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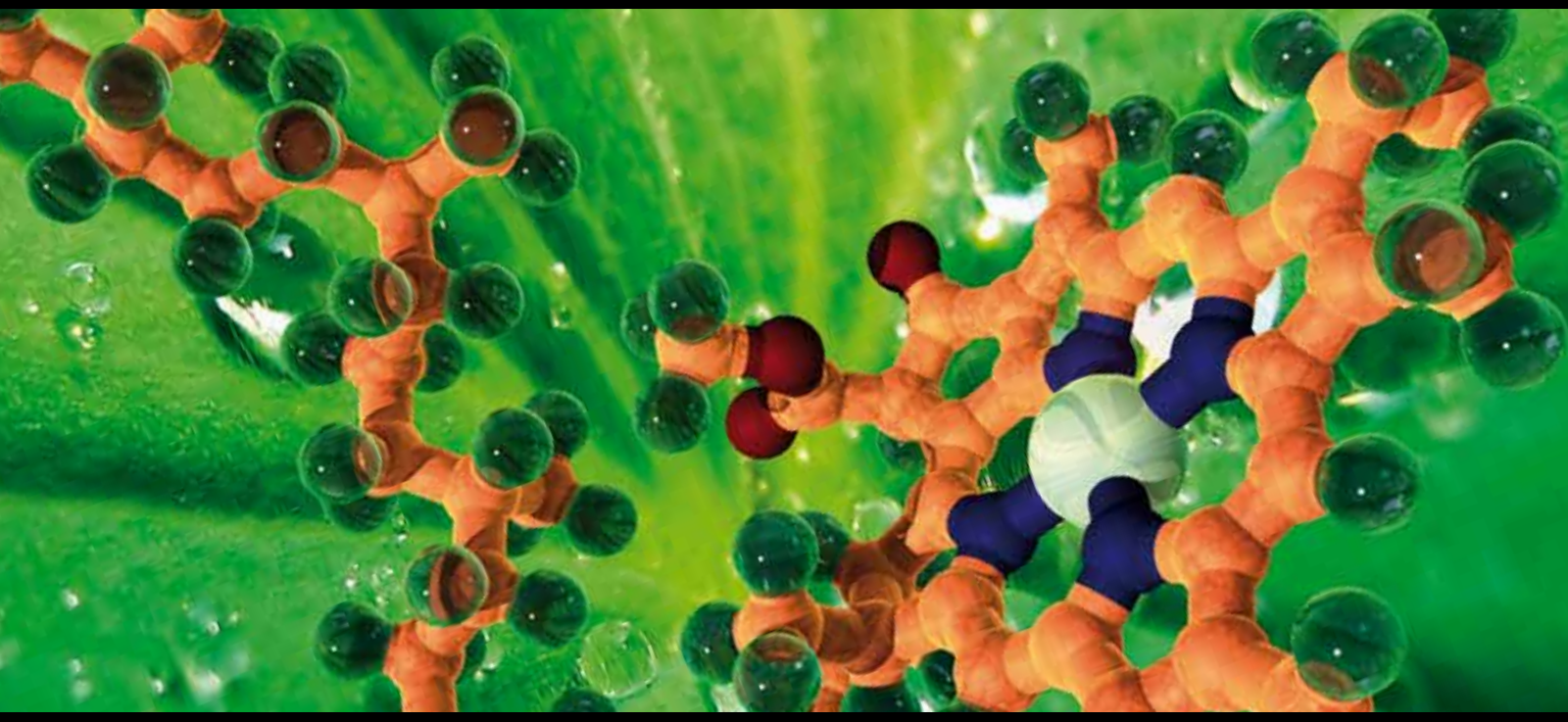
Cardiomyopathies: Classification, Clinical Characterization, and Functional Phenotypes

Guest Editors: Danuta Szczesna-Cordary, Sachio Morimoto, Aldrin V. Gomes, and Jeffrey R. Moore



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Editorial Board

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Editorial

Cardiomyopathies: Classification, Clinical Characterization, and Functional Phenotypes

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Cardiomyopathy is a category of disorders that affect the cardiac muscle and can cause extensive disability in a large and ethnically diverse population. It has been classified by the World Health Organization into three main types, based on anatomical and physiological features: dilated (DCM), hypertrophic (HCM), and restrictive (RCM). While DCM and RCM are associated with a progressive disease phenotype, heart failure, and sudden death, HCM is the leading cause of sudden cardiac death (SCD) in young athletes and is one of the most common forms of heart diseases affecting children. One review article in this issue by C. McCartan et al. discusses how the traditional classification of cardiomyopathies based on morphology has evolved due to rapid advances in our understanding of the genetic and molecular bases for many of these clinical entities. The implications of genetic testing have been discussed as being extremely valuable in the prognosis and treatment of cardiomyopathies. The authors emphasize that such testing may allow clinicians to move beyond unexplained ventricular abnormalities, identify positive patients and their genetic cause, and predict likely outcomes. A similar need for genetic testing has been voiced in another review article in this issue, by R. Parvari and A. Levitas, which addresses the pathogenesis of hereditary DCM. The authors strongly argue that the idea of early identification of the disease-causing mutations as presymptomatic interventions in DCM has proven valuable in preventing morbidity and mortality. DCM may be either sporadic or familial. A diagnosis of familial DCM is assigned when it occurs in at least two closely related family members or there was an occurrence of SCD at a young age. The genetic factor contributing to the manifestation of the disease can be classified either as a disease-causing gene mutation, as the etiology of monogenic disease, or as a disease-associated gene polymorphism that is involved in the pathogenesis of a multifactorial disease.

To date, hundreds of mutations in genes encoding all major sarcomeric proteins have been identified to cause HCM, DCM, or RCM. These include proteins essential for force production (β-cardiac myosin and actin), regulation of cardiac muscle contraction (tropomyosin and troponin), and proteins responsible for maintaining stability of the sarcomere (myosin-binding protein C, titin). The vast majority of cardiomyopathy mutations have been found in MYH7 gene encoding the β-myosin heavy chain (MHC). Mutations in myosin light chains MYL2 and MYL3 encoding the regulatory and essential light chains, respectively, are relatively rare, but they are also associated with malignant outcomes in young individuals. In fact, most of myosin essential light chain (ELC) mutations have been associated with SCD. In one of the articles of this issue, P. S. Andersen et al. identified a novel MYL3 mutation, V79I (Valine → Isoleucine), in a 38-year-old HCM patient with no visible cardiac symptoms. Mutation-positive family members were also asymptomatic, and no information was available on previous occurrences of SCD in two prior generations. At the molecular level, the mutation was predicted to disrupt the interaction of ELC with the MHC, but future biochemical studies are necessary to reveal the possible mechanism of this

V79I-induced HCM. In another paper by S. K. Gollapudi and M. Chandra, two cardiac troponin C (TnC) mutations were studied: an HCM-related, L29Q (Leucine → Glutamine), and a DCM-related, G159D (Glycine → Aspartic acid) mutation. Using mutant reconstituted skinned rat cardiac papillary muscle fibers, the authors provided new evidence for the mechanism by which these two TnC mutations bring about global change in myofilament mechanodynamics. The L29Q mutation caused a small decrease in myofilament Ca^{2+} sensitivity, which was linked to an increase in the tropomyosin-troponin regulatory unit "off" rate. The G159D mutation resulted in an increase in the cross-bridge cycling rates, "f" and "g", with no effects on maximal tension and Ca^{2+} sensitivity. These different molecular effects were predicted to result in two different disease phenotypes in humans. The L29Q-induced HCM phenotype was proposed to evolve due to a compensatory response of the myocardium to decreased tension caused by an attenuation of myofilament Ca^{2+} sensitivity. The G159D-mediated DCM phenotype was hypothesized to be a result of increased cost of tension maintenance, subjecting the heart to a chronic stress that ultimately could lead to ventricular dilatation.

Heart failure is the common end-stage condition of various cardiovascular disorders including cardiomyopathies and is characterized by a progressive decrease in cardiac output combined with insufficient or absent compensatory mechanisms. The interplay between heart development and disease has been the subject of intense research with the characterization of the gene regulatory networks involved in the development of cardiac dysfunction. Another paper in this issue, by A. T. Mikhailov and M. Torrado, focuses on the role of myocardin and myocardin-related transcription factors (MRTF) in heart failure and cardiac hypertrophy. From the combined knowledge of the molecular and functional domains of the molecules as well as the phenotypes of several knockout models, it was concluded that myocardin and MRTF play a central role in cardiac formation, function, and disease and present themselves as potential candidates as therapeutic targets for the treatment of heart failure. Gene-manipulated mouse models that closely recapitulate the clinical phenotypes of human inherited cardiomyopathies are invaluable in demonstrating the true cause of the disease and exploring pathogenic mechanism and effective drugs. Ultrasound imaging has been increasingly applied to identify and characterize structural and functional features of different cardiac phenotypes in mouse models of cardiac disease. The laboratory of Dr. X. P. Huang from Florida Atlantic University is among the first to assess cardiac function using a high-resolution echocardiography on transgenic mice with various cardiomyopathies. In their review article, G. Chen et al. discuss several important technical developments in the conventional echocardiography, pulsed Doppler, and tissue Doppler imaging for assessment of cardiac morphology and function in mouse models of human inherited cardiomyopathies.

In summary, this special issue discusses several aspects of cardiomyopathies related to mutations in myofilament proteins. These papers and reviews as well as our own research using animal models of human heart disease suggest that finding the mutation-specific molecular causes of HCM, DCM, or RCM is absolutely necessary in the development of target-specific rescue strategies to ameliorate or reverse the effects of inherited cardiomyopathies. The classification and clinical characterization of cardiomyopathies will continue to evolve as we unravel these molecular mechanisms by which HCM, DCM, or RCM-associated mutations lead to heart disease and SCD.

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Research Article

Cardiomyopathy-Related Mutations in Cardiac Troponin C, L29Q and G159D, Have Divergent Effects on Rat Cardiac Myofiber Contractile Dynamics

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Previous studies of cardiomyopathy-related mutations in cardiac troponin C (cTnC)—L29Q and G159D—have shown diverse findings. The link between such mutant effects and their divergent impact on cardiac phenotypes has remained elusive due to lack of studies on contractile dynamics. We hypothesized that a cTnC mutant-induced change in the thin filament will affect global myofilament mechanodynamics because of the interactions of thin filament kinetics with both Ca^{2+} binding and crossbridge (XB) cycling kinetics. We measured pCa-tension relationship and contractile dynamics in detergent-skinned rat cardiac papillary muscle fibers reconstituted with the recombinant wild-type rat cTnC ($cTnC_{WT}$), $cTnC_{L29Q}$, and $cTnC_{G159D}$ mutants. $cTnC_{L29Q}$ fibers demonstrated a significant decrease in Ca^{2+} sensitivity, but $cTnC_{G159D}$ fibers did not. Both mutants had no effect on Ca^{2+}-activated maximal tension. The rate of XB recruitment dynamics increased in $cTnC_{L29Q}$ (26%) and $cTnC_{G159D}$ (25%) fibers. The rate of XB distortion dynamics increased in $cTnC_{G159D}$ fibers (15%). Thus, the $cTnC_{L29Q}$ mutant modulates the equilibrium between the non-cycling and cycling pool of XB by affecting the *on/off* kinetics of the regulatory units (Tropomyosin-Troponin); whereas, the $cTnC_{G159D}$ mutant increases XB cycling rate. Different effects on contractile dynamics may offer clue regarding how $cTnC_{L29Q}$ and $cTnC_{G159D}$ cause divergent effects on cardiac phenotypes.

1. Introduction

The presumptive conclusion drawn from the heterogenic nature of human cardiomyopathy suggests a link between the type of mutation and the nature of pathological remodeling of the heart. A growing number of mutations in human cardiac troponin C (cTnC), associated with either hypertrophic cardiomyopathy (HCM) or dilated cardiomyopathy (DCM), makes it a prominent target gene for functional characterization. Thus far, 5 mutations in cTnC are found to be associated with HCM and 6 with DCM. These HCM-linked cTnC mutations include L29Q [1]—A8V, C84Y, and E134D [2]—Q122AfsX30 [3], while the DCM-linked cTnC mutations include E59D/D75Y [4], G159D [5]—Y5H, M103I, D145E, and I148V [6]. cTnC comprises two globular lobes; the amino-(N) and the carboxyl-(C) terminal lobes, which are connected by a flexible linker. The binding of Ca^{2+} to the regulatory site-II of the N-lobe of cTnC is important for triggering structural changes in the regulatory unit (RU), consisting of troponin (Tn) and tropomyosin (Tm).

The binding of Ca^{2+} to cTnC has a strong influence on the rates of transition between *off* and *on* states of RU (Tm-Tn) and the thin filament activation. Moreover, the *on* and *off* kinetic states of RU depend on XB in the force-bearing state through cooperative mechanisms [7, 8]. Therefore, mutations in cTnC lead to the expectation that Ca^{2+} binding kinetics of thin filaments are altered. Two mutations in cTnC are of particular interest to our study: L29Q mutation in the N-lobe, and G159D mutation in the C-lobe. Because of its close proximity to site-II of cTnC, leucine to glutamine substitution at position 29 (L29Q) is considered to have a direct effect on the Ca^{2+} binding properties of cTnC [9–11]. On the other hand, aspartic acid to glycine substitution at position 159 (G159D) is thought to affect interactions

of cTnC with cardiac TnI (cTnI) and cardiac troponin T (cTnT), and possibly Tm [12, 13].

