Cardiac myosin binding protein-C: a novel sarcomeric target for gene therapy

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Abstract Through its ability to interact with both the thick and thin filament proteins within the sarcomere, cardiac myosin binding protein-C (cMyBP-C) regulates the contractile properties of the myocardium. The central regulatory role of cMyBP-C in heart function is emphasized by the fact that a large proportion of inherited hypertrophic cardiomyopathy cases in humans are caused by mutations in cMyBP-C. The primary dysfunction in cMyBP-C-related cardiomyopathies is likely to be abnormal myofilament contractile function; however, currently, there are no effective therapies for ameliorating these contractile defects. Thus, there is a compelling need to design novel therapies to restore normal contractile function in cMyBP-C-related cardiomyopathies. To this end, concepts gleaned from various structural, functional, and biochemical studies can now be utilized to engineer cMyBP-C proteins that, when incorporated into the sarcomere, can significantly improve contractile function. In this review, we discuss the rationale for cMyBP-C-based gene therapies that can be utilized to treat contractile dysfunction in inherited and acquired cardiomyopathies.

Keywords Cardiac myosin binding protein-C · Gene therapy · Hypertrophic cardiomyopathy · Contractile dysfunction

Introduction

It is widely recognized that heart diseases account for the highest morbidity and mortality rates in the USA [28], accounting for ~500,000 deaths every year. The common course

of disease progression results in heart failure (HF) due to diminished cardiac performance which ultimately results in an inability to meet the circulatory demands of organs and peripheral tissues. Current pharmacological therapies for HF have mainly focused on managing the disease symptoms using agents such as β -blockers, angiotensin-converting enzyme inhibitors, angiotensin receptor II antagonists, diuretics, and inotropes (reviewed in [15, 35]). However, there are several drawbacks to these pharmacological therapies which, to date, have not shown long-term improvements in clinical outcomes. First, there are concerns about undesirable side effects on myocardial energetics and intracellular Ca^{2+} transients which may further decompensate the energy-compromised hearts and increase the risk of arrhythmias. Second, the majority of pharmacological therapies are not directed at intracellular targets that cause HF and, therefore, do not have a significant impact on halting the development and/or disease progression [15]. Thus, generating novel therapies that precisely aim at the intracellular targets that directly contribute to HF is a research priority and a major clinical challenge.

Normal cardiac function requires efficient and coordinated cyclical contraction and relaxation of the myocytes, processes which are largely dependent on the function of the sarcomeric proteins and the Ca^{2+} handling machinery. Indeed, the hallmark of HF is impaired contractile function, which, clinically, can be broadly classified into systolic and diastolic dysfunction [2]. Systolic dysfunction is characterized by a decreased pumping capacity of the heart as indicated by a marked reduction in the left ventricular (LV) ejection fraction [2]. On the other hand, diastolic dysfunction is characterized by a decreased ability of the heart to relax during diastole, a process that is largely dependent on the rate of Ca^{2+} sequestration away from the myofilaments back into the sarcoplasmic reticulum (SR). Because HF is commonly associated with a downregulation of the expression of SR Ca^{2+} ATPase

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(SERCA2a) [12], a protein largely responsible for Ca^{2+} reuptake by the SR during diastole, therapies aimed at increasing myocardial expression levels of SERCA2a have shown promise as indicated by significant improvements in cardiac performance in animal models of HF and in human clinical trials [12]. Although manipulation of the Ca^{2+} handling machinery has shown its utility for treatment of diastolic dysfunction, these approaches do not directly address systolic dysfunction and the ability of the myocardium to generate force at the level of the sarcomere. Thus, recently, there has been an increasing emphasis on directly targeting the sarcomeric contractile proteins for improving depressed cardiac contractile function in HF.

