In Vivo Cardiac Myosin Binding Protein C Gene Transfer Rescues Myofilament Contractile Dysfunction in Cardiac Myosin Binding Protein C Null Mice

Sergei Merkulov, PhD; Xiaoqin Chen, MD; Margaret P. Chandler, PhD; Julian E. Stelzer, PhD

Background—Decreased expression of cardiac myosin binding protein C (cMyBPC) in the heart has been implicated as a consequence of mutations in cMyBPC that lead to abnormal contractile function at the myofilament level, thereby contributing to the development of hypertrophic cardiomyopathy in humans. It has not been established whether increasing the levels of cMyBPC in the intact heart can improve myofilament and in vivo contractile function and attenuate maladaptive remodeling processes because of reduced levels of cMyBPC.

Methods and Results—We performed in vivo gene transfer of cMyBPC by direct injection into the myocardium of cMyBPC-deficient (cMyBPC−/−) mice, and mechanical experiments were conducted on skinned myocardium isolated from cMyBPC−/− hearts 21 days and 20 weeks after gene transfer. Cross-bridge kinetics in skinned myocardium isolated from cMyBPC−/− hearts after cMyBPC gene transfer were significantly slower compared with untreated cMyBPC−/− myocardium and were comparable to wild-type myocardium and cMyBPC−/− myocardium that was reconstituted with recombinant cMyBPC in vitro. cMyBPC content in cMyBPC−/− skinned myocardium after in vivo cMyBPC gene transfer or in vitro cMyBPC reconstitution was similar to wild-type levels. In vivo echocardiography studies of cMyBPC−/− hearts after cMyBPC gene transfer revealed improved systolic and diastolic contractile function and reductions in left ventricular wall thickness.

Conclusions—This proof-of-concept study demonstrates that gene therapy designed to increase expression of cMyBPC in the cMyBPC-deficient myocardium can improve myofilament and in vivo contractile function, suggesting that cMyBPC gene therapy may be a viable approach for treatment of cardiomyopathies because of mutations in cMyBPC. (Circ Heart Fail. 2012;5:635-644.)

Key Words: cardiomyopathy ■ echocardiography ■ gene therapy ■ myocardial contraction

Familial hypertrophic cardiomyopathy (FHC) is an inherited autosomal disease that is most commonly caused by mutations in sarcomeric protein genes. Of the 10 different sarcomeric genes identified to cause hypertrophic cardiomyopathy, mutations in the gene encoding cardiac myosin binding protein C (cMyBPC) are among the most common cause of hereditary-linked hypertrophic cardiomyopathy.1–3 FHC impacts ≈1 in 500 individuals; however, several founding mutations in cMyBPC around the world have been recently shown to affect millions of individuals from common ancestry4 who are at significantly greater risk for development of cardiac dysfunction and heart failure compared with individuals who do not carry these mutations. The mechanisms by which cMyBPC mutations cause cardiac disease are still not clear, but the few studies that have examined human tissue samples from patients carrying cMyBPC mutations have found direct evidence for decreased expression of cMyBPC in the heart,5,7 suggesting that in these patients decreased cMyBPC expression may contribute to cardiac dysfunction and hypertrophy.

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A causal link between decreased cMyBPC expression and cardiac dysfunction is yet to be definitively established; however, evidence from mouse models suggests that complete absence of cMyBPC in the heart (cMyBPC−/−) leads to severe contractile dysfunction and left ventricular (LV) hypertrophy,6–11 whereas a ≈25% decrease in cMyBPC expression in hearts of heterozygous cMyBPC-null mice leads to moderate mechanical dysfunction and development of milder hypertrophy than cMyBPC−/− hearts.12,13 It is thought that the molecular mechanism that impairs cardiac contractile function in cMyBPC-deficient mice is an acceleration of cross-bridge kinetics because of a loss in the inhibitory effects of cMyBPC on actomyosin interactions14–16 and decreased stability of strongly bound cross-bridges causing their premature detachment from actin.17 However, it is yet to

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be demonstrated that increasing the levels of cMyBPC in the intact heart can improve contractile function of hearts deficient in cMyBPC. Thus, the goal of this study was to increase the expression of cMyBPC in cMyBPC−/− hearts by transfecting the myocardium in vivo with recombinant viral vectors to determine whether restored levels of cMyBPC in the sarcosome can reverse abnormal cross-bridge behavior at the myofilament level. A secondary goal was to determine whether restored cross-bridge function can improve impaired contractile function in vivo. This proof-of-concept experiment will establish the feasibility of cMyBPC gene therapy in a mouse model of FHC and will provide an initial platform for future research that will develop the clinical application of cMyBPC gene therapy as a strategy for treatment of cMyBPC-related FHC.

Methods
An expanded Methods section is available in the online-only Data Supplement.

cMyBPC Gene Transfer
Lentiviruses encoding the full-length mouse cMyBPC (LcMyBPC) or lacking cMyBPC (vehicle, lentivirus-cytomegalovirus (LCMV) promoter only) were generated using the ViraPower Lentivirus expression system (Invitrogen) according to the manufacturer’s instructions. Adult male cMyBPC−/− and wild-type (WT) mice (SV/129 strain, 8–26 weeks of age) were anesthetized, and a thoracotomy was performed to directly inject the myocardium with lentivirus using a syringe with a fine needle tip. All procedures involving animal care and handling were performed according to institutional guidelines set forth by the Animal Care and Use Committee at Case Western Reserve University.

In Vitro Mechanical Experiments and In Vivo Assessment of Contractile Function
Mechanical measurements of skinned multicellular ventricular myocardium isolated from cMyBPC−/− hearts 21 days and 20 weeks after gene transfer (cMyBPC or vehicle) were performed as previously described.14,15,18,19 Mechanical measurements were also performed on cMyBPC−/− skinned myocardium after reconstitution with recombinant cMyBPC to directly compare the effects of increased cMyBPC expression by in vitro and in vivo techniques. To assess the effects of cMyBPC gene transfer on in vivo cardiac contractile function and morphology, transthoracic echocardiography was performed on a separate group of cMyBPC−/− mice 21 days after cMyBPC gene transfer (cMyBPC or vehicle).

