus (Aurora Scientific) in Krebs-Henseleit buffer (119 mmol/L NaCl; 12 mmol/L Glucose; 4.6 mmol/L KCl; 25 mmol/L NaHCO₃; 1.2 mmol/L KH₂PO₄; 1.2 mmol/L MgCl₂; 1.8 mmol/L CaCl₂, gassed with 95% O₂ / 5% CO₂ (pH 7.4)). ECT constructs were attached with sutures between a model 403A force transducer (Aurora Scientific) and model 322C high speed length controller (Aurora Scientific) (figure 1B). ECT were perfused with 24°C Krebs-Henseleit buffer at a rate of 1 mL/min and field-stimulation initiated at 2 Hz (2.5 ms, 8 - 12.5 V). The longitudinal length of each construct was increased stepwise until maximal twitch force was achieved. ECT were then equilibrated for 10 – 20 minutes or until a stable level of passive tension was achieved while being paced at 2 Hz. The pacing frequency was increased to 4 Hz and the temperature of the perfusate in the chamber adjusted to 37°C prior to force measurements being made at stimulation frequencies of 4 Hz, 5 Hz, 6 Hz, 7 Hz and 8 Hz. Data from force measurements were analyzed and averaged using IonWizard 6.0 software (IonOptix). For each stimulation frequency, force of 80 to 160 successive contractions were collected and averaged. Contraction data was exported to Microsoft Excel and the magnitude and kinetics of force generation calculated.

Statistical Analysis
SPSS software was used to perform statistical analysis. Student’s t-tests were used for two way comparisons, while one way ANOVA with Bonferroni’s correction for post-hoc analysis was used for multiple comparisons. All error bars are standard error of the mean (SEM). Statistical significance was set at p < 0.05.
Supplemental Reference List:


Online Figure I. Hypertrophic Response in WT and cMyBP-C/- ECT Hearts. Heart to body weight ratios (A) in 1 day, 10 day and 35 day old WT (open bars) and cMyBP-C/- (filled bars) mice. mRNA expression levels of Nppa (B), Nppb (C), Myh7 (D), Myh6 (E) in WT and cMyBP-C/- mouse hearts collected from 1 day, 10 day and 35 day old mice. The Myh7:Myh6 ratio is shown in F. *P < 0.05 vs. WT (Student’s T-test; n ≥ 12 for each group in A; n ≥ 6 for each group in B-E). Elevated heart to body weight ratios and increased expression levels of the late hypertrophic response-genes (Myh6 and Myh7) in day 10 and day 35 cMyBP-C/- hearts indicate that hypertrophic remodeling takes place in the first 10 days of life. Elevated expression of the early hypertrophic marker genes (Nppa and Nppb) in neonatal cMyBP-C/- hearts indicates that early hypertrophic signaling precedes overt hypertrophy.
Online Figure II. Beta-Galactosidase Staining of Neonatal Cardiac Cells. WT neonatal cardiac cells in 2D culture transduced with adLacZ at 0 MOI (A); 5 MOI (B); 10 MOI (C); 20 MOI (D); 50 MOI (E) and 100 MOI (F). This data suggest that most cardiomyocytes are transduced with adLacZ at MOI of 10 and above.
Online Figure III. Effect of Adenoviral Transduction on Neonatal Cardiac Cell Survival. MTT assays performed on neonatal mouse cardiac cells transduced with adLacZ (A) and adWT (B) at MOI of 0, 5, 10, 20, 50 and 100. *P<0.05 vs. MOI 0 (one way ANOVA, Bonferroni’s post-hoc analysis; n = 5 for each group). This data indicates that while transduction of cardiomyocytes with adLacZ had a modestly cytotoxic effect, transduction with adWT did not significantly affect cell survival.
Online Figure IV. Effect of Expression of adC in cMyBP-C^−/− ECT on Contractile Kinetics. (A) Time to peak developed twitch force in WT, cMyBP-C^−/−, cMyBP-C^−/− adWT and cMyBP-C^−/− adC^- ECT paced at 6Hz, 7Hz and 8Hz. (B) Time from peak developed twitch force to 50% force decay in WT, cMyBP-C^−/−, cMyBP-C^−/− adWT and cMyBP-C^−/− adC^- ECT paced at 6Hz, 7Hz and 8Hz. WT ECT- open bars; cMyBP-C^−/− ECT- filled bars; cMyBP-C^−/− adWT ECT- light grey bars; cMyBP-C^−/− adC^- ECT- dark grey bars. * P < 0.05 vs. WT and cMyBP-C^−/− adWT (one way ANOVA, Bonferroni's post-hoc analysis; WT n = 7, cMyBP-C^−/− n = 6; cMyBP-C^−/− adWT n = 6; cMyBP-C^−/− adC^- n = 3). This data confirms that restoration of contractile function in cMyBP-C^−/− adWT ECT is the result of expression of exogenous human cMyBP-C, rather than a direct effect of adenoviral transduction.
Online Figure V. Histological Comparison of WT, cMyBP-C<sup>-/-</sup> and cMyBP-C<sup>-/-</sup> adWT ECT. H&E-stained WT (A and D); cMyBP-C<sup>-/-</sup> (B and E) and cMyBP-C<sup>-/-</sup> adWT (C and F) ECT. 2.5x objective (A - C) and 10x objective (D - F). The morphology of WT, cMyBP-C<sup>-/-</sup> and cMyBP-C<sup>-/-</sup> adWT ECT were similar with well-organized, well-aligned cardiomyocytes found predominantly at, or close to, the outer surface of the ECT.
Online Figure VI. Protein and RNA content in ECT. Total protein (A) and RNA (B) yield from WT, cMyBP-C<sup>−/−</sup> and cMyBP-C<sup>−/−</sup> ECT. Total myosin heavy chain protein (C) and alpha myosin heavy chain mRNA (D) levels were assessed by Western blotting and qRT-PCR respectively. WT ECT- open bars; cMyBP-C<sup>−/−</sup> ECT- filled bars; cMyBP-C<sup>−/−</sup> adWT ECT- light grey bars. No significant differences were detected between any groups for any parameters analyzed (one way ANOVA, Bonferroni’s post-hoc analysis; n ≥ 7). Similar protein and RNA yields from WT, cMyBP-C<sup>−/−</sup> and cMyBP-C<sup>−/−</sup> adWT ECT indicate that total cell number is not influenced by ECT genotype. Furthermore, similar total myosin heavy chain protein and Myh6 transcript levels indicate that the cardiomyocyte number were similar in ECT of all three genotypes.
Online Figure VII. Expression of Sarcomeric Proteins in WT and cMyBP-C\textsuperscript{-/} ECT. A- cardiac myosin binding protein-C; B- alpha actinin; C- cardiac troponin T; D- alpha tropomyosin; E- total myosin heavy chain; F- beta myosin heavy chain. Lanes 1-3 – protein from WT ECT; lanes 4-6 – protein from cMyBP-C\textsuperscript{-/} ECT (35µg of total protein were loaded per lane). While cMyBP-C protein was absent in cMyBP-C\textsuperscript{-/} ECT, expression of other sarcomeric proteins was not increased by its ablation. These findings indicate that cardiomyocyte levels are similar in WT and cMyBP-C\textsuperscript{-/} ECT and that cMyBP-C\textsuperscript{-/} ECT has not undergone hypertrophic remodeling.
Online Figure VIII. Expression of Genes Encoding Sarcomeric and Ca\textsuperscript{2+} Handling/Sensing Proteins. Relative expression of *Atpa2*, encoding Serca2A (A); *Ryr2*, encoding cardiac ryanodine receptor (B); *Slc8a1*, encoding sodium-calcium exchanger (C); *Tnni1*, encoding slow skeletal troponin I (D); *Tnni3*, encoding cardiac troponin I (E); *Tnnt2*, encoding cardiac troponin T (F); *Tpm1*, encoding alpha tropomyosin (G); *Actc1*, encoding cardiac alpha actin (H) and *Actn2*, encoding alpha actinin (I). WT ECT- open bars; cMyBP-C-/- ECT- filled bars; cMyBP-C-/- adWT ECT- light grey bars. Relative expression levels are represented as a percentage of *Actb* (beta actin) expression levels. No significant differences were detected between any groups for any gene analyzed (one way ANOVA, Bonferroni’s post-hoc analysis; n = 4). These findings indicate that expression levels of sarcomeric protein encoding genes, as well as genes encoding proteins involved in Ca\textsuperscript{2+} sensing/handling are similar in WT, cMyBP-C-/- and cMyBP-C-/- ad WT ECT.
Online TABLE I

Contractile function of WT, cMyBP-C<sup>−/−</sup> and cMyBP-C<sup>−/−</sup> adWT ECT

* P < 0.05 (one way ANOVA, Bonferroni’s post-hoc analysis)

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