The first mutation, L29Q, in cTnC was discovered in a 60-year old male, who was diagnosed with concentric hypertrophy in the left ventricle [1]. However, the patient showed no signs of diastolic and systolic dysfunction [1]. On the other hand, the proband of the G159D *mutation* was diagnosed with DCM at the age of 21 [5]. The proband displayed sudden heart failure symptoms and required a heart transplant two months after diagnosis [5]. Previous *in vitro* studies of L29Q and G159D mutations reported contrasting findings, making it difficult to correlate the functional effects observed to the known cardiac phenotypes. Functional studies on the L29Q mutant reported an increase [10], a decrease [11], or no change in the myofilament Ca^{2+} sensitivity [14, 15]. Studies of G159D mutant also resulted in diverse findings on Ca^{2+} sensitivity; with one group reporting an increase [16], several groups reporting a decrease [14, 17, 18], and other groups reporting no effect at all [19, 20]. Such discrepancies may be primarily related to the use of heterologous tissue/proteins, extracted from pig, bovine, rabbit, human, rat, and mouse species. A cursory look at the proteins from these species reveal multiple amino acid differences, which render it difficult to use them in an assay that is designed to test the effect of a single amino acid exchange. Therefore, to permit an unambiguous understanding of the functional effects of point mutations in cTnC, it is imperative to minimize heterogeneity in experimental conditions.

An important question is whether the effects of cTnC mutations go beyond that of previously observed mild-to-moderate changes in myofilament Ca^{2+} sensitivity [10, 11, 14, 16–18]. Because of the interactions of thin filament kinetics with both Ca^{2+} binding and XB cycling kinetics, we predict that a cTnC mutant-induced effect on the thin filament would affect the other kinetic paradigms; the net effect is a change in the overall mechanodynamics of the whole myofilament system. Therefore, we hypothesized that a cTnC mutant-induced change will be expressed as a change in global myofilament mechanodynamics. To test our hypothesis, we studied the functional effects of L29Q and G159D cTnC mutations on contractile dynamics in detergent-skinned rat cardiac papillary muscle fibers reconstituted with homologous rat cardiac Tn subunits. L29Q substitution caused a small but significant decrease in Ca^{2+} sensitivity, while G159D mutation resulted in no effect. The rate constant that governs the length-mediated XB recruitment dynamics was faster in both L29Q and G159D mutants. The rate constant that describes the length-mediated XB distortion dynamics was faster in the G159D mutant. We discuss these data in terms of cTnC mutant-induced effect on global myofilament mechanodynamics.

2. Methods

2.1. Preparation of Detergent-Skinned Cardiac Papillary Muscle Fiber Bundles. All animals used in this study received proper care and treatment in accordance with the guidelines set by the Washington State University Institutional Animal Care and Use Committee. Papillary muscle fiber bundles from Sprague-Dawley rat hearts were prepared using the procedure described previously [21]. Briefly, rats were anaesthetized using isoflurane until they were deeply sedated; the depth of anesthesia was confirmed using a pedal withdrawal reflex. Hearts were quickly excised and placed into an ice-cold relaxing solution of pCa 9.0 [22]. Papillary bundles were removed from left ventricles of rat hearts and were further dissected into thinner bundles measuring 2.0-3.0 mm in length and 150–200 μm in cross-section. Thinner fiber bundles were detergent-skinned overnight in relaxing solution that contained 1% Triton-X-100 [23].

2.2. Expression and Purification of Recombinant Rat Cardiac Tn Subunits. Recombinant *c-myc* tagged rat cardiac troponin T (*c-myc* RcTnT), troponin I (RcTnI), and troponin C (RcTnC), were all cloned into a pSBETa vector, and were expressed in BL21∗DE3 cells (Novagen, Madison, WI) for protein synthesis. L29Q and G159D substitutions in RcTnC were generated using site-directed DNA mutagenesis techniques and were cloned into a pSBETa vector. BL21∗DE3 cells were lysed and the proteins (*c-myc* RcTnT, RcTnI, and RcTnC) were purified using ion-exchange chromatography techniques, as described previously [22, 24, 25]. In brief, BL21∗DE3 cells of each protein preparation (~4 liters) were spun down and sonicated in 50 mM Tris (pH 8.0 at 4°C), 6 M urea, 5 mM EDTA, 0.2 mM PMSF, 5 mM benzamidine-HCl, 10 mM leupeptin, 1 mM pepstatin, 5 mM bestatin, 2 mM E-64, and 1 mM DTT. The insoluble fraction in each preparation was removed by centrifugation. *c-myc* RcTnT was purified by fractionation of the supernatant from the culture preparation using ammonium sulfate. The pellet from the 70% ammonium sulfate cut was first dissolved in 50 mM Tris (pH 8.0 at 4°C), 6 M urea, 1 mM EDTA, 0.2 mM PMSF, 4 mM benzamidine-HCl, and 1 mM DTT, and then purified by chromatography on a DEAE-fast Sepharose column [22]. RcTnT was eluted from the column using a linear NaCl gradient. RcTnI was purified by directly loading the supernatant from the RcTnI culture preparation onto a CM cation-exchange column and eluted using a linear NaCl gradient. Complete details on cTnI purification can be found in the study by Guo et al. [24]. Wild-type (WT) RcTnC, RcTnC-L29Q, and RcTnC-G159D were purified by loading the supernatant from each RcTnC culture preparation onto a DE-52 anion-exchange column and were eluted using a linear KCl gradient [25]. More details on the purification of cTnC can be found in the study by Pan and Johnson [25]. All fractions containing pure proteins were pooled and dialyzed thoroughly against deionized water containing 15 mM β-mercaptoethanol. Dialyzed proteins were lyophilized and stored at −80°C.

2.3. Reconstitution of Recombinant Rat Cardiac Tn Subunits into Detergent-Skinned Rat Cardiac Muscle Fiber Bundles. The reconstitution of recombinant cardiac Tn subunits into muscle fiber bundles was performed using a protocol described previously [22]. Briefly, we first prepared an extraction solution containing RcTnT-RcTnI by dissolving *c-myc* RcTnT (1.5 mg/mL, W/V) and RcTnI (1.0 mg/mL,

W/V) in 50 mM Tris-HCl (pH 8.0), 6 M urea, 1.0 M KCl, 10 mM DTT, and 0.2 μM PMSF. *c-myc* tagged RcTnT was used in our preparation so that the incorporation of exogenously added Tn could be probed using an antibody against the *c-myc* epitope. Previous studies have shown that presence of 11 amino acid *c-myc* epitope at the N-terminus of cTnT has no effect on the normal function of the heart [26–28]. High salt and urea in the extraction solution containing *c-myc* RcTnT-RcTnI were removed by successive dialysis against the following buffers that contain steadily decreasing salt and urea concentrations: 50 mM Tris-HCl (pH 8.0 at 4°C), 4 M urea, 0.7 M KCl, 1 mM DTT, 4 mM benzamidine-HCl, 0.4 mM PMSF, and 0.01% NaN_3 followed by 50 mM Tris-HCl (pH 8.0 at 4°C), 2 M urea, 0.5 M KCl, 1 mM DTT, 4 mM benzamidine-HCl, 0.4 mM PMSF, and 0.01% NaN_3. The extraction solution was then extensively dialyzed against the extraction buffer (50 mM BES (pH 7.0 at 20°C), 180 mM KCl, 10 mM BDM, 5 mM EGTA, 6.27 mM $MgCl_2$, 1.0 mM DTT, 4 mM benzamidine-HCl, 0.2 mM PMSF, and 0.01% NaN_3). Detergent-skinned papillary muscle fibers were first treated with this *c-myc* RcTnT + RcTnI protein sample, followed by RcTnC-WT or RcTnC mutant proteins (3 mg/mL) to complete the reconstitution procedure. In our study, detergent-skinned fiber bundles reconstituted with *c-myc* RcTnT + RcTnI + RcTnC-L29Q are referred to as "$cTnC_{L29Q}$ fibers" and those reconstituted with *c-myc* RcTnT + RcTnI + RcTnC-G159D are referred to as "$cTnC_{G159D}$ fibers." Fiber bundles reconstituted with *c-myc* RcTnT + RcTnI + RcTnC-WT are referred to as "$cTnC_{WT}$ fibers" and served as controls in this study.

2.4. SDS-PAGE and Western Blot. We ran 12.5% SDS PAGE to determine the incorporation of cTnC. First, we used 2% SDS solution (10 μL/fiber) to digest the reconstituted fibers for SDS-PAGE, as described previously [29]. SDS-digested fibers were mixed with an equal volume of gel-loading buffer that contained 125 mM Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 0.01% bromophenol blue, and 50 mM β-mercaptoethanol. Digested fibers were run on 12.5% SDS-PAGE to separate the proteins according to their molecular weights [29, 30].

For Western blot analysis, proteins from 12.5% SDS-PAGE were transferred onto a PVDF membrane and cTnC was probed using an anti-TnC primary antibody (Clone M5092922, Fitzgerald Industries International, Concord, MA). The resulting protein profiles from the Western blot were used to assess the incorporation of exogenous cTnC mutants in the reconstituted fibers.

2.5. pCa Solutions. For pCa titrations, the fiber was bathed in different pCa solutions ranging from 4.3 to 9.0. The maximal Ca^{2+}-activating solution (pCa 4.3) contained the following (in mM concentrations): 50 BES, 5 NaN_3, 10 phosphoenol pyruvate (PEP), 10 EGTA, 10.11 $CaCl_2$, 6.61 $MgCl_2$, 5.95 Na_2ATP, and 31 K-propionate. The relaxing solution (pCa 9.0) contained the following (in mM concentrations): 50 BES, 5 NaN_3, 10 PEP, 10 EGTA, 0.024 $CaCl_2$, 6.87 $MgCl_2$, 5.83 Na_2ATP, and 51.14 K-propionate. In addition, pCa solutions contained the following cocktail of protease inhibitors (in μM concentrations): 10 Leupeptin, 1 Pepstatin, 10 PMSF, 20 A_2P_5, and 10 Oligomycin. The reagent concentrations of all pCa solutions were calculated based on a program developed by A. Fabiato and F. Fabiato [31]. The pH of each solution was adjusted to 7.0 using KOH.

2.6. Measurements of Isometric Steady-State Force and ATPase Activity. Isometric steady-state force was measured using methods described previously [21, 22]. Briefly, detergent-skinned muscle fiber was attached between a force transducer and a servo motor using aluminum clips and was submerged in a chamber containing pCa 9.0 solution. The baseline SL of the fiber was adjusted to 2.2 μm using laser diffraction technique [32]. The fiber was then activated with a series of pCa solutions starting from pCa 4.3 to 9.0 and the steady-state tension elicited by the fiber in each pCa solution was recorded. These tension values in various pCa solutions were normalized with its respective value in pCa 4.3. The normalized tension values were plotted against pCa to construct pCa-tension relationships for each muscle fiber. All measurements from muscle fibers in this study were performed at 20°C and at a SL of 2.2 μm.

Ca^{2+}-activated maximal ATPase activity (pCa 4.3) was measured in reconstituted muscle fibers during isometric steady-state using an assay described previously [28, 33, 34]. In brief, a near UV light was projected through the muscle chamber which was split 50 : 50 for intensity detection at 340 nm and 400 nm wavelengths. Light intensity of the beam at 340 nm was sensitive to NADH, and thus a change in the UV absorbance at 340 nm can be directly correlated to the oxidation of NADH (i.e., ATP usage) through enzymatically coupled reactions [33, 34]. Light intensity of the beam at 400 nm was insensitive to NADH and, therefore, served as the reference signal. An analog divider and log amplifier produced a signal proportional to the amount of ATP consumed (i.e., amount of NADH oxidized) in the muscle chamber solution. After each recording, the UV absorbance signal of NADH was calibrated by multiple rapid injections of 25 pmol of ADP into the bathing solution, with a motor-controlled calibration pipette. Tension cost was estimated by dividing the maximal ATPase activity by the maximal tension in each muscle fiber.

2.7. Mechanodynamic Studies. To measure dynamic force-length relationships, we applied sinusoidal muscle length (ML) changes of constant amplitude (±0.5% of ML) to maximally activated muscle fibers [21, 35]. Two chirps, one with frequencies ranging from 0.1 to 4 Hz for a time period of 40 s, and the other with frequencies ranging from 1 to 40 Hz for a time period of 5 s, were administered to emphasize low- and high-frequency force components. The recruitment-distortion (R-D) model was fitted to the overall force response (including both low- and high-frequency components), as described previously [35]. The R-D model predicts a change in muscle force, $\Delta F(t)$, corresponding to

a change in muscle length, $\Delta ML(t)$, based on the following equation:

$$\Delta F(t) = \underbrace{E_0 \eta(t)}_{\text{recruitment}} + \underbrace{E_\infty x(t)}_{\text{distortion}}. \quad (1)$$

In the equation above, $\eta(t)$ and $x(t)$ are the variables that describe dynamic changes in crossbridge (XB) recruitment and distortion due to changes in ML, respectively. E_0 and E_∞ are stiffness coefficients that are proportional to the number of XB in the states, $\eta(t)$ and $x(t)$, respectively.

The R-D model was fitted to the total force response elicited by the fiber with $\Delta ML(t)$ as the input to estimate four important model parameters—E_0, b, E_∞, c [35]. Previously, we showed that a big advantage of the R-D model is that the total force response (Figure 1(a)) could be uniquely separated into two components: (1) force response due to low-frequency recruitment component (b, E_0; Figure 1(b)) and (2) force response due to high-frequency distortion component (c, E_∞; Figure 1(c)). This feature of the R-D model was successfully used previously to elicit the dynamic features of the respective force components in constantly activated muscle fibers [21, 35].