Myofibrillar Protein Content and RNA Analysis
The cMyBPC content was measured in individual fibers isolated from LcMyBPC-treated cMyBPC−/− LV that were used for mechanical experiments. No significant differences in the relative abundance or phosphorylation status of myofilament proteins were detected between groups (Figure 1). As expected, cMyBPC was not detected in fibers isolated from untreated and vehicle-treated cMyBPC−/− hearts; however, fibers isolated from LcMyBPC-treated cMyBPC−/− hearts (21 days after cMyBPC gene transfer) expressed cMyBPC at a level of 97±7% of the cMyBPC content of fibers isolated from WT hearts. Expression of cMyBPC in LcMyBPC-treated hearts was stable and maintained at a high level (87±12% of the cMyBPC content of WT fibers) 20 weeks after gene transfer (Table 1). Expression of β-myosin heavy chain was slightly elevated in skinned myocardium isolated from untreated cMyBPC−/− hearts (16±5%; P<0.05) compared with WT myocardium and was nearly absent in myocardium isolated from LcMyBPC-treated cMyBPC−/− hearts (4±3%; not significant) (Figure 1).

Mechanical Properties of Skinned Myocardium Isolated from cMyBPC−/− Hearts After In Vivo cMyBPC Gene Transfer and Reconstitution With Recombinant cMyBPC
The steady-state mechanical properties of skinned myocardium isolated from WT, cMyBPC−/−, LcMyBPC-treated cMyBPC−/− hearts (21 days and 20 weeks post gene transfer), vehicle-treated cMyBPC−/− hearts, and cMyBPC−/− fibers reconstituted with recombinant cMyBPC are summarized in Table 1.

Fluorescence Imaging Immunohistochemistry
Immunofluorescent detection of cMyBPC was performed by confocal microscopy on skinned myocardium and whole heart sections isolated from WT, cMyBPC−/−, and virus-treated cMyBPC−/− mice 21 days after gene transfer.

Statistical Analysis
Skinned fiber mechanical data were analyzed as previously described.16,17 Comparisons of in vitro and in vivo measurements between groups were performed using a 1-way ANOVA with Tukey post hoc test. Statistical significance was defined as P<0.05.

Results
Myofilament Protein Expression and Phosphorylation in WT and Virus-Treated cMyBPC−/− Myocardium Used for In Vitro Mechanical Studies
The cMyBPC content of individual fibers isolated from LcMyBPC-treated cMyBPC−/− LV that were used for mechanical experiments was not different than the cMyBPC content of fibers isolated from WT LV or cMyBPC−/− fibers after in vitro reconstitution using recombinant cMyBPC (Table 1). In contrast, no cMyBPC expression was detected in skinned myocardium isolated from vehicle-treated cMyBPC−/− LV (ie, LCMV) (Figure 1). The sarcomeric localization of cMyBPC in skinned myocardium isolated from LcMyBPC-treated cMyBPC−/− hearts and cMyBPC−/− myocardium reconstituted with cMyBPC was probed by immunohistochemistry and showed similar staining patterns to WT myocardium, suggesting that in vivo and in vitro cMyBPC reconstitution resulted in proper cMyBPC incorporation within the sarcomere (Figure 2). Myofilament protein expression and phosphorylation were assessed in myocardium isolated from the region of the viral injection site (mid-LV to apex) of WT, cMyBPC−/−, and virus-treated cMyBPC−/− hearts that were used for mechanical experiments. No significant differences in the relative abundance or phosphorylation status of myofilament proteins were detected between groups (Figure 1). As expected, cMyBPC was not detected in fibers isolated from untreated and vehicle-treated cMyBPC−/− hearts; however, fibers isolated from LcMyBPC-treated cMyBPC−/− hearts (21 days after cMyBPC gene transfer) expressed cMyBPC at a level of 97±7% of the cMyBPC content of fibers isolated from WT hearts. Expression of cMyBPC in LcMyBPC-treated hearts was stable and was maintained at a high level (87±12% of the cMyBPC content of WT fibers) 20 weeks after gene transfer (Table 1). Expression of β-myosin heavy chain was slightly elevated in skinned myocardium isolated from untreated cMyBPC−/− hearts (16±5%; P<0.05) compared with WT myocardium and was nearly absent in myocardium isolated from LcMyBPC-treated cMyBPC−/− hearts (4±3%; not significant) (Figure 1).
Skinned myocardial preparations were isolated from the region of the viral injection site in all groups of mice. There were no differences in steady-state force generation at maximal and submaximal activating [Ca\(^{2+}\)] or in the steepness of the force-pCa relationship (Hill coefficient, \(n_0\)) in any of the groups (Figure 3). Consistent with previous studies,\(^{14,15}\) cMyBPC−/− skinned myocardium displayed dramatically accelerated rates of force development (\(k_f\)) (Figure 3), stretch-induced force decay (\(k_d\)) and delayed force development (\(k_d\)) at submaximal Ca\(^{2+}\) activations, and greater stretch-induced force decay (P\(_f\) amplitude) and stretch activation amplitude (P\(_a\)) compared with WT myocardium (Table 2). Skinned myocardium isolated from LcMyBPC-treated cMyBPC−/− hearts displayed dramatically slower cross-bridge kinetics (\(k_s\), \(k_{rel}\), and \(k_d\)) compared with untreated cMyBPC−/− myocardium, both 21 days and 20 weeks after cMyBPC gene transfer (Table 2; Figures 3 and 4). In contrast, skinned myocardium isolated from LCMV-treated cMyBPC−/− hearts displayed similar stretch activation properties to untreated cMyBPC−/− myocardium (Table 2).

The effects of increased exogenous cMyBPC content in cMyBPC−/− myocardium by in vivo gene transfer on contractile function were also directly compared with in vitro reconstitution of cMyBPC−/− myocardium with recombinant cMyBPC. Acute increases in cMyBPC content in the cMyBPC−/− sarcomere by in vivo and in vitro methods resulted in similar effects on contractile function because incubation of cMyBPC−/− skinned myocardium with recombinant cMyBPC did not alter steady-state force generation (Table 1) but dramatically slowed cross-bridge kinetics such that they became indistinguishable from WT myocardium (Figure 3).