The sarcomere as a therapeutic target

The sarcomere is the basic contractile unit of the myocyte, and its functional properties are the primary determinants of in vivo cardiac performance. The force and speed of cardiac muscle contraction is determined by the cyclical interactions of actomyosin cross-bridges (XBs), which are, in turn, modulated by the thin and thick filament regulatory proteins. Force generation is dependent on the number of strongly bound XBs which is determined by the level of Ca^{2+} binding to troponin C, the phosphorylation status of regulatory contractile proteins, and inherent XB properties (such as stiffness, duty ratio, etc.) which together influence the unitary force of individual XBs and the duration of their force-producing state. Contractile dysfunction is often the principal initial insult in HF, and therefore, the sarcomere has become an attractive therapeutic target. In this regard, cardiac myosin activators are a new class of drugs that are being developed to treat systolic HF and have been shown to improve cardiac contractility in ongoing clinical trials [18, 32]. Myosin activators directly affect XB properties and enhance contractile force by favoring XB transitions from the weakly to strongly bound state by accelerating the rate of actin-dependent phosphate release, thereby enhancing the number of force-generating XBs at a given time [18, 32]. By shifting the XB equilibrium towards the strongly bound state and inhibiting nonproductive actin-independent phosphate release, myosin activators also minimize wasteful ATP hydrolysis [31, 32], and because these compounds do not alter Ca^{2+} transient properties, they also reduce the energetic costs associated with the intracellular Ca^{2+} cycling [31]. Therefore, directly targeting the sarcomere to augment the XB transition rates towards a strongly bound state is beneficial to the function of diseased hearts that are usually energetically compromised.

Apart from the use of cardiac myosin activators, sarcomere-directed gene therapies have also been proposed for correcting contractile dysfunction in HF (reviewed in [7]). The rationale for using gene therapy is that the cardiac

myocytes can be specifically targeted, and the genetic material packaged within the viral vectors can be transcribed to generate peptides or proteins that can be specifically directed to bind or affect precise molecular targets within the sarcomere. In this regard, the sarcomere is uniquely suited for gene transfer approaches because it maintains a stoichiometric relationship between contractile proteins; therefore, it is possible to target and replace an endogenous defective contractile protein with an exogenously derived “normal” contractile protein without altering the total quantity of the protein in the sarcomere (reviewed in [7]). Of course, a prerequisite to successful gene therapy is an efficient and long-term cardiac-specific transduction of the desired gene product, which is partly dependent on the application (i.e., either preclinical or clinical), the viral vector utilized, the delivery method chosen, and the size of the gene of interest. A detailed description of various vector platforms and delivery methods available for cardiac gene therapy is beyond the scope of this review and is the focus of other reviews [7, 12].

The first cardiac myofilament protein targeted by acute gene transfer was cardiac troponin I (cTnI) because of its important role in regulating Ca^{2+} sensitivity of force generation under normal and pathological conditions. It was demonstrated that gene transfer of genetically engineered cTnI with a histidine substitution for alanine at position 164 conferred cardioprotection in conditions of myocardial ischemia and hypoxia, which are common following a myocardial infarct, by preventing decreases in myofilament force generation induced by decreased intracellular pH, similar to the properties of slow skeletal TnI [7, 8]. Cardiac myosin heavy chain (MHC), the heart’s molecular motor, has also been explored as a therapeutic target because in some forms of HF, the expression of the slow β -MHC isoform is increased at the expense of the fast α -MHC isoform, thereby compromising contractile performance. Recent studies show that acute gene transfer of α -MHC into the predominantly β -MHC myocytes isolated from failing rabbit and human hearts improved myofilament contractile function without altering intracellular Ca^{2+} transient amplitudes [16], suggesting a utility of α -MHC as a positive inotrope.

Although these recent studies have shown promise for the utility of sarcomere-based gene therapies for the treatment of acquired forms of HF, this approach has not been rigorously investigated to treat inherited cardiomyopathies. However, because inherited cardiomyopathies principally arise due to mutations in sarcomeric proteins, they may be ideal targets for gene therapy. In particular, hypertrophic cardiomyopathy (HCM) affects ~0.2 % of humans [19] and is characterized by LV hypertrophy and impaired diastolic function, presenting with a broad clinical spectrum that includes a high rate of sudden cardiac deaths in young adults. Although mutations in 11 different sarcomeric genes cause HCM, ~40 % have been mapped to MYBPC3, the gene that encodes cardiac myosin