2.8. Crossbridge Model Scheme. In this study, we interpreted our experimental data using a reduced three-state model, as illustrated in Figure 2 [36]. In brief, the model describes thin filament activation using three kinetic processes:

(1) Ca^{2+} binding to the thin-filament regulatory unit (RU; Tm-Tn);

(2) RU switching between *on* and *off* states;

(3) XB cycling between attached and detached states.

To describe these respective processes, the total XB population is subdivided into two pools: a non-cycling pool (N_{nc}) and a cycling pool ($N_{c\text{-nfb}}$ and $N_{c\text{-fb}}$). The kinetic processes 1 and 2 are lumped into a single kinetic step that represents RU *on*/*off* kinetics. The *on*/*off* kinetics of RU are strongly affected by the interactions of thin filament regulatory processes with Ca^{2+} binding/dissociation kinetics of cTnC. The preferred state of the RU is "*off*" when Ca^{2+} is not bound to cTnC, whereas, the preferred state is "*on*" when Ca^{2+} is bound to cTnC. When RU are *off* (i.e., no activator Ca^{2+}), the interactions of myosin head with actin are inhibited due to the steric blocking of actin sites by RU. In this scenario, all XB will populate in the non-cycling N_{nc} state. The binding of Ca^{2+} to cTnC will switch the RU *on* by removing their steric blocking effect on actin sites, favoring the entry of XB into the cycling, $N_{c\text{-nfb}}$ and $N_{c\text{-fb}}$, states. The rate at which the XB transition from non-cycling to cycling pool (i.e., RU *on* kinetics) is represented by k_{on}, whereas, the rate at which XB transition from cycling to non-cycling pool (i.e., RU *off* kinetics) is represented by k_{off}. It is important to note that k_{on} and k_{off} are affected by both Ca^{2+} binding/dissociation kinetics and force-bearing XB. XB within the cycling pool may exist in two distinct states, a non-force-bearing state, $N_{c\text{-nfb}}$, and a force-bearing state, $N_{c\text{-fb}}$. In the cycling pool, XB alternate between $N_{c\text{-nfb}}$ and $N_{c\text{-fb}}$ states according to the rate constants, f and g.

In the context of this reduced three-state XB model scheme, the R-D model parameters—E_0, b, E_∞, and c—specifically represent the following: E_∞ is the magnitude of instantaneous length-mediated increase in stiffness due to the rapid distortion of XB in the $N_{c\text{-fb}}$ state; E_0 is the magnitude of length-mediated increase in stiffness caused by an increase in the number of XB in the $N_{c\text{-fb}}$ state; recruitment rate constant, b incorporates various length-sensing mechanisms including thin filament overlap, XB attachment, and its amplification by cooperativity (indicated by dashed arrow in Figure 2). In other words, the RU *on*/*off* rates, k_{on} and k_{off}, and XB cycling kinetics defined by rate constants, f and g, all coalesce into a single rate constant, b. Parameter c governs the distortion dynamics and has a strong dependence on the XB detachment rate, g [35].

2.9. Data Analysis. Data are shown as mean ± SEM. pCa required to elicit half maximal tension, pCa_{50}, and the Hill coefficient, n_H, were estimated by fitting the Hill's equation to normalized tension data. Our data included three groups: $cTnC_{WT}$, $cTnC_{L29Q}$, and $cTnC_{G159D}$ fibers. Statistical differences between $cTnC_{WT}$, $cTnC_{L29Q}$, and $cTnC_{G159D}$ fibers were analyzed using one-way ANOVA. Minimal statistical significance was set at $\alpha = 0.05$.

3. Results

3.1. Incorporation of Recombinant Mutant cTnC Proteins into Myofibers. Figure 3 shows the cTnC protein profiles from various reconstituted muscle fiber groups. The Western blot against anti-cTnC primary antibody confirmed the absence of native cTnC in the cTnT-cTnI treated fiber bundles, clearly demonstrating a near-complete removal of endogenous Tn units using our exchange procedure (Figure 3; *lane* 3). Furthermore, the Western blot confirmed that the recombinant $cTnC_{L29Q}$ (*lane* 4) and $cTnC_{G159D}$ (*lane* 5) mutants were incorporated properly in the reconstituted fiber bundles.

3.2. Effect of $cTnC_{L29Q}$ and $cTnC_{G159D}$ on Ca^{2+}-Activated Maximal Tension, Maximal ATPase Activity, and the Magnitude of Myofiber Dynamic Stiffness. We first assessed the effects of $cTnC_{L29Q}$ and $cTnC_{G159D}$ mutants on Ca^{2+}-activated maximal tension and maximal ATPase activity. To determine the maximal tension, reconstituted fibers were bathed in pCa 4.3 solution until they reached a steady-state isometric force. The isometric steady-state force was then converted to tension by expressing it as force per cross-sectional area. The Ca^{2+}-activated maximal tension values (in $mN \cdot mm^{-2}$) in $cTnC_{WT}$, $cTnC_{L29Q}$, and $cTnC_{G159D}$ fibers were 57 ± 2 ($n = 15$), 56 ± 2 ($n = 10$), and 53 ± 2 ($n = 14$), respectively. These data demonstrate that the Ca^{2+}-activated maximal tension values in $cTnC_{L29Q}$- and $cTnC_{G159D}$-reconstituted fibers were similar to that of $cTnC_{WT}$-reconstituted fibers. Thus, both $cTnC_{L29Q}$ and $cTnC_{G159D}$ had no impact on Ca^{2+}-activated maximal tension. Our observations in maximal tension agree well with many previous *in vitro* studies of L29Q [10, 14, 15] and

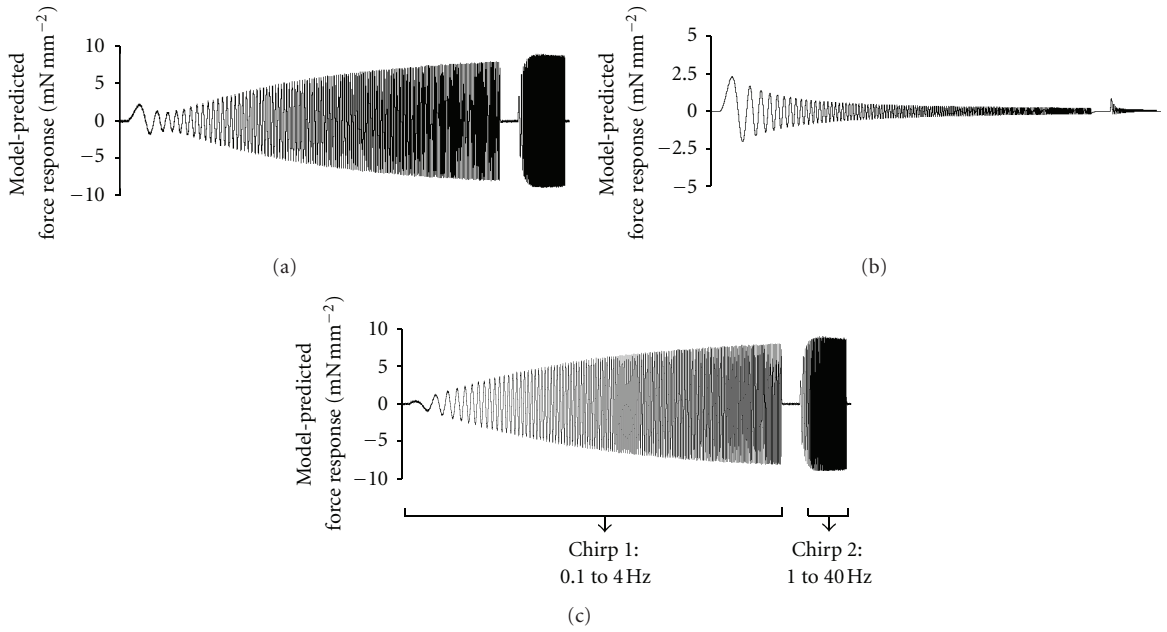

Figure 1: Representative model-predicted force response to chirp-length perturbation for a control-rat fiber (i.e., $cTnC_{WT}$ fiber). (a) Total force response that includes force components due to low-frequency XB recruitment component, $E_0{}^*\eta(t)$, and high-frequency XB distortion component, $E_\infty{}^*x(t)$. (b) Force response due to low-frequency XB recruitment component, $E_0{}^*\eta(t)$. (c) Force response due to high-frequency XB distortion component, $E_\infty{}^*x(t)$. E_0 and E_∞ represent stiffness magnitudes that scale the contributions of recruitment and distortion components to total stiffness. $\eta(t)$ and $x(t)$ are variables that describe the dynamic changes in the XB recruitment and distortion components due to changes in muscle length. Two chirps were administered to emphasize low- and high-frequency components of the force response: from 0.1 to 4 Hz over 40 s in chirp 1 and from 1 to 40 Hz over 5 s in chirp 2.

G159D [14, 16, 19, 20] mutations, confirming that both these mutants did not affect the maximal tension. Thus, our tension data further substantiated our conclusion from the Western blot (Figure 3) that the reconstitution of $cTnC_{L29Q}$ and $cTnC_{G159D}$ mutants into detergent-skinned fibers was normal.

We also measured the maximal ATPase activity in reconstituted muscle fibers using a procedure described in Methods. The Ca^{2+}-activated maximal ATPase values (in pmol · mm^{-3} · s^{-1}) in $cTnC_{WT}$, $cTnC_{L29Q}$, and $cTnC_{G159D}$ fibers were 193 ± 9 (n = 9), 187 ± 8 (n = 8), and 219 ± 13 (n = 9), respectively. Although the maximal ATPase activity was not significantly different between various groups, $cTnC_{G159D}$ fibers demonstrated an increasing trend in the maximal ATPase consumption (by 13.4%) when compared to that of $cTnC_{WT}$ fibers.

We also estimated the magnitudes of XB distortion dynamics (E_∞) and recruitment dynamics (E_0) in $cTnC_{WT}$-, $cTnC_{L29Q}$-, and $cTnC_{G159D}$-reconstituted fibers. In previous studies, we have demonstrated that E_∞ is a measure of the number of strongly-bound XB and E_0 is a measure of the number of newly-recruited XB due to an increase in muscle length [22]. Both E_0 and E_∞ estimates in $cTnC_{L29Q}$- and $cTnC_{G159D}$-reconstituted fibers were not significantly different from those of $cTnC_{WT}$-reconstituted fibers. E_0 estimates (in mN mm^{-3}) in $cTnC_{WT}$, $cTnC_{L29Q}$, and $cTnC_{G159D}$ fibers were 173 ± 10 (n = 15), 151 ± 10 (n = 10), and 148 ± 11 (n = 14), respectively. The corresponding E_∞ estimates (in mN mm^{-3}) were 786 ± 32 (n = 15), 750 ± 47 (n = 10), and 788 ± 43 (n = 14), respectively. Thus, both $cTnC_{L29Q}$ and $cTnC_{G159D}$ had no effect on the number of strongly-bound XB and the number of newly-recruited XB due to a change in muscle length. Collectively, our results from the Western blot, Ca^{2+}-activated maximal tension, and the magnitude of XB distortion dynamics indicate that both cTnC mutants incorporated properly into the myofibers and that they had no effect on either the number of strongly-bound XB or maximal tension.

3.3. Effect of $cTnC_{L29Q}$ and $cTnC_{G159D}$ on Myofilament Ca^{2+} Sensitivity and Cooperativity. To determine if $cTnC_{L29Q}$ and $cTnC_{G159D}$ mutants altered myofilament Ca^{2+} sensitivity (pCa_{50}) and cooperativity (n_H), we fitted the Hill's equation to normalized tension data obtained at different pCa. Figure 4 illustrates a comparison of pCa-tension relationships between $cTnC_{WT}$, $cTnC_{L29Q}$, and $cTnC_{G159D}$ fibers. When compared to $cTnC_{WT}$ fibers, $cTnC_{L29Q}$ fibers showed a small but significant decrease in pCa_{50}, as indicated by a rightward shift in the pCa-tension relationships ($P < 0.001$; Figures 4 and 5(a)). However, estimates of pCa_{50} in $cTnC_{G159D}$ fibers were not different from those of $cTnC_{WT}$-reconstituted fibers (Figures 4 and 5(a)), suggesting that $cTnC_{G159D}$ did not affect myofilament Ca^{2+} sensitivity. These data demonstrate that $cTnC_{L29Q}$ and $cTnC_{G159D}$ behave differently, with respect to their effect on myofilament Ca^{2+} sensitivity. n_H was unaltered in fibers reconstituted

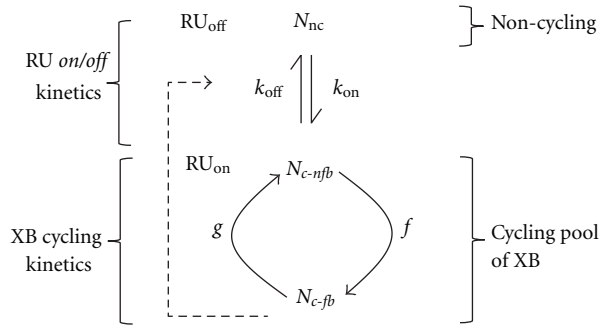

Figure 2: Reduced three-state crossbridge (XB) model scheme depicting regulatory unit (RU; Tm-Tn) kinetics and XB cycling kinetics. This scheme is adapted from Campbell [36]. k_{on} and k_{off}, represent the RU *on/off* rates and are functions of Ca^{2+} bound to cTnC. Once turned on by the binding Ca^{2+}, RU permits the transition of XB from the non-cycling (N_{nc}) pool to the cycling pool of XB. The cycling pool of XB includes two states of XB: cycling non-force-bearing ($N_{c\text{-nfb}}$) and cycling force-bearing ($N_{c\text{-fb}}$). The transition between non-cycling and cycling pools is mainly regulated by k_{on}/k_{off} kinetics of RU. The influence of the force-bearing XB on the RU *on/off* kinetics is represented by the feedback arrow (dashed line). f and g represent the rate constants governing forward transition, $N_{c\text{-nfb}} \rightarrow N_{c\text{-fb}}$, and backward transition, $N_{c\text{-fb}} \rightarrow N_{c\text{-nfb}}$, respectively.