### Effects of cMyBPC Gene Transfer on In Vivo Cardiac Function

The effects of cMyBPC gene transfer on in vivo cardiac contractile function were analyzed by echocardiography in a separate group of WT, cMyBPC−/−, LcMyBPC-treated cMyBPC−/− mice and are presented in Table 3. Consistent with previous findings,\(^{8–11}\) cMyBPC−/− hearts displayed significant increases in LV chamber dimensions at end systole and end diastole, as well as increases in posterior wall thickness and LV mass-to-bodyweight ratios, compared with WT hearts (Table 3). Furthermore, cMyBPC−/− hearts displayed diminished fractional shortening and shortened systolic ejection time and prolonged isovolumic relaxation times, indicating impaired systolic and diastolic function, respectively (Table 3). As expected, vehicle-treated cMyBPC−/− hearts showed no differences in contractile function and LV morphology compared with untreated cMyBPC−/− hearts (data not shown); however, LcMyBPC-treated cMyBPC−/− hearts showed improvements in cardiac contractile function and reduced LV dimensions compared with untreated cMyBPC−/− hearts (Table 3; Figure 5). The effects of cMyBPC gene transfer on cMyBPC expression, in vivo cardiac contractility, and LV morphology of cMyBPC−/− hearts were variable. The average cMyBPC content of LcMyBPC-treated cMyBPC−/− hearts assessed by in vivo echocardiography was 60±17% of the cMyBPC content of WT hearts (range, 38%–96%) (Figure 5). Hearts expressing high levels of cMyBPC displayed contractile function and LV wall dimensions that were similar to WT hearts (Figure 5), whereas hearts expressing cMyBPC at lower levels showed some functional improvements but modest changes in wall dimensions. Overall, averaged data of all LcMyBPC-treated hearts (n=19) showed statistically significant improvements in contractile function and cardiac morphology compared with untreated cMyBPC−/− hearts (Table 3).

The expression and localization of cMyBPC in the myocardium of LcMyBPC-treated cMyBPC−/− hearts were further analyzed by immunohistochemistry in serial sections cut from the apex, mid-LV, and base. LcMyBPC-treated hearts that showed significant improvements in in vivo function exhibited robust cMyBPC expression (Figure 5) throughout the myocardium, even in regions of the LV distal from the injection site, whereas in LcMyBPC-treated hearts that did not show marked improvements in in vivo function, cMyBPC expression was mostly localized to regions proximal to the viral injection site and was usually absent in the distal base region (data not shown).

### Discussion

The majority of treatment modalities for heart failure are mostly focused on delaying or preventing mechanisms that

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**Table 1. Steady-State and Dynamic Mechanical Properties of Skinned Fibers Isolated From WT and cMyBPC−/− Hearts After Viral Treatment and In Vitro cMyBPC Reconstitution**

<table>
<thead>
<tr>
<th>Total cMyBPC Content, %</th>
<th>(F_{\text{rel}}, \text{mN/mm}^2)</th>
<th>(F_{\text{max}}, \text{mN/mm}^2)</th>
<th>(pCa_{50})</th>
<th>(n_0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (5)</td>
<td>100±2</td>
<td>0.67±0.10</td>
<td>19.35±1.22</td>
<td>5.78±0.01</td>
</tr>
<tr>
<td>cMyBPC−/− (4)</td>
<td>0±0*</td>
<td>0.49±0.13</td>
<td>20.36±1.43</td>
<td>5.81±0.01</td>
</tr>
<tr>
<td>cMyBPC−/−+LCMV (3)</td>
<td>0±0*</td>
<td>0.56±0.18</td>
<td>19.25±1.75</td>
<td>5.79±0.02</td>
</tr>
<tr>
<td>cMyBPC−/−+LcMyBPC (21 days) (5)</td>
<td>97±7</td>
<td>0.63±0.12</td>
<td>20.67±1.19</td>
<td>5.81±0.02</td>
</tr>
<tr>
<td>cMyBPC−/−+LcMyBPC (20 weeks) (3)</td>
<td>87±12</td>
<td>0.64±0.23</td>
<td>19.57±2.02</td>
<td>5.79±0.02</td>
</tr>
<tr>
<td>cMyBPC−/−+cMyBPC (5)</td>
<td>98±9</td>
<td>0.69±0.22</td>
<td>21.08±2.17</td>
<td>5.79±0.02</td>
</tr>
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</table>

*WT indicates wild-type; cMyBPC, cardiac myosin binding protein C; total cMyBPC (%), percent cMyBPC content in individual fibers; \(F_{\text{rel}}\), Ca\(^{2+}\)-independent force at pCa 9.0; \(F_{\text{max}}\), maximal Ca\(^{2+}\)-activated force at pCa 4.5; pCa\(_{50}\), pCa required for half-maximal force generation; \(n_0\), Hill coefficient for force-pCa relationship; cMyBPC−/−+LCMV, vehicle-treated cMyBPC−/−; cMyBPC−/−+LcMyBPC, LcMyBPC-treated cMyBPC−/− 21 days or 20 weeks after gene transfer; cMyBPC−/−+cMyBPC, in vitro reconstitution of cMyBPC−/− myocardium with recombinant cMyBPC.

Data are means±SE. Skinned ventricular fibers (20–25 per group) were isolated from 3 to 5 mice per group (indicated in parentheses). All data presented for each subgroup were collected from the number of mice indicated in the left column.