binding protein-C (cMyBP-C) [24]. So far, nearly 197 HCM mutations—ranging from missense mutations, nonsense mutations, splice site donor/acceptor mutations, insertions, and deletions—have been identified in MYBPC3 [14], with phenotypes ranging from a complete absence of symptoms to LV hypertrophy, progressive HF, stroke, and sudden cardiac death [25]. Most of cMyBP-C mutations cause reading frame shifts and formation of premature stop codons which would be predicted to produce truncated proteins [14]. However, recent studies [20, 33] on human myocardial biopsies from patients carrying these mutations have not identified truncated cMyBP-C proteins, but rather a decrease in total cMyBP-C content in the myocardium (haploinsufficiency), suggesting that truncated cMyBP-C proteins are not incorporated into the sarcomere and are degraded by cell surveillance mechanisms such as nonsense-mediated mRNA decay and ubiquitin–proteasome system [1].

It is unclear if cMyBP-C haploinsufficiency is the only disease mechanism related to cMyBP-C mutations, because there is evidence that missense mutations may incorporate into the sarcomere and act as “poison peptides” [9, 14]. Furthermore, a recent study has shown that mutations in cMyBP-C that are predicted to cause haploinsufficiency are likely to result in HCM, and missense mutations that may incorporate into the sarcomere are more likely to result in dilated cardiomyopathies (DCMs) [34], suggesting that the disease mechanisms for different mutations are distinct. Irrespective of the molecular mechanisms for cMyBP-C mutations, because cMyBP-C is a critical modulator of cardiac contractile function, the initial dysfunction in cMyBP-C mutation carriers is likely to be an abnormal myofilament function and force generation. In this regard, animal models have shown that a complete absence of cMyBP-C (cMyBP-C^{−/−}) causes severe contractile dysfunction and LV hypertrophy [13, 23], while moderate reductions in cMyBP-C (cMyBP-C^{+/-}) cause milder contractile dysfunction with little or no LV hypertrophy [4, 10, 13].

The precise molecular mechanisms by which decreased cMyBP-C expression or incorporation of mutant cMyBP-C into the sarcomere cause disease are not known. Normal function of cMyBP-C within the sarcomere involves its interactions with both myosin and actin [6, 27, 29, 30]; interactions of cMyBP-C with myosin S2 restrict the mobility of the myosin heads, thereby decreasing actomyosin interactions and XB recruitment, and interactions with actin act as a brake that slows XB detachment, which together slows ATP turnover rate [6, 27]. Thus, even subtle changes in the interactions of cMyBP-C with myosin, actin, or both would be expected to significantly affect XB behavior. In this regard, we recently showed that even modest decreases (25–30 %) in cMyBP-C expression in the sarcomere, similar to that observed in samples isolated from cMyBP-C mutation carriers [20, 33], accelerate the rate of cooperative XB recruitment at low Ca²⁺ activations due to the loss of the inhibitory effects of

cMyBP-C on the myosin heads, thereby enhancing the likelihood of strong XB formation and force generation [4]. Similarly, incorporation of cMyBP-C expressing an E258K missense mutation into the sarcomere resulted in accelerated XB kinetics and perturbed force generation due to disrupted cMyBP-C N-terminal interactions with the myosin S2 region [9]. Taken together, these studies suggest that abnormal cMyBP-C function directly contributes to myofilament contractile dysfunction underscoring the importance of devising novel therapeutic interventions that directly target cMyBP-C for improving contractile function in HF.