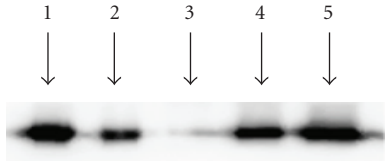

Figure 3: Western blot analysis of detergent-skinned rat cardiac muscle fibers reconstituted with $cTnC_{WT}$, $cTnC_{L29Q}$ or $cTnC_{G159D}$. Muscle protein samples were separated on 12.5% SDS-PAGE. Proteins from gel were transferred to a PVDF membrane and RcTnC was probed with an anti-TnC primary antibody (Fitzgerald M5092922). Protein profiles in *lanes* 1–5 represent the following: *lane* 1, purified RcTnC; *lane* 2, fibers reconstituted with *c-myc* RcTnT + RcTnI + RcTnC-WT; *lane* 3, fibers reconstituted with *c-myc* RcTnT + RcTnI; *lane* 4, fibers reconstituted with *c-myc* RcTnT + RcTnI + RcTnC-L29Q; *lane* 5, fibers reconstituted with *c-myc* RcTnT + RcTnI + RcTnC-G159D.

with either $cTnC_{L29Q}$ or $cTnC_{G159D}$, suggesting that the myofilament cooperativity was unaffected by both cTnC mutants (Figure 5(b)).

3.4. Effect of $cTnC_{L29Q}$ and $cTnC_{G159D}$ on XB Detachment Kinetics. Myofilament Ca^{2+} sensitivity may also be affected by changes in XB detachment kinetics, g. For example, an increase in g may also decrease myofilament Ca^{2+} sensitivity. To examine whether cTnC mutations affected the XB detachment kinetics, we estimated length-mediated XB distortion dynamic, c, and tension cost, TC, in detergent-skinned muscle bundles reconstituted with $cTnC_{WT}$, $cTnC_{L29Q}$, or $cTnC_{G159D}$ mutants. Previously, we have shown that TC is

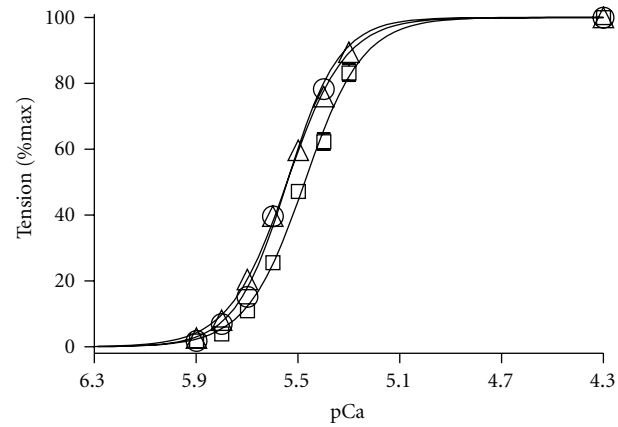

Figure 4: Comparison of normalized pCa-tension relationships in detergent-skinned fibers reconstituted with $cTnC_{WT}$, $cTnC_{L29Q}$, or $cTnC_{G159D}$. Isometric steady-state tensions elicited by each fiber in various pCa solutions were normalized with its respective value in pCa 4.3 solution. The normalized tensions were plotted against pCa to construct the pCa-tension relationship. The Hill equation was fitted to the normalized pCa-tension relationships to estimate myofilament Ca^{2+} sensitivity (pCa_{50}) and the Hill coefficient (n_H). pCa_{50} and n_H values are shown in Figures 5(a) and 5(b), respectively. The curves presented here are Hill fits to pCa-tension relationships in $cTnC_{WT}$ (◯), $cTnC_{L29Q}$ (□), and $cTnC_{G159D}$ (△) fibers, respectively. Values are expressed as mean ± SEM. Number of fibers tested in each group is as follows: $cTnC_{WT}$, $n = 15$; $cTnC_{L29Q}$, $n = 10$; $cTnC_{G159D}$, $n = 14$.

strongly correlated to c and that both have a strong dependence on the XB detachment rate, g [35]. Thus, changes in c and TC may convey important effects of $cTnC_{L29Q}$ and $cTnC_{G159D}$ on XB detachment kinetics. Our estimates of c and TC in $cTnC_{L29Q}$ fibers were not significantly different from those of $cTnC_{WT}$ fibers (Figures 6(a) and 6(b), resp.). However, estimates of c and TC in $cTnC_{G159D}$ fibers were significantly higher by 15% ($P < 0.01$; Figure 6(a)) and 26% ($P < 0.01$; Figure 6(b)), respectively, suggesting that the $cTnC_{G159D}$ mutant increased XB detachment rate. It is important to note that although the maximal tension and maximal ATPase activity of $cTnC_{G159D}$ fibers were not significantly different from those of $cTnC_{WT}$ fibers, the TC was significantly higher. The reason for this is that the maximal tension is slightly lower (by 7%) and the maximal ATPase is slightly higher (by 13.4%), making the TC significantly higher in $cTnC_{G159D}$ fibers when compared to that of $cTnC_{WT}$ fibers.

3.5. Effect of $cTnC_{L29Q}$ and $cTnC_{G159D}$ on the Rate of XB Recruitment Dynamics. cTnC mutant-induced effect on myofilament Ca^{2+} sensitivity may indicate an effect on RU *on*/*off* kinetics. Because changes in RU *on*/*off* kinetics have an impact on the rate of XB recruitment dynamics, we measured the rate constant of XB recruitment dynamics, b, using dynamic muscle fiber stiffness measurements in $cTnC_{WT}$-, $cTnC_{L29Q}$-, and $cTnC_{G159D}$-reconstituted fibers. As illustrated in Figure 7, our observations show that b speeds by 26.5% ($P < 0.01$) in $cTnC_{L29Q}$ and by

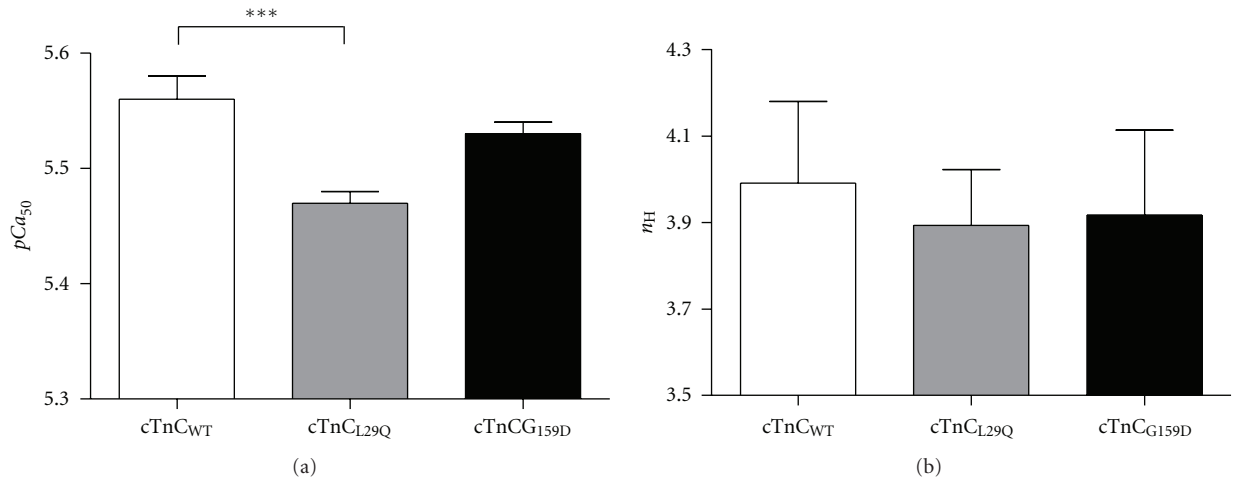

Figure 5: Comparison of myofilament Ca^{2+} sensitivity (pCa_{50}) and cooperativity (n_H) of pCa-tension relationships in detergent-skinned fibers reconstituted with cTnC$_{WT}$, ccTnC$_{L29Q}$, or cTnC$_{G159D}$. (a) Effects of RcTnC mutants on pCa_{50} (b) Effects of RcTnC mutants on n_H. The Hill equation was fitted to the normalized pCa-tension relationships to estimate pCa_{50} and n_H. One-way ANOVA was used to compare pCa_{50} and n_H estimates in cTnC$_{L29Q}$ and cTnC$_{G159D}$ fibers with the data from cTnC$_{WT}$ fibers as controls. Values are expressed as mean ± SEM. Number of fibers tested in each group is as follows: cTnC$_{WT}$, $n = 15$; cTnC$_{L29Q}$, $n = 10$; cTnC$_{G159D}$, $n = 14$. Minimal statistical significance was set at $\alpha = 0.05$. $^{***}P < 0.001$.

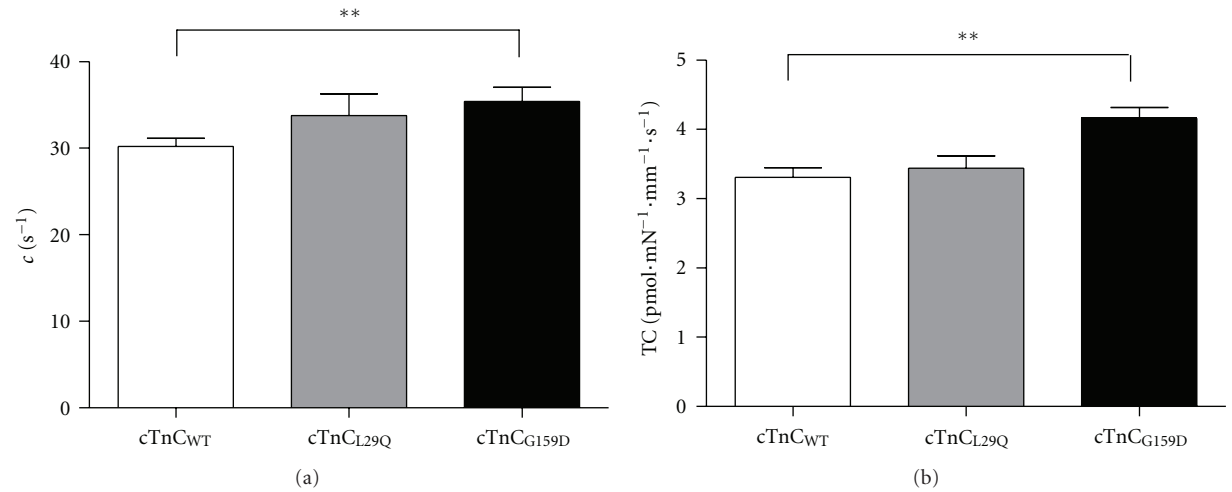

Figure 6: Comparison of XB distortion rate constant, c, and tension cost, TC, in detergent-skinned fibers reconstituted with cTnC$_{WT}$, cTnC$_{L29Q}$, or cTnC$_{G159D}$. (a) Effects of RcTnC mutants on c. c was estimated by fitting the R-D model to the force responses from muscle fibers to chirp-length perturbations [35]. (b) Effects of RcTnC mutants on TC. TC was estimated by dividing the maximal ATPase activity with the maximal tension elicited by the muscle fiber. One-way ANOVA was used to compare estimates of c and TC in cTnC$_{L29Q}$ and cTnC$_{G159D}$ fibers with the data from cTnC$_{WT}$ fibers as controls. Values are reported as mean ± SEM. Number of fibers tested in each group is as follows: cTnC$_{WT}$, $n = 15$; cTnC$_{L29Q}$, $n = 10$; cTnC$_{G159D}$, $n = 14$. Minimal statistical significance was set at $\alpha = 0.05$, $^{**}P < 0.01$.

25.3% ($P < 0.05$) in cTnC$_{G159D}$ fibers. Therefore, our data suggest that both cTnC$_{L29Q}$ and cTnC$_{G159D}$ mutants affect thin filament processes that mediate the length-dependent effects on the rate of XB recruitment dynamics.