*Significantly different from WT, \(P<0.001\).
Effects of cMyBPC Gene Transfer on Myofilament Contractile Function

The main purpose of this investigation was to determine whether in vivo gene transfer of cMyBPC into the cMyBPC-deficient myocardium can reverse the acceleration of cross-bridge kinetics at the myofilament level. Our data demonstrate that LcMyBPC-treated cMyBPC−/− myocardium displays high expression of cMyBPC without changes in the expression or phosphorylation of other myofilament proteins (Figure 1). Some studies using skinned myocardium isolated from cMyBPC knockout mice have reported decreases\(^8\) or increases\(^\text{22,23}\) in Ca\(^{2+}\) sensitivity of force, but other studies\(^\text{11,13,16,24}\) have shown that the absence of cMyBPC does not affect isometric steady-state force generation. It is possible that differences in the reported effects of cMyBPC on force generation may be related to alterations in the expression of thin filament proteins in the different mouse models, such as troponin I isoforms\(^\text{25}\) that can also affect submaximal force generation and differences in study protocols, that is, types of myocardial preparations used and experimental temperature. Here, we found that cMyBPC ablation did not affect steady-state isometric force generation. Because force generation is proportional to the number of strongly bound cross-bridges and the time cross-bridges are in strongly bound states, accelerated cross-bridge attachment and detachment because of cMyBPC ablation\(^\text{14,24}\) promotes cross-bridge transitions to force-generating states but also reduces the time cross-bridges remain in strongly bound states, with the net effect being relatively preserved duty ratios and force generation. Consistent with this argument, increased expression of cMyBPC in cMyBPC−/− myocardium did not alter steady-state Ca\(^{2+}\)-independent or Ca\(^{2+}\)-dependent isometric force generation (Figure 3). In contrast, cross-bridge kinetics were dramatically slowed in LcMyBPC-treated cMyBPC−/− myocardium, both 21 days and 20 weeks after gene transfer (Figures 3 and 4), such that \(k_\alpha\) and rates of force relaxation and development after acute stretch were similar to WT. Importantly, the salutary effects of viral-driven cMyBPC expression on contractile function in cMyBPC−/− myocardium were not transient but rather were maintained during a period of 20 weeks. Furthermore, using a complementary in vitro approach, we demonstrate that cMyBPC reconstitution of skinned myocardium isolated from cMyBPC−/− hearts with recombinant cMyBPC results in a similar slowing of cross-bridge kinetics\(^\text{24}\) as cMyBPC...
gene transfer in vivo (Figure 3), confirming that increased cMyBPC expression was responsible for changes in contractile function at the myofilament level in LcMyBPC-treated cMyBPC−/− myocardium.

The content of cMyBPC in myocardial preparations isolated from LcMyBPC-treated cMyBPC−/− hearts and cMyBPC−/− fibers incubated with recombinant cMyBPC in vitro was similar to the cMyBPC content of WT fibers. However, none of the fibers analyzed after in vivo or in vitro cMyBPC reconstitution expressed cMyBPC at greater levels than WT fibers. These results indicate that there may be limited numbers of cMyBPC-binding sites that dictate the localization of cMyBPC within the thick filament and suggest that acute introduction of exogenous cMyBPC in the cMyBPC-deficient sarcomere results in a physiological cMyBPC stoichiometry. This observation is also in agreement with earlier studies, demonstrating that cMyBPC is arranged regularly along the thick filament and is only present in specific regions within the A-band.

The mechanism by which incorporation of exogenous cMyBPC into cMyBPC−/− myocardium results in normal cross-bridge behavior likely involves restored interactions
of the cMyBPC N-terminal domains, with the S2 region of myosin near the myosin lever arm region that inhibits binding of myosin cross-bridges to actin, thereby slowing rates of cross-bridge attachment. In addition, cMyBPC may prolong the strongly bound state of cross-bridges to prevent their premature detachment from actin after completion of the cross-bridge powerstroke and augment the structural stability and longitudinal rigidity of the sarcomere via interactions of the C-terminal domain of cMyBPC with titin and the tail region of myosin.

**Effects of cMyBPC Gene Transfer on In Vivo Contractile Function**

Accelerated cross-bridge kinetics because of a lack of cMyBPC in the sarcomere lead to a truncated duration of systolic ejection and impair LV relaxation leading to diastolic dysfunction. Our in vitro studies demonstrated cMyBPC gene transfer reversed myofilament contractile dysfunction in skinned myocardium; therefore, it was of interest to study the effects of cMyBPC gene transfer on whole-organ cardiac contractile function in cMyBPC−/− mice. The cMyBPC

### Table 2. Stretch Activation Parameters of Skinned Fibers Isolated From WT and cMyBPC−/− Hearts After Viral Treatment

<table>
<thead>
<tr>
<th></th>
<th>$k_{df, S^{-1}}$</th>
<th>$k_{rel, S^{-1}}$</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (5)</td>
<td>23.59±1.23</td>
<td>271±21</td>
<td>0.04±0.01</td>
<td>0.10±0.01</td>
<td>0.06±0.01</td>
</tr>
<tr>
<td>cMyBPC−/− (4)</td>
<td>35.49±1.40*</td>
<td>470±20*</td>
<td>−0.03±0.01†</td>
<td>0.10±0.01</td>
<td>0.13±0.01†</td>
</tr>
<tr>
<td>cMyBPC−/−+LCMV (3)</td>
<td>34.96±1.92*</td>
<td>443±32*</td>
<td>−0.04±0.02†</td>
<td>0.10±0.02</td>
<td>0.14±0.02†</td>
</tr>
<tr>
<td>cMyBPC−/−+LcMyBPC (21 days) (5)</td>
<td>24.94±1.79</td>
<td>280±27</td>
<td>0.04±0.02</td>
<td>0.11±0.01</td>
<td>0.07±0.02</td>
</tr>
<tr>
<td>cMyBPC−/−+LcMyBPC (20 weeks) (3)</td>
<td>26.03±2.41</td>
<td>318±36</td>
<td>0.04±0.02</td>
<td>0.11±0.02</td>
<td>0.07±0.02</td>
</tr>
</tbody>
</table>

*WT indicates wild-type; cMyBPC, cardiac myosin binding protein C; cMyBPC−/−+LCMV, vehicle-treated cMyBPC−/−; cMyBPC−/−+LcMyBPC, LcMyBPC-treated cMyBPC−/− 21 days or 20 weeks after gene transfer.