cMyBP-C as a target for treatment of inherited cardiomyopathies

Several studies have implicated cMyBP-C and its phosphorylation status as important mediators of pathology and function in acute and chronic HF [17, 29]; however, inherited mutations in cMyBP-C are the most established links to the direct roles of cMyBP-C in HF. Inherited cardiomyopathies are good candidates for gene therapy, as often as there is a defect in a single gene that is the primary cause of contractile dysfunction. Stoichiometric replacement of the defective gene with a normal functioning gene driven by an exogenous promoter can be a suitable approach to correct sarcomeric dysfunction, such that the progression of the disease is slowed or even reversed. In this context, we recently conducted a proof-of-principle study [21] to test the hypothesis that an increased cMyBP-C expression in vivo in cMyBP-C-deficient hearts can rescue myofilament dysfunction and improve in vivo contractile function. As an initial approach, in vivo cMyBP-C gene transfer was performed on cMyBP-C^{−/−} hearts by direct myocardial injection of lentiviral vectors designed to drive the expression of full-length cMyBP-C. Following cMyBP-C gene transfer, increased myocardial expression of cMyBP-C corrected the hypercontractile myofilament function of the cMyBP-C null myocardium, as evidenced by slowing of the rate of cooperative XB recruitment at submaximal Ca²⁺ activations in skinned myocardium isolated from these hearts such that they were indistinguishable from the function of wild-type (WT) skinned myocardium (Fig. 1) [21]. The levels of exogenously driven cMyBP-C in the myocardium isolated from lentivirus-treated cMyBP-C^{−/−} hearts did not exceed that of the WT cMyBP-C levels or the cMyBP-C levels of isolated skinned cMyBP-C^{−/−} myocardium following reconstitution with recombinant cMyBP-C [21], confirming that the exogenous cMyBP-C was properly incorporated into the sarcomere within the correct stoichiometry (Fig. 2).

Interestingly, improved myofilament contractile function due to cMyBP-C gene transfer in cMyBP-C^{−/−} hearts also resulted in concomitant improvements in whole-organ-level

Fig. 1 Effects of cMyBP-C gene transfer on XB function and in vivo systolic function. *Lane 1* indicates untreated WT; *lane 2* untreated cMyBP-C^{-/-}; *lane 3* cMyBP-C^{-/-}, 21 days following cMyBP-C gene transfer; and *lane 4* WT, 21 days following cMyBP-C gene transfer. **a** The rate constant of delayed force development (k_{df}) which denotes the rate constant of stretch-induced (1 % of initial muscle length) XB

recruitment was recorded in the skinned myocardium isolated from untreated and cMyBP-C-transfected hearts at ~50 % of maximal Ca^{2+} activation [21]. **b** Systolic ejection time was measured by in vivo echocardiography in untreated and cMyBP-C-transfected hearts [21]. All values are expressed as mean $\pm$ SE. ** $P < 0.05$, significantly different from WT

function as well as partial regression of maladaptive LV remodeling [21]. In particular, exogenous cMyBP-C incorporation into the cMyBP-C^{-/-} sarcomere likely restored normal interactions between cMyBP-C and myosin, thereby inhibiting unregulated XB interactions and prolonging the strongly bound state of XBs by preventing a premature detachment from actin by enhancing sarcomere stability and rigidity [26]. The molecular changes in XB function induced by cMyBP-C expression in the sarcomere resulted in significant increases in the abbreviated systolic ejection phase [21], which is the in vivo functional hallmark of the cMyBP-C^{-/-} heart [23]. It is possible that a normalization of systolic function in the cMyBP-C^{-/-} hearts also synchronized systolic and diastolic function which reduced hemodynamic load on the heart, thereby contributing to a partial regression of LV hypertrophy. In the context of potential clinical applications, because it is recognized that contractile dysfunction in cMyBP-C mutation carriers precedes maladaptive compensatory changes in LV geometry and hypertrophy [22], early stage gene therapy intervention may be a conducive procedure for correcting contractile dysfunction and halting disease progression.

Because the majority of cMyBP-C mutation carriers are heterozygous rather than homozygous, with one mutant and

one functional allele, it is also important to evaluate the utility of acute cMyBP-C gene transfer into hearts expressing normal amounts of cMyBP-C to establish that the exogenous cMyBP-C expression does not interfere with the function of endogenous cMyBP-C. Therefore, we recently conducted a parallel study (unpublished) to our cMyBP-C^{-/-} study [21] where we transfected WT hearts in vivo with the same lentivirus constructs that drove the expression of exogenous cMyBP-C. The exogenous protein was myc tagged to differentiate between the expression of endogenous and exogenous cMyBP-C proteins in the WT hearts. It was confirmed that viral driven exogenous cMyBP-C incorporates into the sarcomere properly (Fig. 2) and does not interfere with in vivo cardiac function as measured by echocardiography (Fig. 1). In vitro mechanical studies on skinned myocardium isolated from WT hearts 3 weeks following cMyBP-C gene transfer revealed no differences in steady state force measurements or dynamic XB kinetics compared to untreated WT skinned myocardium (Fig. 1). Expression of exogenous cMyBP-C was ~60 % of the total cMyBP-C in the heart which is consistent with a recent study showing that the half-life of cMyBP-C in the sarcomere is ~2 weeks [3]. Collectively, these studies show that cMyBP-C gene transfer can be effectively used to normalize cMyBP-C expression in cMyBP-C deficient hearts and