4. Discussion

Experiments presented here provide new evidence for the mechanism by which TnC mutations bring about global change in myofilament mechanodynamics. To our knowledge, this is the first study that addresses important unresolved questions. (1) What is the effect of TnC mutations on XB recruitment and distortion dynamics? (2) How does the interplay between Ca^{2+} binding kinetics and XB cycling kinetics produce a global change in myofilament mechanodynamics? Global myofilament kinetics is governed by interactions between Ca^{2+} binding kinetics and XB cycling kinetics [7, 8, 36]. We have tested this interplay

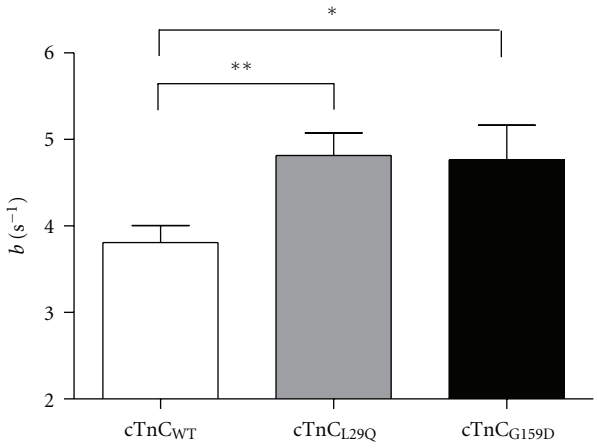

Figure 7: Comparison of XB recruitment dynamic, b, in detergent-skinned fibers reconstituted with $cTnC_{WT}$, $cTnC_{L29Q}$, or $cTnC_{G159D}$. b was estimated by fitting the R-D model to the force responses from muscle fibers to chirp-length perturbations [35]. One-way ANOVA was used to compare estimates of b in $cTnC_{L29Q}$ and $cTnC_{G159D}$ fibers with the data from $cTnC_{WT}$ fibers as controls. Values are expressed as mean ± SEM. Number of fibers tested in each group is as follows: $cTnC_{WT}$, $n = 15$; $cTnC_{L29Q}$, $n = 10$; $cTnC_{G159D}$, $n = 14$. Minimal statistical significance was set at $\alpha = 0.05$. $^*P < 0.05$; $^{**}P < 0.01$

effect by measuring pCa-tension relationship and myofiber dynamic stiffness in rat cardiac muscle fibers reconstituted with L29Q and G159D cTnC mutants. New data from our study provides a mechanistic basis for the functional effects observed in humans containing L29Q and G159D mutations in cTnC.

Our finding that the L29Q mutation elicited a small but significant decrease in myofilament Ca^{2+} sensitivity, pCa_{50} (~0.09 units; Figure 5(a)), is consistent with the report from a previous study [11]. Furthermore, our observation that pCa_{50} remained unaltered by the G159D mutation is also in agreement with previous studies which employed reconstituted assays [19, 20]. However, there are significant discrepancies between our observations and other studies, which reported contrasting findings on pCa_{50} for either L29Q [10, 14, 15] or G159D mutation [14, 16–18]. Some of these discrepancies between our study and others [10, 14–18] may be attributed to many experimental variants, including but not limited to the type of proteins used (i.e., homologous or heterologous), reconstitution techniques employed, phosphorylation status of cTnI in the reconstituted system, species-specific differences (mouse, rat, pig, rabbit, human), and so forth.

Our study showed a decrease in myofilament Ca^{2+} sensitivity with the L29Q mutation, while a previous study showed no effect [15]. On the other hand, our finding that G159D mutation had no effect on Ca^{2+} sensitivity is in contrast with two previous studies which showed a decrease [17, 18]. Such discrepancies between our study and the aforementioned studies may be likely due to the use of heterologous proteins. For example, while Neulen et al. [15] reconstituted human cardiac Tn subunits into mouse cardiac myofilaments, Mirza et al. [17] and Robinson et al. [18] used rabbit skeletal (F-actin and myosin) and human cardiac (TnC/I/T and Tm) muscle proteins in their *in vitro* ATPase assays. The use of such a heterologous reconstituted system to understand the functional effect of a single site mutation in cTnC makes it difficult to ascribe the findings directly to the specific substitution introduced. Our study avoids this issue through reconstitution of rat papillary muscle fibers with homologous recombinant rat cardiac Tn subunits.

The method used for reconstituting the recombinant proteins into the thin filament may also play a role in such discrepancies. For example, two previous investigations used CDTA treatment to selectively extract the endogenous cTnC subunits in their experimental preparations and to reconstitute them with recombinant cTnC mutants [14, 15]. In this regard, our study differs in that we removed all endogenous cardiac Tn subunits from rat papillary muscle fibers and reconstituted them with recombinant rat cardiac Tn subunits. Furthermore, because Dweck et al. [14] and Neulen et al. [15] confined the extraction and reconstitution to cTnC in their studies, it may be possible that phosphorylation of endogenous cTnI in their experimental preparations might be different from our preparations (reconstituted with nonphosphorylated cTnI). In addition, functional effects can also be attributed to the use of lower rodents (rats in our study) versus larger animals (pigs in the study by Dweck et al. [14] and humans in the study by Dyer et al. [16]). These possible factors may likely explain the discrepancies observed between our study and the aforementioned studies of L29Q and G159D mutations.

The first question that needs to be addressed in our study is, "how does the L29Q mutation brings about a small change in Ca^{2+} sensitivity?" The L29Q mutation may affect myofilament Ca^{2+} sensitivity via either a direct effect on Ca^{2+} binding to site-II of TnC or an indirect allosteric effect on the overall configuration of the regulatory unit (RU; Tn-Tm). Evidence pertaining to these claims comes from previous Ca^{2+} binding affinity studies of L29Q mutation, which suggest a possible L29Q-induced destabilization of helix A in cTnC. This effect of L29Q on helix A may affect the Ca^{2+} binding properties at site II [10] and/or the interaction between cTnC and cTnI [11]. Regardless of the way the L29Q mutation affects myofilament Ca^{2+} sensitivity, we expect that the kinetics of *on*/*off* transition of the RU will be affected. Based on the XB model scheme shown in Figure 2, we think that a decrease in Ca^{2+} sensitivity may be linked to RU *on*/*off* kinetics, k_{on} and k_{off}. Because force-bearing XB have an effect on *on*/*off* kinetics of RU, we first established that the infinite frequency stiffness (E_∞) was not altered in L29Q-reconstituted fibers. Previously, we have demonstrated that E_∞ and Ca^{2+}-activated maximal tension (T_{max}) are both measures of the number of parallel force-bearing XB [35]. Our observations that E_∞ and T_{max} were unaltered suggested that the L29Q mutation did not affect the number of force-bearing XB. Another mechanism that may decrease myofilament Ca^{2+} sensitivity is through an augmenting effect on XB detachment rate, g. Our observation that both the rate constant for XB distortion dynamics (c) and tension cost (TC) were unaltered suggested that the L29Q mutation

did not affect g (Figures 6(a) and 6(b), resp.). Our finding is consistent with a previous study which showed that the velocity of unloaded shortening was unaltered by the L29Q mutation [15]. Collectively, these observations suggest that the L29Q mutation may affect the equilibrium between the non-cycling and cycling XB pools by affecting the k_{on}/k_{off} of RU (Figure 2).

An impact on k_{on}/k_{off} of RU will affect the dynamics of XB recruitment [22, 35]. To determine the effects on XB dynamics, we assessed the length-mediated effects on XB recruitment rate, b, in L29Q-reconstituted fibers. b increased by 26.5% in L29Q-reconstituted fibers. It is important to note that dynamics of XB recruitment are affected by various length-sensing mechanisms, including changes in the thin filament overlap and XB cycling kinetics [35]. In addition, XB themselves affect the balance between RU *on*/*off* states via cooperative mechanisms (dashed line with feedback arrow in Figure 2). Therefore, b is a function of the RU *on*/*off* rates, k_{on} and k_{off}, as well as the rate parameters that define XB cycling kinetics, f and g [35]. Because g was unaffected by the L29Q mutation, an increase in b may be associated with an increase in any of the following rate constants—k_{off}, k_{on}, and f (Figure 2). However, an increase in k_{on} or f is unlikely because such effects would increase the number of force-bearing XB and the T_{max}, effects that are not observed in L29Q-reconstituted fibers. Thus, we predict that an increase in b in L29Q-reconstituted fibers is probably due to an increase in the RU *off* rate, k_{off}. Because k_{on} and k_{off} are functions of Ca^{2+} bound to RU, an increase in k_{off} is consistent with a decrease in pCa_{50} observed in L29Q-reconstituted fibers. A higher k_{off} stabilizes XB in the N_{nc} state, thereby reducing their transitioning into the cycling pool, $N_{c\text{-}nfb}$ (Figure 2). Because the population of XB in the cycling pool depends on the net effect of k_{on} and k_{off}, an increase in k_{off} acts to reduce XB recruitment, resulting in a lower tension in L29Q fibers at submaximal Ca^{2+} activations. However, at maximal Ca^{2+} activation, all the troponin units have near-maximal Ca^{2+} bound to TnC, thereby increasing the probability of more force-bearing XB (i.e., f). Thus, the net effect of k_{off} on f will be minimal at maximal Ca^{2+}-activation, resulting in no effect on the number of force-bearing XB and the maximal force.

The G159D mutation had no effect on pCa_{50}, T_{max}, and E_{∞}. Thus, its effect on the heart must come from another source of primary myofilament defect that remains poorly understood. Our measurements in G159D-reconstituted fibers showed that both the rate constant for XB distortion dynamic, c, and TC were significantly higher in G159D fibers (Figures 6(a) and 6(b)). Because both c and TC have a strong dependence on g [21, 35], higher c and TC are indicative of an increase in the rate of XB detachment in G159D fibers. In addition, G159D fibers also demonstrated a significant increase in the rate constant for XB recruitment dynamic, b (Figure 7). As discussed before, an increase in b may be associated with an increase in any of the rate constants—k_{on}, k_{off}, f, and g. However, an unaltered pCa_{50} supports the notion that the RU *on*/*off* rates, k_{on} and k_{off}, are not affected by the G159D mutation. Therefore, an increase in b may be attributed to an increase in g alone or an increase in both f and g. An increase in g alone is unlikely because such an effect would reduce the number of force-bearing XB, which would decrease both T_{max} and myofilament Ca^{2+} sensitivity. Therefore, a plausible interpretation is that the G159D mutant may increase both f and g, causing XB to cycle faster. Although conjectural, the following explanation may shed some light on how the G159D mutation affects the dynamics of myofilament activation. The location of the mutant in the C-domain of cTnC suggests that it is unlikely to have a direct impact on Ca^{2+}-mediated regulatory steps. One possible mechanism by which a mutation in the C-domain of cTnC may affect XB recruitment dynamics is by altering the dynamics of XB-mediated effects on RU. Because part of the C-domain (adjacent to G159D) is in close contact with the IT arm of cardiac Tn [13, 37], it is possible that this mutation affects the global configuration of the IT arm in such a way that the status of RU is affected. The IT arm is formed by several key interactions, including that between cTnT and cTnI and hydrophobic interactions between cTnI and the C-domain of cTnC. Thus, it is reasonable to postulate that the IT arm of the troponin complex is affected by the G159D mutation.

Our study adds the following new data to our existing knowledge on the L29Q and G159D mutant-cardiac phenotype relationships. The L29Q mutation caused a small decrease in myofilament Ca^{2+} sensitivity, which was linked to an increase in RU *off* rate. Such an effect can be linked to the cardiac phenotype (i.e., hypertrophy) in L29Q proband by considering the fact that cardiac muscle cells operate under a very narrow range of physiological $[Ca^{2+}]_{free}$. Therefore, any significant decrease in myofilament Ca^{2+} sensitivity may cause a significant decrease in tension at that given $[Ca^{2+}]_{free}$. Thus, the hypertrophy associated with the L29Q substitution may be a compensatory response to overcome a decrease in tension caused by an attenuation of myofilament Ca^{2+} sensitivity. On the other hand, G159D mutation resulted in an increase in the XB cycling rate that is, f and g, with no effects on maximal tension and Ca^{2+} sensitivity. Such increases in the XB cycling rate causes an increase in the cost of tension maintenance, subjecting the heart to a chronic stress to meet this increased energy demand. This functional consequence may be an impetus for the ventricular dilatation associated with the G159D mutation.

Acknowledgments

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Review Article

Cardiomyopathy Classification: Ongoing Debate in the Genomics Era

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Cardiomyopathies represent a group of diseases of the myocardium of the heart and include diseases both primarily of the cardiac muscle and systemic diseases leading to adverse effects on the heart muscle size, shape, and function. Traditionally cardiomyopathies were defined according to phenotypical appearance. Now, as our understanding of the pathophysiology of the different entities classified under each of the different phenotypes improves and our knowledge of the molecular and genetic basis for these entities progresses, the traditional classifications seem oversimplistic and do not reflect current understanding of this myriad of diseases and disease processes. Although our knowledge of the exact basis of many of the disease processes of cardiomyopathies is still in its infancy, it is important to have a classification system that has the ability to incorporate the coming tide of molecular and genetic information. This paper discusses how the traditional classification of cardiomyopathies based on morphology has evolved due to rapid advances in our understanding of the genetic and molecular basis for many of these clinical entities.

1. Introduction

Cardiomyopathies are a broad spectrum of diseases that affect the muscle or myocardium of the heart. This results in a failure of the heart to provide adequate oxygenated blood to the body and remove carbon dioxide and other waste products. The heart is an extremely specialised, richly innervated muscular pump that is designed to beat continuously, without stopping for the entire lifespan of its owner. To put this in perspective, a human heart beating at 70 bpm will beat approximately 2.5 billion times during a 70-year lifespan.

The official definition of cardiomyopathy by the American Heart Association in 2006 is as follows.

> *"Cardiomyopathies are a heterogeneous group of diseases of the myocardium associated with mechanical and/or electrical dysfunction that usually (but not invariably) exhibit inappropriate ventricular hypertrophy or dilatation and are due to a variety of causes that frequently are genetic. Cardiomyopathies either are confined to the heart or are part of generalized systemic disorders, which may lead to cardiovascular death or progressive heart failure-related disability [1]."*

2. Classification

There are many ways to classify cardiomyopathies. Previously, a cardiomyopathy was defined as "a heart muscle disease of unknown cause" [3] and was broken down according to their pathophysiological phenotype into dilated cardiomyopathy, hypertrophic cardiomyopathy, or restrictive cardiomyopathy. Since this first classification, major advances have meant that this overly simplistic system needed a more indepth approach to incorporate new clinical entities such as arrhythmogenic right ventricular dysplasia (ARVD) [3, 4].