Data are mean±SE. Skinned ventricular fibers (20–25 per group) were isolated from 3 to 5 mice per group (indicated in parentheses). All data presented for each subgroup were collected from the number of mice indicated in the left column. Rate constants were calculated from force transients in response to stretches of 1% of muscle length at approximately half-maximal levels of activation. Stretch activation parameters are described in the online-only Data Supplement.

*Significantly different from WT, P<0.001.
†Significantly different from WT, P<0.01.
The gene transfer technique used here (i.e., a single injection of LcMyBPC into the LV wall) was not specifically designed to transduce the whole cMyBPC−/− myocardium and resulted in variable cMyBPC expression. However, in some cMyBPC−/− hearts, high cMyBPC expression was achieved, even in regions distal to the injection site (Figure 5), which resulted in dramatically improved systolic and diastolic contractile function (Figure 5), as evidenced by increased ejection times and shortened isovolumic relaxation times (Table 3). Prolonged ejection times and accelerated diastolic filling in LcMyBPC-treated cMyBPC−/− hearts may be explained by the molecular effects of cMyBPC on cross-bridge function, which prolong the lifetime of the strongly bound cross-bridge state and inhibit cross-bridge rebinding to actin during the isovolumic relaxation phase of diastole, respectively.

Previous studies have shown that transgenic cMyBPC expression of ≈40% on a cMyBPC-null background reversed abnormal contractile function and hypertrophy in the mouse heart, suggesting that complete reconstitution of cMyBPC is not required to rescue the cMyBPC-null phenotype. Here also, despite the heterogeneity of the efficiency of myocardial transduction by in vivo cMyBPC gene transfer, we observed consistent improvements in contractile function in cMyBPC−/− hearts, which graded with myocardial cMyBPC expression. Reductions in LV wall thickness were observed in LcMyBPC-treated cMyBPC−/− mice and were corroborated by decreases in the abundance of mRNA of molecular markers of hypertrophy (online-only Data Supplement Figure). However, significant improvements in LV morphology were most notable in cMyBPC−/− hearts expressing high levels of cMyBPC, whereas weaker expression of cMyBPC localized to the viral injection region did not consistently result in improvements in LV morphology. It is possible that, unlike transgenic expression of cMyBPC that presumably results in a uniform distribution of
Figure 5. Effects of cardiac myosin binding protein C (cMyBPC) gene transfer on in vivo cardiac function of cMyBPC−/− hearts. Two-dimensional echocardiography images acquired in M-mode along the parasternal short-axis view showing contractility of (A) wild-type (WT), (B) lentiviruses encoding the full-length cMyBPC (LcMyBPC)-treated cMyBPC−/−, and (C) untreated cMyBPC−/− hearts. Total cMyBPC expression (normalized to α-actinin levels) as quantified by Western blotting of left ventricular homogenates of WT (lane 1), LcMyBPC-treated cMyBPC−/− (lane 2), and cMyBPC−/− (lane 3) hearts that were studied by in vivo echocardiography. ESD indicates end-systolic dimension, EDD, end-diastolic dimension.
cMyBPC in different regions of the heart, focal expression of cMyBPC by acute gene transfer in the cMyBPC−/− heart is insufficient to fully rescue myocardial dysfunction.

Implications for Treatment of cMyBPC-Related FHC
Recent studies have shown that gene transfer interventions designed to enhance sequestration of Ca$$^{2+}$$ into the sarcoplasmic reticulum to accelerate cardiac relaxation by increased expression of parvalbumin or SERCA2a [sarco/endoplasmic reticulum calcium ATPase 2a isoform] may be effective for correcting contractile dysfunction and pathological remodeling in heart failure. However, there are few studies that have tested this approach in vivo in an animal model of FHC. Although the precise mechanisms by which mutations in cMyBPC lead to contractile dysfunction and LV hypertrophy are still being debated, there is evidence that reduced amounts of cMyBPC in the myocardium may be a common feature that contributes to contractile dysfunction in cMyBPC-related FHC. Therefore, this proof-of-concept study demonstrates the feasibility and use of in vivo cMyBPC gene transfer to increase levels of cMyBPC in the sarcomere and improve contractile function in a mouse model of FHC. Importantly, our data show that increasing cMyBPC expression in cMyBPC-deficient hearts may have therapeutic application to delay the emergence of FHC or reverse the pathological course of the disease. The development of cMyBPC gene therapy may be especially useful in the treatment of patients with compound heterozygous or homozygous cMyBPC mutations, which may result in significant reductions in the content of cMyBPC in the sarcomere, and are associated with severe cardiac dysfunction and high incidences of death at a young age.

Limitations
Although this study suggests that cMyBPC gene therapy was effective in improving contractile function in a mouse model of cMyBPC-related FHC, several limitations require further consideration. Lentivirus vectors were used here because they can stably transduce nondividing cardiac myocytes and integrate into the host genome, thereby providing long-term gene expression, and have a sufficient cloning capacity to accommodate the cMyBPC cDNA. However, some questions remain regarding lentivirus biosafety, with concerns of potential insertional mutagenesis events, which were not investigated here. Furthermore, consistent and efficient global myocardial transduction of cMyBPC in vivo in larger mammalian hearts will require multiple direct injections of the LV wall or the use of systemic delivery techniques. Thus, further experiments will be required to refine in vivo lentivirus delivery methods and other viral gene delivery platforms, such as adenovirus-associated virus, which have shown promise in human clinical trials. Should also be considered for myocardial cMyBPC delivery. The efficiency of cMyBPC gene transfer should also be examined in mouse models that express varying levels of endogenous cMyBPC similar to some patients with cMyBPC insufficiency because native cMyBPC may compete with the exogenous cMyBPC for binding sites in the thick filament.

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Disclosures
Dr Stelzer holds a provisional patent for cardiac myosin binding protein C gene delivery for correction of contractile dysfunction in hypertrophic cardiomyopathy. The other authors have no conflicts to disclose.