Fig. 2 Sarcomeric cMyBP-C incorporation following cMyBP-C gene transfer. Confocal images ($\times 100$ magnification) of myofibrils isolated from cMyBP-C^{-/-} (a) and WT (b) hearts 21 days following cMyBP-C gene transfer showing the localization of exogenous cMyBP-C in the

myocardium. Fluorescent detection using antibodies directed against cMyBP-C (a) and myc-tagged cMyBP-C (b) (in red), and α -actinin (in green) was employed to identify cMyBP-C incorporation into the sarcomere

could have clinical applicability because it effectively replaces mutant cMyBP-C proteins that are incorporated into the sarcomere without interfering with the function of endogenous cMyBP-C.

Future directions

Contractile dysfunction is a central feature of HF, and because cMyBP-C is a key sarcomeric protein that regulates both the speed and force of cardiac muscle contraction, it has the potential to become a novel therapeutic target. The development of cMyBP-C as a therapeutic target for heart disease is still in its infancy, but recent animal studies [21] have shown that cMyBP-C gene therapy may have utility for treatment of inherited mutations that lead to a decreased cMyBP-C expression in the heart by normalizing cMyBP-C levels in the sarcomere or by replacing mutant cMyBP-C proteins that are incorporated into the sarcomere. The ability of the sarcomere to stoichiometrically integrate exogenous proteins makes it an ideal target for viral-based gene delivery for treatment of cardiac contractile dysfunction [7]. Although it is well established that cMyBP-C mutations are a major cause of HCM and DCM [34], there is growing evidence that aberrant or depressed cMyBP-C phosphorylation contributes to the pathology of acquired forms of heart disease [5]. In particular, systolic HF which is characterized by depressed LV pumping capacity leads to compensatory downregulation and desensitization of β -adrenergic receptors, which, at the myofilament level, results in dephosphorylation of sarcomeric proteins such as cMyBP-C and cTnI [17]. It has been shown that in normal donor human hearts, the majority (~80 %) of cMyBP-C is at least partially phosphorylated, whereas in failing hearts, the majority of cMyBP-C becomes unphosphorylated [17]. Currently, the clinically approved inotropic agents for systolic HF may result in only temporary increases in cardiac contractility while eliciting undesirable side effects [15]. Because cMyBP-C phosphorylation accelerates the rate of XB recruitment and force generation as well as XB detachment and force relaxation, it is conceivable that the gene delivery of pseudo-phosphorylated cMyBP-C, where the phosphorylatable serines are mutated to aspartic acids to mimic the charge of phosphorylation, into the failing heart can serve as a sarcomere-based positive inotrope which can accelerate pressure development during systole and enhance cardiac relaxation during diastole, without invoking increases in intracellular Ca^{2+} levels or ATP utilization. Recent studies have demonstrated that the functional effects of phosphorylation of individual cMyBP-C residues are not equivalent [11, 29]. Thus, a more detailed understanding of the molecular mechanisms that govern the effects of cMyBP-C phosphorylation on interactions with actin and myosin will enable the development and delivery of designer cMyBP-Cs that can more precisely fine-tune contractile function in the heart based

on the modification of individual residues. Similarly, further studies are required to gain a more detailed understanding of how inherited mutations in cMyBP-C cause contractile dysfunction which will enable us to design patient-specific personalized gene therapies to prevent and/or blunt the progression of disease in different cardiomyopathies.

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Conflict of interest Dr. Stelzer holds a provisional patent for cMyBP-C gene delivery for correction of contractile dysfunction in hypertrophic cardiomyopathy. The other authors have no conflicts to disclose.

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