Therefore in 1995, a task force established by the WHO/ISFC compiled a new system which included ARVD

Table 1: Summary of AHA 2006 classification [1].

Primary cardiomyopathies	Secondary cardiomyopathies
Genetic (hypertrophic cardiomyopathy; conduction abnormalities: prolonged QT syndrome; Brugada syndrome)	Infiltrative (amyloidosis and Gaucher disease)
Mixed (dilated cardiomyopathy; restrictive cardiomyopathy)	Storage (haemochromatosis and Fabry's disease)
Acquired (inflammatory myocarditis, peripartum, stress cardiomyopathy—"broken heart syndrome" or tako-tsubo)	Toxicity (drugs, alcohol, heavy metals, and chemicals/chemotherapy)
	Inflammatory (sarcoidosis) endocrine (diabetes mellitus; thyroid disorders; hyperparathyroidism), cardiofacial (Noonan syndrome, lentiginosis) neuromuscular/neurological, nutritional deficiencies, and autoimmune and collagen disorders

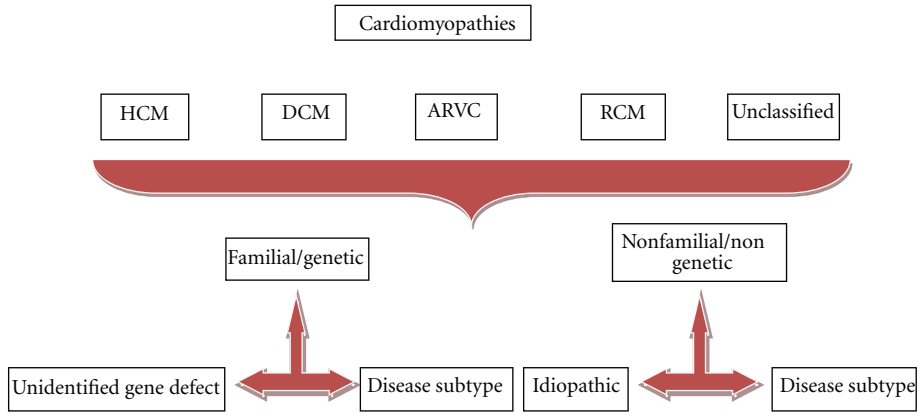

Figure 1: Summary of ESC 2008 Classification [2]. DCM: dilated cardiomyopathy; HCM: hypertrophic cardiomyopathy; ARVC: arrhythmogenic right ventricular cardiomyopathy; RCM: restrictive cardiomyopathy.

and unclassified cardiomyopathies (e.g., fibroelastosis, noncompacted myocardium, systolic dysfunction with minimal dilatation, and mitochondrial involvement). The term "specific cardiomyopathy" was used to describe heart muscle disorders which are as a result of cardiac or systemic diseases such as coronary artery disease, valvular heart disease, or hypertension [61].

A more complete classification based on the AHA definition above divides cardiomyopathies into (1) primary cardiomyopathies, which affect the heart alone, and (2) secondary cardiomyopathies, which are the result of a systemic illness affecting many other parts of the body. These are then further broken down into subgroups within these two broad categories incorporating new genetic and molecular insights (Table 1).

Distinguishing between primary and secondary cardiomyopathies can be challenging as many diseases classified as primary can have extra cardiac components, and many secondary cardiomyopathies can mainly or exclusively affect the heart. Whether or not this is the best method of classification has generated some debate within the literature [62, 63]. With our growing understanding of the genomic and molecular markers made available by modern laboratory research, a new approach had been proposed to address this overlap based on the causative mutation implicated in causing the disease [64, 65]. The working group from the European Society of Cardiology (ESC) in 2008 defined cardiomyopathy as follows.

> *"A myocardial disorder in which the heart muscle is structurally and functionally abnormal, in the absence of coronary artery disease, hypertension, valvular disease and congenital heart disease sufficient to cause the observed myocardial abnormality [2]."*

While diagnosis of cardiomyopathy rarely begins with the identification of a genetic mutation, it is rational to incorporate genetic mutation testing within a framework of classification as it is important while formulating a plan of treatment and also in informing families of their prognosis (Figure 1).

2.1. Hypertrophic Cardiomyopathy (HCM). Hypertrophic cardiomyopathy (HCM) has been defined by the presence of myocardial hypertrophy incongruent with the haemodynamic stress required for the degree of hypertrophy and the exclusion of infiltrative diseases such as amyloidosis and storage diseases [2, 66, 67].

In the absence of hypertension and valve disease, left ventricular hypertrophy (LVH) occurs in approximately 1 : 500 of the general population [68]. In day-to-day clinical practice it is very difficult to differentiate between pathologies using minimally invasive techniques such as cardiac echo or cardiac magnetic resonance imaging (MRI). Histological demonstration (on myocardial biopsy) of myocyte hypertrophy in the definition of HCM is unreliable due to the patchy

Table 2: Genes associated with hypertrophic cardiomyopathy.

Gene	Protein	Function	Reference
β-MHC	β-Myosin heavy chain	Sarcomere protein	[5]
α-MHC	α-Myosin heavy chain	Sarcomere protein	[6, 7]
cMYBPC	Cardiac myosin-binding protein C	Sarcomere protein	[8, 9]
cTnI	Cardiac troponin I	Sarcomere protein	[10]
cTnT	Cardiac troponin T	Sarcomere protein	[11]
cTnC	Cardiac troponin C	Sarcomere protein	[12]
α-TM	α-Tropomyosin	Sarcomere protein	[11]
MLC-1	Myosin essential light chain	Sarcomere protein	[13]
MLC-2	Myosin regulatory light chain	Sarcomere protein	[7]
ACTC	Actin	Sarcomere protein	[14]
TTN	Titin	Sarcomere protein	[15, 16]
	Metabolic phenocopies		
PRKAG2	AMP kinase		[17]
LAMP2	Lysosome membrane protein		[18]

nature of the abnormality within the myocardium. The position statement from the ESC [2] contained the following "the presence of intramyocardial storage material is not an exclusion criterion for HCM.... Instead, hypertrophic cardiomyopathies are simply defined by the presence of increased ventricular wall thickness or mass in the absence of loading conditions (hypertension, valve disease) sufficient to cause the observed abnormality." The "potential inaccuracy" in not fully excluding infiltrative disease or demonstrating myocyte hypertrophy on biopsy is justified by leading to increased emphasis in the clinical picture and a promise of better minimally invasive diagnostic strategies.

If the HCM is familial, then it is usually transmitted in an autosomal dominant pattern of inheritance caused by mutations within genes that encode for various proteins of the cardiac sarcomere. Currently, there are over 500 mutations in 13 genes that have been identified that cause HCM and 50% of these are familial [66, 69–72] (Table 2).

Pathologically, left ventricular (LV) cavity size is normally reduced and this can progress to LV dilatation and heart failure, albeit in a minority of patients. There are many patterns of hypertrophy and all are consistent with a diagnosis of HCM but concentric hypertrophy is more suggestive of a systemic cause such as glycogen storage disease. Moreover, mutations in the genes encoding for cardiac troponins can be associated with mild phenotypes but, conversely, a high incidence of cardiac death [73]. The normal physiological hypertrophy that occurs in highly competitive athletes is uncommon (less than 2% of male athletes) [74], but it is important not to miss HCM in these individuals as the risk of sudden death is unacceptably high [75] and causes great distress to both families and communities who have been affected.

2.2. Restrictive Cardiomyopathy. Restrictive cardiomyopathies have a diverse range of aetiology; however, all are recognised as having distinct haemodynamic features separating them from other forms of cardiomyopathy. Restrictive cardiomyopathies in general are defined as showing normal ventricular size (nondilated and nonhypertrophied) with impaired haemodynamic function, elevated filling pressures, and diastolic dysfunction, and in most cases normal systolic function [76, 77].

Presentation can include symptoms of both right and left sided failure; decreased exercise tolerance, dyspnoea, peripheral oedema, and palpitations are the most common symptoms [77]. Due to the contrast in both aetiology and treatment options and the similarities in haemodynamics, it is important to recognise the difference between restrictive cardiomyopathy and constrictive pericarditis. Usually, this is defined with a variety of investigatory modalities with both haemodynamic and morphological assessment and includes echocardiography and pericardial imaging [78].

Various aetiologies have been identified as causing restrictive cardiomyopathy and range from idiopathic (primary) restrictive cardiomyopathy, to systemic conditions including infiltrative, noninfiltrative, and storage disorders, as well as endomyocardial disorders, various medications, and iatrogenic causes [79]. Familial restrictive cardiomyopathies are usually inherited in an autosomal dominant fashion, the genetic basis of which remains to be identified, and are noted to be relatively rare [80]. Hereditary conditions known to cause a restrictive cardiomyopathy include haemochromatosis, glycogen storage diseases, Fabry's disease, Gaucher's disease, and Hurler syndrome.

Prognosis in symptomatic patients is quite poor, depending on aetiology. Idiopathic restrictive cardiomyopathy has been associated with a significant difference in 10-yr survival when compared to expected survival in groups matched for age and sex [81]. In comparison with other forms of cardiomyopathy, restrictive cardiomyopathy is relatively uncommon, though it still demonstrates an appreciable incidence in some population groups, namely, Asia, South and Central America [79].

2.3. Dilated Cardiomyopathy (DCM). DCM is a common cause of congestive cardiac failure (CCF) and is defined by the presence left ventricular systolic dysfunction with left

Table 3: Genes associated with dilated cardiomyopathy.

Gene	Protein	Function	Reference
		Autosomal dominant	
ACTC	Cardiac actin	Sarcomere protein	[19]
DES	Desmin	Dystrophin-associated glycoprotein complex	[20]
SGCD	δ-Sarcoglycan	Dystrophin-associated glycoprotein complex	[21]
MYH7	β-Myosin heavy chain	Sarcomere protein	[22, 23]
TNNT2	Cardiac troponin T	Sarcomere protein	[22, 24, 25]
TPM1	α-Tropomyosin	Sarcomere protein	[26]
TTN	Titin	Sarcomere structure	[27]
VCL	Metavinculin	Intercalated discs	[28]
MYBPC	Myosin-binding protein C	Sarcomere protein	[23]
MLP/CSRP3	Muscle LIM protein	Z discs	[29]
ACTN2	α-Actinin-2	Sarcomere structure	[30]
MYH6	α-Myosin heavy chain	Sarcomere protein	[31]
ABCC	SUR2A	Cardiac K channel	[32]
LMNA	Lamin A/C	Nuclear membrane protein	[33]
PLN	Phospholamban	Sarcoplasmic reticulum Ca regulator	[34, 35]
ZASP/LBD3	Cypher	Cytoskeletal assembly	[36]
		X linked	
DMD	Dystrophin	Dystrophin-associated glycoprotein complex	[37, 38]
TAZ/G4.5	Tafazzin		[39, 40]
		Recessive	
TNNI3	Troponin I	Sarcomere protein	[41]

ventricular dilatation the absence of coronary artery disease or other causes such as hypertension or valvular pathology [2]. The right ventricle may be involved but is not necessary for the diagnosis. The exact prevalence of DCM in the general population is unknown, but it clearly varies with age and geography and is the most common diagnosis in patients referred for cardiac transplantation [82, 83]. Around 30–50% of cases have a familial component [71, 84], and more than 30 genes have been identified, to date, that cause DCM (Table 3). Most are inherited in an autosomal dominant fashion although some can be autosomal recessive, X-linked or mitochondrial. The actual frequency of familial DCM is probably underestimated.

The 2009 HFSA [71] has released guidelines on the diagnosis and treatment of patients with DCM. A careful family history of three or more generations of family members should be elicited including unexplained heart failure and sudden death in family members before the age of 60 without any symptoms of coronary artery disease. The diagnosis of familial DCM can be made when there are three or more close family members with unexplained DCM. Screening of family members can then take place; this should happen with or without genetic testing and is supported by the fact that many patients can be asymptomatic despite being affected. The 2009 HFSA made the following recommendations for screening: full history, focusing on symptoms of heart failure (dyspnoea, syncope, presyncope, and palpitations); physical examination; ECG; Echo; CK MM. First-degree relatives who have negative findings on initial screening should be rescreened in three- to five-year intervals, but if there are any abnormal findings during the initial screen, the patient should be rescreened in one year.

Peripartum cardiomyopathy is a specific subgroup of dilated cardiomyopathy defined as the development of heart failure with evidence of left ventricular dysfunction, within the last month of pregnancy to within 5 months of delivery, without other identifiable cause or underlying cardiac condition [85, 86]. Groups of women presenting during the earlier stages of pregnancy have been identified and with similar epidemiological characteristics and with similar disease progression and outcomes. The earlier time frame of presentation has been postulated to represent part of a spectrum of peripartum cardiomyopathy [87]. In the group of women presenting in the early stages of pregnancy, search for underlying cardiac conditions (valvular, ischaemic, and myocardial) should be approached. Peripartum cardiomyopathy affects approximately 1 : 4000 women across the US and Europe each year, with higher rates noted across the African continent [88]. The condition usually presents with dyspnoea, cough, peripheral oedema, orthopnoea, paroxysmal nocturnal dyspnoea, generalised fatigue, and chest discomfort. Investigations including new ECG finding of arrhythmia, chest X-ray with foetal shielding (if required for diagnosis of pulmonary oedema) showing cardiomegaly, pulmonary venous congestion and interstitial oedema, and an elevated BNP or NT-proBNP level further suggest the presence of peripartum cardiomyopathy [89]. Echocardiographic evidence demonstrating LV enlargement, a LV end-systolic dimension greater than 2.7 cm/m^2 of body surface area, LVEF less than 45% and/or fractional

Table 4: Genes associated with ARVD.