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**CLINICAL PERSPECTIVE**

Mutations in cardiac myosin binding protein C (cMyBP-C) are among the most common causes of inherited hypertrophic cardiomyopathy, accounting for >40% of all known cases worldwide. Although the precise disease mechanisms of cMyBP-C-related hypertrophic cardiomyopathy are not fully understood, decreased expression of cMyBP-C in the heart because of cMyBP-C haploinsufficiency has been implicated as a common consequence of mutations in cMyBP-C. Because cMyBP-C is a crucial regulator of cardiac muscle contraction, the primary molecular defect of decreased cMyBP-C expression in the myocardium is abnormal cross-bridge function and force generation at the myofilament level. Indeed, cMyBP-C-null (cMyBP-C−/−) mice display accelerated cross-bridge kinetics accompanied by impaired in vivo contractile function and left ventricular hypertrophy. Thus, development of novel sarcomere-specific treatments to correct abnormalities in cardiac muscle contractile function because of mutations in cMyBP-C is critical to delay or prevent hypertrophic cardiomyopathy. In the present study, we demonstrate that skinned myocardium isolated from cMyBP-C−/− hearts after in vivo cMyBP-C gene transfer displays improved contractile properties compared with untreated cMyBP-C−/− myocardium, which were indistinguishable from wild-type myocardium and correlated with increases in cMyBP-C expression. In the intact cMyBP-C−/− heart, in vivo cMyBP-C gene transfer resulted in overall improvements in contractile function and left ventricular morphology compared with untreated cMyBP-C−/− hearts, despite heterogeneous cMyBP-C expression. Although modifications are required to improve methods of cMyBP-C delivery to the heart, this proof-of-concept study demonstrates that cMyBP-C gene therapy may be a viable approach for treatment of cardiomyopathies related to mutations in cMyBP-C.
In Vivo Cardiac Myosin Binding Protein C Gene Transfer Rescues Myofilament Contractile Dysfunction in Cardiac Myosin Binding Protein C Null Mice
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Detailed Methods

Preparation and Purification of Recombinant Lentivirus Vectors

A 3.8 kilobase cDNA fragment encoding the complete cDNA copy of murine cMyBPC was cloned from a recombinant vector pET 28b (Promega) using PCR TOPO cloning kit (pCR 8/GW/TOPO TA Cloning Kit, K2500-20SC, Invitrogen) in accordance with the manufacturer’s instructions. The forward primer sequence was 5’-atgccggagc cagggaagaa accag-3’ and the reverse primer sequence was 5’-tcactgaggaactcgcacctccag-3’. To construct the recombinant lentivirus (LcMyBPC) we used a lentivirus preparation kit from Invitrogen (Catalogue no. K4934-00). The cMyBPC cDNA and CMV promoter were cloned into the lentivirus destination vector pLenti6.4/R4R2/V5-DEST using the Gateway approach according to the protocol provided by Invitrogen. Briefly, three plasmids CMV/topo, cMyBPC/topo and pLenti6.4 were mixed in one tube following addition of LR recombinase. After 16 hours incubation at room temperature the reaction mix was used for transformation of Stbl3 E.coli competent cells (Invitrogen). Selected clones were propagated and plasmid DNA was purified using a QIagen kit and analyzed by digestion and sequencing. A lentivirus destination vector containing a CMV promoter and cMyBPC cDNA was propagated and DNA purified by MAXI Endotoxin free Kit (QIagen).

Lentivirus particles were produced by co-transfection of 293T cells with the destination vector pLenti6.4/R4R2/V5-DEST containing cMyBPC cDNA driven by CMV promoter and Viral Power Plasmid Mix (Invitrogen) using Lipofectamine 2000 as the transfecting agent. Briefly, 9 µg of Viral Power Mix and 4 µg DEST vector were mixed in a tube containing 1.5 mL
of Opti-MEM reduced serum media and 40 µl Lipofectamine 2000 were added to another tube containing 1.5 mL of Opti-MEM reduced serum media. The contents of both tubes were combined and incubated for 20 minutes at room temperature. Then Lipofectamine-DNA complexes were added to 293T cells on tissue culture plates and incubated overnight in a 95% O₂ and 5% CO₂ environment at 37°C. The following morning Lipofectamine containing medium was replaced with complete 293T medium (DMEM, 10%FBS, 1%P/S). Twenty four hours later medium containing virus particles was collected and virus particles were isolated by filtering the medium through 0.45 µm syringe filter (Millipore Millex-HV low protein binding Durapore (PVDF) (Catalogue no. SLHV033RS) attached to a 60ml syringe. 30 ml aliquots of virus particles were added to 2 ml of 20% sucrose solution in 38.5 ml centrifuge tubes (Beckman, #355642), and ultracentrifuged at 25000 rpm for 2.5 hours at 4°C, using a SW28 rotor. The resulting pellet was resuspended in 60 µl cold HBSS (Invitrogen #14025), aliquoted in eppendorff tubes and stored at -80°C until use. Virus titter was determined by a Lenti-GOStix kit (Clontech) and SYBR-based qRT-PCR kit (ABM, LV900) according to the manufactures’ instructions using an Applied Biosystems RT-PCR platform.

**Myocardial In Vivo cMyBPC Gene Transfer**

Adult male cMyBPC<sup>−/−</sup> and wild-type (WT) mice of the SV/129 strain (8-26 weeks of age) were used in this study. Mice were anesthetized with 1-2% isoflurane, intubated and placed on artificial ventilation (80-100 breaths/minute). A thoracotomy was performed over the third intercostals space of the left thorax to expose the heart. A syringe with a 30 gauge fine needle was inserted into the LV free wall in between the muscle layers of the LV approximately equidistant from the apical tip and the mid-LV. The tip of the needle was oriented almost parallel to the apex-base plane of the LV and at ~50% wall depth in between the epicardial and
endocardial layers of the myocardium. The tip of the needle was carefully advanced beyond the mid-LV towards the LV base and 20-25µl of lentivirus was slowly released into the myocardium (2x10^9 CFU/ml) as the syringe was slowly regressed towards the apex. After virus injection the syringe was withdrawn the chest was closed and negative pleural pressure reestablished before extubation. All procedures involving animal care and handling were performed according to institutional guidelines set forth by the Animal Care and Use Committee at Case Western Reserve University.

Expression and Purification of Recombinant cMyBPC

Purified recombinant cMyBP-C was generated as previously described.2,3 The full length mouse cMyBP-C DNA sequence containing an 11–amino acid N-terminal FLAG-tag epitope were cloned into tpFastBac1 plasmids (Invitrogen) and used for transposition of expression cassettes into bacmids. Baculovirus strains were prepared according to the manufacturer’s instructions, and were used to infect Sf9 cell monolayers. Cells were collected 80–96 h after infection, and recombinant cMyBP-C was extracted and purified on anti-FLAG M2 agarose columns (Sigma).

Solutions for Skinned Fiber Experiments

Solution compositions were calculated using the computer program of Fabiato4 and stability constants listed by Godt & Lindley5 corrected to pH 7.0 and 22°C. All solutions contained (in mM) 100 N,N-bis (2 hydroxy-ethyl)-2-aminoethanesulfonic acid (BES), 15 creatine phosphate, 5 dithiothreitol, 1 free Mg^{2+}, and 4 MgATP. pCa 9.0 solution contained 7 EGTA and 0.02 CaCl_2; pCa 4.5 contained 7 EGTA and 7.01 CaCl_2; and pre-activating solution contained 0.07 EGTA. Ionic strength of all solutions was adjusted to 180 mM with potassium propionate. Solutions
containing different amounts of Ca\textsuperscript{2+}\textsubscript{free} were prepared by mixing appropriate volumes of solutions of pCa 9.0 and pCa 4.5.

**Apparatus and Experimental Protocols for Skinned Fiber Experiments**

Skinned multicellular ventricular myocardium for mechanical experiments was prepared and attached to the arms of a position motor and force transducer as previously described.\textsuperscript{6-10} Motor position and force signals were sampled using SL Control software\textsuperscript{11} and saved to computer files for later analysis. All mechanical measurements were performed at 22°C and sarcomere length was set to 2.1 µm. Mechanical measurements were performed on skinned myocardium isolated from cMyBPC\textsuperscript{-/-} hearts following treatment with LcMyBPC, and skinned myocardium isolated from LCMV treated cMyBPC\textsuperscript{-/-} hearts as well as untreated cMyBPC\textsuperscript{-/-} and WT hearts. For *in vitro* reconstitution experiments skinned myocardium was isolated from cMyBPC\textsuperscript{-/-} hearts and was incubated with purified recombinant cMyBPC for 1.5 hours at 22°C in pCa 9.0 solution containing 0.2 mg/ml before the measurement of mechanical properties.\textsuperscript{3}

*Force-pCa Relationships:* Methods for obtaining and analysis of force-pCa relationships are described in detail elsewhere.\textsuperscript{6,7} Briefly, the sarcomere length of each myocardial preparation was adjusted to ~2.1 µm in relaxing solution prior to measurement of steady state isometric force in solutions of varying free [Ca\textsuperscript{2+}]. The difference between steady-state force and the force baseline obtained after the 20% slack step was measured as the total force at that free [Ca\textsuperscript{2+}]. Active force was then calculated by subtracting Ca\textsuperscript{2+}-independent force in solution of pCa 9.0 from the total force and was normalized to the cross-sectional area of the preparation, which was calculated from the width of the preparations assuming a cylindrical cross-section. Force-pCa relationships were constructed by expressing submaximal force (P) at each pCa as a fraction of maximal force (P\textsubscript{o}) determined at pCa 4.5, i.e., P/P\textsubscript{o}. The apparent cooperativity in the activation
of force development was inferred from the steepness of the force-pCa relationship and was quantified using a Hill plot transformation of the force-pCa data. The force-pCa data were fit using the equation, \( \frac{P}{P_o} = \frac{[Ca^{2+}]^n}{(k^n + [Ca^{2+}]^n)} \), where \( n \) is the Hill coefficient, and \( k \) is the \([Ca^{2+}] \) required for half-maximal activation (i.e., pCa \(_{50}\)).

Rate of Force Development: The rate constant of force development \( (k_{tr}) \) was measured in skinned myocardium as a measure of the rate of transitions of cross-bridges between weak-binding, non-force-generating states and strong-binding, force-generating states, using a release-restretch protocol \( (k_{tr}) \). Each skinned preparation was transferred from relaxing to an activating solution containing Ca\(^{2+}\) (sufficient for half maximal activation) and allowed to generate steady-state force. The myocardial preparation was rapidly (< 2 ms) slackened by 20% of its original length, resulting in a rapid reduction of force to near zero, i.e., < 5% of steady isometric force. This was followed by a brief period of unloaded shortening (10 ms) after which the preparation was rapidly restretched to its original length. The apparent rate constant of force development \( (k_{tr}) \) was estimated by linear transformation of the half-time of force redevelopment, i.e., \( k_{tr} = \frac{0.693}{t_{1/2}} \), as described previously. The apparent rate constant of force development \( (k_{tr}) \) was estimated by linear transformation of the half-time of force redevelopment, i.e., \( k_{tr} = 0.693/t_{1/2} \).