Locus	Gene	Protein	Function	References
ARVD1	TGFB3	Transforming growth factor β3	Cell signalling	[42, 43]
ARVD2	RYR2	Ryanodine receptor 2	Sarcoplasmic reticulum calcium channel	[44, 45]
ARVD3	Not known	[46]		
ARVD4	Not known	[47]		
ARVD5	LAMR1	Extracellular matrix glycoprotein	Cell signalling, adhesion, and migration	[48, 49]
ARVD6	PTPLA	Protein-tyrosine phosphatase-like member A	Fatty acid synthesis	[50, 51]
ARVD7	DES; ZASP	Desmosomal protein; PDZ domain protein	Dystrophin-associated glycoprotein complex, and Cytoskeletal assembly	[52, 53]
ARVD8	DSP	Desmoplakin	Anchoring of intermediate filaments	[53, 54]
ARVD9	PKP2	Plakophilin 2	Cell adhesion	[55, 56]
ARVD10	DSG2	Desmoglein 2	Calcium-binding transmembrane glycoprotein	[57, 58]
ARVD11	DSC2	Desmocollin 2	Calcium-dependent glycoprotein	[59, 60]

shortening less than 30 percent, conclude the presence of heart failure. The use of cardiac MRI in the diagnosis and evaluation of peripartum cardiomyopathy is currently being explored and the presence or lack thereof of late gadolinium enhancement as a prognostic feature in peripartum cardiomyopathy [90, 91]. The aetiology of peripartum cardiomyopathy has been unclear for many years; however, new research into an inflammatory or immunological basis, and the role of prolactin in the development of the disease has shed new light on the causative mechanisms that may be behind this condition. Familial clustering of peripartum cardiomyopathy has been identified; however, on screening other family members and with further genetic testing, this clustering may represent a subset of undiagnosed familial dilated cardiomyopathy. TNF alpha, and other proinflammatory cytokines have been shown to be elevated in a large number of peripartum cardiomyopathy cases and similarly some studies have suggested a role for autoantibodies against normal human cardiac tissues proteins and further research is required in this area [92]. Higher levels of CRP, Fas/Apo-1, TNF alpha and IL-6 have been demonstrated in some population groups with peripartum cardiomyopathy and have implicated a role for inflammatory mediator in the disease process [93, 94]. The use of immunoglobulin and antitumour necrosis factor agents as therapy for peripartum cardiomyopathy has been trialled based on these observations in several smaller pilot studies [95, 96]. Evidence implicating myocarditis as a causative factor is varied however may suggest the presence of myocarditis in 7.8–8.8% of cases [97]. The role of an altered form of prolactin in the pathophysiology of peripartum cardiomyopathy has been explored of late in animal models. Mice with cardiac tissue-specific STAT3 knockout have shown an increased cleavage of prolactin (a pituitary hormone released cyclically in varying degrees in the pregnant state) mediated by cathespsin D to its proapoptotic and antiangiogenic form, 16 kDa prolactin and have subsequently demonstrated the development of peripartum cardiomyopathy [98]. In light of this research, further preliminary studies have taken place on the use of bromocriptine as a therapy for women developing peripartum cardiomyopathy this small trials conducted thus far have shown a mortality benefit [99]. Peripartum cardiomyopathy, although rare, is an important entity affecting the pregnant woman, with significant morbidity and mortality consequences. Recent research into the pathophysiology behind the disease may allow for further subclassification of this disease, and hence earlier diagnosis, and new novel therapies in its treatment.

2.4. Arrhythmogenic Right Ventricular Dysplasia (ARVD). ARVD is a heart muscle disease which, pathologically, consists of progressive fibrofatty replacement of the right ventricular musculature which may or may not involve the left ventricle. It predisposes towards malignant arrhythmias originating from the right ventricle and is a major cause of sudden death in young athletes [100]. Major and minor criteria of ARVD diagnosis have been compiled, and the diagnosis can be made if there are two major, one major and one minor or four minor criteria present [101]. Diagnosis and risk stratification are extremely important as there are proven life saving interventions which are available to the clinician [102].

It is a familial disease in around 50% of cases and is usually transmitted in an autosomal dominant fashion [103]. The first gene, ARVD1, coding for a desmosome protein, was discovered in 1994 [42], and since then multiple causative genes relating to the desmosome have been discovered, indicating that ARVD is a disease of the desmosome [55, 57, 104–107] (Table 4).

Genetics is obviously important as it adds certainty to the diagnosis, but given the incomplete penetrance of the disease the established diagnostic criteria are essential.

3. Discussion

The 2009 Heart Failure Society of America (HFSA) genetic evaluation of cardiomyopathy practice guideline and 2005 American College of Cardiology/American Heart Association (ACC/AHA) HF guidelines include recommendations with regard to genetic counselling and genetic testing in patients and families with certain cardiomyopathies. As far

as treatments are concerned, gene therapy is still quite young and transferring concepts from animal models to human therapies is yet to be seen. Therapeutic interventions of the future are likely to focus on the signaling events from abnormal gene to protein and finally clinical phenotype, and the modification of the genetic and environmental factors mediating this process [108].

As sudden cardiac death is a possible first presenting complaints for patients with dilated cardiomyopathy especially those with *LMNA* gene defects (where penetrance rates are noted to be very high over 30 years and associated with high rates of sudden death) and in particular *SCN5A* defects, early implantation of ICD may be considered in these populations, especially in the setting of family history of sudden cardiac death or implantable cardiac defibrillator usage [109].

Gene therapy in animal models of heart failure aimed at improving sarcoplasmic calcium transport has been investigated with therapeutic promise and may lead to further application in human model of restrictive cardiomyopathy [110].

In patients with ARVC, genotyping and early ICD implantation as primary prevention may be indicated in patients with Naxos disease and recessive forms of ARVC [111].

3.1. The Impact of Genetic and Genomic Approaches on Current and Future Clinical Application. Screening of family members of patients with dilated cardiomyopathy, hypertrophic cardiomyopathy, and arrhythmogenic right ventricular is recommended as family members are frequently asymptomatic and disease progression is often quite short and although asymptomatic early noninvasive investigations may prove abnormal [112, 113].

Due to the commonality of autosomal dominant inheritance of hypertrophic cardiomyopathy and the high degree of penetrance associated with many of the gene mutations, it is recommended that first-degree relatives are regularly screened for inheritance of the disease [114]. With the likely increase in amount of genetic testing the impact on family members of patients with an inheritable disease is likely to be affected significantly, particularly with many defects identified showing varying degrees of expression and penetrance. The role of genetic counselling will become more and more important as further genetic variants are identified with unknown pathological and prognostic significance.

For example, patients with gene mutations of desmosomal components (those most commonly seen in ARVC), penetrance is low and there is commonly age related variability in expression and therefore, early identification holds an unknown prognostic significance for patients in question [115, 116].

4. Conclusion

Classification systems in all branches of science are designed to allow categorisation within a consistent framework thereby imparting a degree of homogeneity to satisfy researchers and clinicians alike. Over the years, many classification systems have been put forward for cardiomyopathy based on origin, structural abnormality, functional status, and etiology. Not surprisingly, this has failed to some degree. From a purely functional viewpoint, cardiomyopathy is not a static condition but can move from one functional group to another due to cardiac remodelling. Similarly, using etiology has limitations given that similar genotypes can express different phenotypes depending on where the disease is in its natural history. Despite these shortcomings, a genetic diagnosis does offer some definite advantages. Karibe et al. reported a novel tropomyosin mutation that was associated with a mild phenotype but had a poor prognosis when contrasted to other mutations in the gene (13 deaths in 26 affected family members) [117]. Genetic testing in such cases allows identification of patients which would benefit from primary ICD implantation as well as a definitive diagnosis in conjunction with traditional methods. Similarly, patients with mutations in the gene for cardiac myosin-binding protein C can have a favourable clinical course due to the fact that the cardiomyopathy may not be expressed until later in life. Therefore, prolonged lifetime screening for family members who do not have the mutation can be avoided by genetic testing within these individuals [118]. There has been some debate on the value of genetic testing, with the debate focusing on the ability of genetic testing to accurately predict clinical course [119]; however, genetic testing allows clinicians to move beyond unexplained ventricular abnormalities and definitively identify not only who has the disease but what the cause is and what are the likely outcomes. Changing the natural history of a disease starts with accurate diagnosis.

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Review Article

The Mutations Associated with Dilated Cardiomyopathy

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Cardiomyopathy is an important cause of heart failure and a major indication for heart transplantation in children and adults. This paper describes the state of the genetic knowledge of dilated cardiomyopathy (DCM). The identification of the causing mutation is important since presymptomatic interventions of DCM have proven value in preventing morbidity and mortality. Additionally, as in general in genetic studies, the identification of the mutated genes has a direct clinical impact for the families and population involved. Identifying causative mutations immediately amplifies the possibilities for disease prevention through carrier screening and prenatal testing. This often lifts a burden of social isolation from affected families, since healthy family members can be assured of having healthy children. Identification of the mutated genes holds the potential to lead to the understanding of disease etiology, pathophysiology, and therefore potential therapy. This paper presents the genetic variations, or disease-causing mutations, contributing to the pathogenesis of hereditary DCM, and tries to relate these to the functions of the mutated genes.

1. Introduction

Disorders of the heart leading to heart failure are leading causes of morbidity and mortality. Cardiomyopathy is a heterogeneous disease caused by functional abnormality of cardiac muscle and classified as primary or secondary cardiomyopathy [1]. Secondary cardiomyopathy is caused by extrinsic factors, including infection, ischemia, hypertension, and metabolic disorders, whereas the diagnosis of primary cardiomyopathy is based on exclusion of secondary cardiomyopathy and there are several different clinical types [2, 3]. Dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM) are the two major cardiomyopathies. DCM is characterized by a dilated ventricular cavity with systolic dysfunction; the clinical symptom of DCM is heart failure, which is often associated with arrhythmia and sudden death. On the other hand, HCM, a major cause of heart failure and sudden death in the young and, is characterized by left ventricular hypertrophy, often asymmetric, accompanied by myofibrillar disarrays and reduced compliance (diastolic dysfunction) of the cardiac ventricles.

The diagnosis of dilated cardiomyopathy (DCM) is based on the presence of left ventricular enlargement and reduced systolic dysfunction with an ejection fraction <50% or, more stringently, <45%. In children cardiomyopathy is a rare disorder, yet it is a common cause of heart failure and heart transplantation (HTx) [4]. The Pediatric Cardiomyopathy Registry (PCMR) estimates the annual incidence of pediatric primary cardiomyopathy in the United States to be 1.13 cases per 100,000 children aged 18 years or younger. Most cases are dilated (50%) or hypertrophic (42%) phenotypes [5]. Annual incidence is significantly higher in infants less than 1 year of age (8.34 cases per 100,000), and approximately 40% of symptomatic children died from heart failure or required HTx. Many (65%) cardiomyopathy cases are idiopathic after detectable causes have been excluded. Cardiomyopathy can be part of a multisystem disease process or may be acquired. The PCMR provided the following data on etiology of dilated cardiomyopathy (DCM): idiopathic (66%), myocarditis (16%), neuromuscular disorders (9%), familial (5%), inborn errors of metabolism (4%), and malformation syndrome (1%) [6]. However, the registry excluded patients

with toxin-induced cardiomyopathy; anthracycline exposure is an important cause of cardiac dysfunction in children who receive chemotherapy for neoplastic disease [7, 8]. In the adult population, DCM is the most common form of non-ischemic cardiomyopathy and a major cause of the heart failure leading to sudden cardiac death. It commonly manifests after middle age and is clinically a very heterogeneous disease, ranging from symptomless to severe heart failure. On diagnosis the most common DCM cause in the US, ischemic heart disease due to coronary artery disease (CAD), needs to be excluded in men over 40 years and women over 45 years (or at younger ages if risk factors are present, for example, cigarette smoking, diabetes, hypertension, a strong family history of early coronary disease). Less common causes of DCM that need to be excluded include structural heart disease (congenital or valvular), thyroid disease, iron overload, exposure to cardiotoxins such as anthracyclines, chest radiation, and other much less common conditions, including those accompanying inflammatory arthritides, myocarditis (e.g., giant cell myocarditis), and protozoal infections (e.g., Chagas disease). HCM may occasionally show characteristics of DCM (reduced systolic function, some dilatation) late in its course. Extensive literature, not reviewed here, is available for HCM (e.g., [9]). Idiopathic dilated cardiomyopathy represents approximately one-half of all cases, accounts for more than 10,000 deaths in the U.S. annually, and is the primary indication for cardiac transplantation. Sudden cardiac death (SCD) is a major public health problem in the western world, with over 200,000 deaths reported annually in the U.S. alone [10]. The underlying mechanism is ventricular tachyarrhythmia in the overwhelming majority. However, the underlying substrate varies: ischemic heart disease in 75–80% cases, idiopathic cardiomyopathy in 10–15%, and 1–5% due to rare monogenic mutations in cardiac ion channels or associated proteins [11].