Stretch Activation Experiments: To measure dynamic cross-bridge behavior in response to acute stretch (stretch activation) a rapid stretch (~10 muscle lengths s\(^{-1}\)) of 1% of muscle length was imposed on fibers that were activated to develop submaximal forces of ~50% \( P_o \). The method used for measuring the stretch activation variables have been described in detail. The amplitudes of the phases of the stretch activation responses were measured as follows: \( P_1 \), measured from pre-stretch steady-state force to the peak of phase 1, \( P_2 \), measured from pre-stretch steady-state force to the minimum force value at the end of phase 2, \( P_3 \), measured from pre-stretch steady-state force to the minimum force value at the end of phase 3.
state force to the peak value of delayed force, and P_{df}, difference between P_{3} and P_{2}. All amplitudes were normalized to the pre-stretch Ca^{2+} activated force to allow comparisons between preparations. Apparent rate constants were calculated for stretch induced force decay ($k_{rel}$, s$^{-1}$) between the peak of P_{1} and the minimum of P_{2} and for delayed force development ($k_{df}$, s$^{-1}$) from the beginning of force re-uptake following, i.e., the trough of P_{2} to the completion of delayed force development.8

**In Vivo Echocardiography**

Transthoracic echocardiography was performed 21 days after LV virus injection of cMyBPC$^{-/-}$ mice by using a Sequoia C256 system (Siemens) with a 15-MHz transducer. Echocardiography was also performed on untreated WT and cMyBPC$^{-/-}$ mice, and cMyBPC$^{-/-}$ mice injected with a vector containing only the CMV promoter but not the cMyBPC cDNA (LCMV, i.e., vehicle), which served as controls. Mice were lightly anesthetized with isoflurane (1-2%) and maintained supine on a heated warming pad with ECG limb electrodes. Two-dimensionally guided M-mode imaging of the LV were performed via parasternal and foreshortened apical windows. LV dimensions during end diastole (EDD) and end systole (ESD) were measured at the mid ventricle using ultrasonograph software, as well as posterior wall thickness (PWT) in diastole and systole. Isovolumic relaxation times (IVRT) and ejection time (ET) were measured as indices of LV chamber relaxation and contraction, respectively. Fractional shortening (FS) was calculated as (EDD-ESD)/EDD x 100, where ESD is the LV dimension in systole.12,13 End-diastolic and end-systolic dimensions were measured using ultrasonograph software.

**Myofibrillar Protein Content and RNA Analysis**

Whole heart tissue homogenates were prepared from WT, cMyBPC$^{-/-}$, and cMyBPC$^{-/-}$ mice following cMyBPC gene transfer for analysis of myofibrillar protein content and
phosphorylation. To detect phosphorylated proteins, 10 or 12% SDS-PAGE gels were stained with Pro-Q Diamond following the protocol of the vendor (Molecular Probes)\(^9\,10\), and to detect myofibrillar proteins, the gels were stained with Coomassie. Quantification of gels of myofibrillar protein content and phosphoproteins was performed on a Typhoon scanner (GE). The product of the area and mean raw optical density vs volume loaded were generated and a first order linear regression was fitted to the data points to determine the slope of the relationship between optical density and volume loaded as described previously.\(^9\) Expression of cMyBPC in WT, cMyBPC\(^{-/-}\), and virus treated cMyBPC\(^{-/-}\) hearts was also analyzed by Western blot using a cMyBPC specific antibody (Santa Cruz, CA). Individual skinned myocardial preparations isolated from untreated and virus treated cMyBPC\(^{-/-}\) hearts for mechanical experiments were also probed for cMyBPC content by SDS-PAGE. Myosin heavy chain (MHC) content in LV isolated from WT, cMyBPC\(^{-/-}\), and virus treated cMyBPC\(^{-/-}\) hearts was assessed by SDS-PAGE (6% acrylamide), and the relative proportions of \(\alpha\) and \(\beta\) MHC isoforms were determined by densitometric analysis of silver-stained gels as previously described.\(^6\) To determine expression levels of hypertrophic marker genes following cMyBPC gene transfer, reverse transcription PCR was performed using transcript-specific oligonucleotides to atrial natriuretic factor (ANF) and alpha-skeletal actin (\(\alpha\)-sk actin), and the values were expressed normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) values.\(^10\)

**Fluorescence Imaging and Immunohistochemistry**

Hearts were excised from WT, cMyBPC\(^{-/-}\), and virus treated cMyBPC\(^{-/-}\) mice 21 days post gene transfer, and transverse serial sections (5 \(\mu\)m) were prepared from the apex, mid-LV, and base for immunofluorescent detection of cMyBPC by confocal microscopy (Olympus Fluoview 1000) at 60X magnification. cMyBPC detection was accomplished by incubation of frozen heart
sections with a primary polyclonal antibody raised in rabbit designed to recognize the N-terminal domain of cMyBPC (Santa Cruz, CA) (1:400 dilution), and a goat anti-rabbit secondary fluorophore antibody (Texas Red, Invitrogen) (1:250 dilution). Immunofluorescent detection of cMyBPC was also performed on skinned myocardial preparations isolated from WT and cMyBPC<sup>−/−</sup> LV following cMyBPC gene transfer for mechanical experiments, and cMyBPC<sup>−/−</sup> fibers following reconstitution with recombinant cMyBPC, for visualization of localization of cMyBPC within the sarcomere. Skinned myocardium was prepared by homogenization, centrifuged in relaxing solution and washed with fresh relaxing solution three times prior to being dispersed onto polylysine-coated glass slides and fixed with 4% paraformaldehyde. Unspecific binding sites were then blocked by incubation in 5% nonspecific goat serum, followed by incubation with primary antibodies for cMyBPC (Santa Cruz 1:50 dilution) and mouse anti-α-actinin (Invitrogen, 1:1000 dilution) overnight at 4 °C and then with secondary antibodies (4 h at room temperature). After final washing, the slides were mounted with and imaged on Olympus Fluoview 1000 confocal microscope equipped with a 40X and 100X lens, using appropriate excitation and emission wavelengths.
**Supplemental Figure. Cardiac hypertrophy analysis.** The relative abundance of mRNA in of
the molecular markers of cardiac hypertrophy (values normalized to glyceraldehyde 3-phosphate
dehydrogenase, GAPDH abundance) atrial natriuretic factor (ANF) and alpha-skeletal actin (α-sk actin) were measured in ventricular tissues isolated from hearts of wild-type (WT), untreated
cMyBPC^−/− (cMyBPC^−/−), LcMyBPC treated cMyBPC^−/− (LcMyBPC^−/−, 21 days post gene
transfer), and vehicle treated (LCMV) cMyBPC^−/− hearts. Values are expressed as means ± SE,
from 4-8 hearts per group. *Significantly different from WT, P<0.05. †significantly different
than cMyBPC^−/−, P<0.05.
Supplemental References


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