2. Familial DCM

DCM may be either sporadic or familial. A diagnosis of familial DCM is assigned when it occurs in at least two closely related family members or there was a sudden cardiac death at a young age [12]. The genetic factor contributing to the manifestation of the disease can be classified either as a disease causing gene mutation as the etiology of monogenic disease or as a disease-associated gene polymorphism that is involved in the pathogenesis of multifactorial disease. It is conceivable that full biological function in healthy subjects is impaired only to the threshold for the development of disease by both genetic factors and environmental factors. In the monogenic disease, most of the dysfunction is caused by a disease-causing gene mutation, although additional genetic factors or modifier genes might also contribute to the pathogenesis, and other environmental factors including gender, age, and life-style factors may be involved in disease development. The ratio of disease development among carriers of the disease-causing mutation is defined as penetrance, which is determined by factors other than the disease-causing mutation. The genetic factor is composed of multiple disease associated gene polymorphisms in the multifactorial disease. In general, the contribution of genetic factors to the disease is approximately 60–100% in the monogenic disease, whereas it is approximately 20–30% in the multifactorial disease. Family history of the same disease is indicative of genetic factors in the disease, and family history or aggregation of the disease can be seen in both the monogenic and multifactorial disease. The family history of monogenic disease can be explained by the Mendelian rule of inheritance, whereas the aggregation of multifactorial disease does not follow this rule. However, it is sometimes difficult to distinguish monogenic disease from multifactorial disease when the size of the family is small or the penetrance of the disease is low. It has been reported that as much as 35% of DCM patients have a family history, mainly consistent with autosomal dominant inheritance, although some familial cases can be explained by autosomal recessive or X-linked recessive trait [13–15]. In clear contrast, more than half of HCM patients have a family history consistent with an autosomal dominant genetic trait. Although genetic factors seem to play an important role in the pathogenesis of DCM, hitherto reported mutations explain only a minority of familial DCM [16].

Autosomal dominant inheritance is the most common inheritance form, presenting usually in the second or third decade of life [17, 18]. Mutations in 26 genes of 30 chromosomal loci were identified (presented in OMIM [19] 115200). Most of the mutated genes encode for structural components of the heart muscle, implying that the disease is caused by damage to force generation, sensing, and transmission, as will be detailed below. Mutation in other genes affects the structure of the nucleus (lamin A/C, OMIM 150330; *LAP2*, OMIM 188380), ion regulation (*SCN5A*, a sodium channel, OMIM 600163), *ABCC9*, regulatory K(ATP) channel subunit, (OMIM 601439), Ca^{2+} metabolism (phospholamban, OMIM 172405), a transcription coactivator (*EYA4*, OMIM 603550), an RNA-binding protein of the spliceosome (RBM20, OMIM 613171) that regulates the splicing of titin as well as 30 additional genes with conserved splicing regulation between humans and rats [20], the extracellular matrix protein laminin 4 (*LAMA4*, OMIM 600133), and the intracellular serine threonine kinase that interacts with integrins (*ILK*, OMIM 602366) and may mediate the signaling of LAMA4 [21] (presented in Table 1). DCM with recessive inheritance was described eight times and the affected genes identified in seven of the cases. In two of them (OMIM 611880 and OMIM 611705), the mutated genes encode cardiac structural proteins: cardiac troponin I (*TNNI3,* OMIM 191044) and titin (*TTN*, OMIM 188840), respectively. Mutations in two other-genes affect modifications of structural proteins. The gene encoding dolichol kinase (*DOLK*, OMIM 610746) which O-mannosylates alpha-dystroglycan [22] may cause syndromic or nonsyndromic DCM (OMIM 610768); mutations in fukutin (*FKTN*, OMIM 611615, 607440), which may be involved in the glycosylation of alpha-dystroglycan in skeletal muscle cause DCM.,The fifth gene, identified in a late-onset DCM, encodes GATA zinc finger domain-containing

Table 1: Disease genes for primary cardiomyopathy.

Clinical type	Inheritance	Gene name (symbol)	Encoded protein	Function
HCM/DCM/RCM	AD	Myosin heavy chain 7, (*MYH7*)	Cardiac beta-myosin heavy chain	Contraction
HCM/DCM/atrial septal defect type 3	AD	Myosin heavy chain 6, (*MYH6*)	Cardiac alpha-myosin heavy chain	Contraction
HCM/DCM/RCM /LVNC	AD	Troponin T2, cardiac (*TNNT2*)	Cardiac troponin T	Calcium sensitivity
HCM/DCM/LVNC	AD	Tropomyosin 1 (*TPM1*)	Alpha-tropomyosin	Calcium sensitivity
HCM/DCM	AD	Myosin binding protein 3, cardiac (*MYBPC3*)	Cardiac myosin-binding protein C	Calcium sensitivity
HCM/DCM/RCM	AD, AR	Troponin I3, cardiac (*TNNI3*)	Cardiac troponin I3	Calcium sensitivity
HCM/DCM	AD	Actin alpha cardiac (*ACTC1*)	Alpha cardiac actin	Contraction
HCM/DCM	AD/AR	Titin (*TTN*)	Titin	Connection
HCM/DCM	AD	Troponin C1, cardiac (*TNNC1*)	Cardiac troponin C type 1 slow	Calcium sensitivity
HCM/DCM	AD	Cystein- and glycine-rich protein 3 (*CSRP3*)	Cardiac LIM protein	Connection
HCM/DCM	AD	Titin cap (*TCAP*)	Telethonin (tcap)	Connection
HCM/DCM	AD	Vinculin (*VCL*)	Metavinculin	Connection
HCM/DCM	AD	Ankyrin repeat domain containing protein (*ANKRD1*)	Ankyrin repeat domain 1 containing protein	Connection
DCM/RCM	AD	Desmin (*DES*)	Desmin	Connection
DCM	AD	Lamin A/C (*LMNA*)	Lamin A/C	Nuclear scaffold
DCM	AD	Sarcoglycan-delta (*SGCD*)	Delta sarcoglycan	Connection
DCM	AD	Actinin alpha 2 (*ACTN2*)	Alpha actinin 2	Connection
DCM/LVNC	AD	Lim domain binding 3 (*LDB3*)	Zasp/CYPHER	Binds protein kinase C
DCM	AD	Phospholamban (*PLB*)	Phospholamban	Calcium sensitivity
DCM	AD	Presenilin 1 (*PSEN1*)	Presenilin 1	Calcium sensitivity
DCM	AD	Presenilin 2 (*PSEN2*)	Presenilin 2	Calcium sensitivity
DCM	AD	ATP binding cassette C9 (*ABCC9*)	K_{ATP} channel	Ion regulation
DCM	AD	Sodium channel voltage-gated 5A (*SCN5A*)	Cardiac Na channel	Ion regulation
DCM/HCM	AD	Muscle-restricted coiled-coil (*MURC*)	Muscle-restricted coiled-coil	Connection
DCM/HCM	AD	Crystallin-alpha B (*CRYAB*)	Alpha B crystallin	Connection/stress response
DCM	AD	Four and a half Lim domains 2 (*FHL2*)	Four and a half Lim domains 2	Connection
DCM	AD	Laminin alpha 4 (*LAMA4*)	Laminin alpha 4	Connection
DCM	AD	Nebulette (*NEBL*)	Nebulette	Myofibrinogenesis
DCM/HCM/RCM	AD	Myopalladin (*MYPN*)	Myopalladin	Connection
DCM	AD	RNA-binding motif protein 20 (*RBM20*)	RNA-binding motif protein 20	Splicing
HCM/DCM	AD	Nexilin (*NEXN*)	Nexilin	Connection
DCM	AD	Bcl2-associated athanogene 3 (*BAG3*)	Bcl2-associated athanogene 3 protein	Chaperone activity
DCM	XR	Dystrophin (*DMD*)	Dystrophin	Connection
DCM	XR	Emerin (*EMD*)	Emerine	Nuclear membrane anchorage to the cytoskeleton
DCM/LVNC	XR	Tafazzin (*TAZ*)	Tafazzin	Metabolism
DCM	XR	Fukutin (*FKTN*)	Fukutin	Connection
DCM/ARVC	AR	Desmoplakin (*DSP*)	Desmoplakin	Connection
DCM	AR	Dolichol kinase (*DOLK*)	Dolichol kinase	Connection

Table 1: Continued.

Clinical type	Inheritance	Gene name (symbol)	Encoded protein	Function
DCM	AR	GATA zinc finger domain-containing protein 1 (*GATAD1*)	GATA zinc finger domain -containing protein 1	Gene expression
DCM/ARVC	AR/AD	Plakoglobin (*JUP*)	Junction plakoglobin	Connection
DCM	AR	Flavoprotein (*SDHA*)	Flavoprotein	Metabolism

AD: autosomal dominant; AR: autosomal recessive; ARVC: arrhythmogenic right ventricular cardiomyopathy; DCM: dilated cardiomyopathy; HCM: hypertrophic cardiomyopathy; RCM: restrictive cardiomyopathy; LVNC: left ventricular noncompaction; XR-X-linked recessive.

protein 1 (*GATAD1*, OMIM 614518) which binds to a histone modification site that regulates gene expression [23]. The sixth gene identified in a fatal congenital DCM (OMIM 300069) is tafazzin, involved in cardiolipin metabolism and ATP production (OMIM 300394 [24]). Similarly we have identified a mutation in the SDHA encoding the flavoprotein subunit of Complex II of the mitochondrial respiratory chain [25]. Mutations in *SDHA*, *tafazzin,* and *FKTN* were found to cause additional syndromes (Leigh syndrome (OMIM 256000), Barth (OMIM 30206), and muscular dystrophy dystroglycanopathy (OMIM 236670), resp.).

As in general in genetic studies, identification of the mutated genes has a direct clinical impact for the families and population involved. Identifying causative mutations immediately amplifies the possibilities for disease prevention through carrier screening and prenatal testing. This often lifts a burden of social isolation from affected families, since healthy family members can be assured of having healthy children. Presently, genetic testing for DCM is limited by the low yield of mutations found overall. Very recently, *TTN* (encoding titin protein) truncating mutations were reported to be a common cause of dilated cardiomyopathy, occurring in approximately 25% of familial cases of idiopathic dilated cardiomyopathy and in 18% of sporadic cases where they may represent de novo mutations [26]. Thus, screening for mutations in this gene would be recommended for DCM patients and if a mutation is found to their family. Mutation screening of the *LMNA* gene is worthwhile in the subset of families with DCM and conduction-system disease. For other families with DCM, participation in research studies investigating new candidate genes could be considered an option (reviewed in [27, 28]). Detailed recommendations for mutation screening are provided [29, 30]. Briefly, a thorough family history should be taken on every individual with DCM to identify at-risk family members in order to recommend that they be screened. This may not be conclusive since complexities of incomplete penetrance and variable expression can easily preclude the diagnosis from those with limited knowledge of clinical genetics. All first-degree relatives (including parents) of patients should undergo clinical (echocardiographic and ECG) screening. This screening leads to the diagnosis of familial disease in 12% to 15% of apparently isolated DCM cases, demonstrating its greater sensitivity versus family history alone and genetic analysis.

Even though the genetics technologies have advanced considerably, mainly the high-throughput sequence capabilities, the sequencing of the human genome, has not resulted in major advances in understanding or therapy of the most common morbid diseases present throughout the world [27, 31–33]. Most human diseases that are associated with substantial morbidity and mortality today are the result of manifold interactions between multiple genetic loci, as well as epigenetic factors, combined with environmental forces and nutritional components impinging on the organism. Familial studies, specifically families showing Mendelian inheritance, hold the enormous potential not only for reducing the complexity resulting from the involvement of multiple genes and environment but also for the identification of the genomic locus harboring the mutation and reducing sifting through many variations of unknown effect by limiting the search for the mutation to a smaller DNA interval. Indeed the mutations in the genes discussed in the following section were identified in such families. In the following sections we offer insight into the effected function aiming to understand the disease etiology, pathophysiology, and therefore potential therapy. The presymptomatic interventions of DCM have proven valuable in preventing morbidity and mortality [30].

3. Transmission of Force and Regulation of Calcium Sensitivity and Muscle Contractility

Dystrophin (OMIM 300377) was the first gene identified to cause X-linked DCM in the Duchenne-type and the Becker-type muscular dystrophy, both of which are often complicated by cardiac dysfunction in the later phase of the clinical course. Dystrophin is a sarcolemmal protein that plays a key role in the anchoring of muscle cells. Mutations in the genes of other proteins involved in anchoring are known to cause DCM. These include dystrobrevin (*DTNA*, OMIM 601239), integrin (e.g., OMIM 613204), and metavinculin (OMIM 193065) that form a sarcolemma complex linking dystrophin and the Z-disc. In addition, the laminin (a heterotrimeric extracellular matrix protein consisting of three chains, OMIM 156225) and dystroglycan complex links muscle cells to the extracellular matrix [34]. Furthermore, fukutin is involved in the glycosylation of α-dystroglycan [35]. The mutations are proposed to disrupt anchoring and hence abrogate the transmission of the force generated by muscle contraction [35].

Muscle contraction, which is caused by the interaction between an actin filament and myosin heavy chain, is regulated by the concentration of intracellular Ca^{2+} that

is released from the sarcoplasmic reticulum (SR) via the ryanodine receptor and reuptake to the SR via SR Ca^{2+}-ATPase (*SERCA*), which is regulated by phospholamban (*PLB*). When the concentration of Ca^{2+} is increased or decreased, muscle contracts or relaxes, respectively. It has been reported that DCM is caused by a *PLB* mutation [36], by which uptake of Ca^{2+} to the SR is impaired such that release of Ca^{2+} from the SR per beat is decreased. There are two sensing systems for fine tuning of Ca^{2+}-dependent muscle contraction: regulation by the troponin complex and regulation by myosin light chain. The fine-tuning system controls the magnitude of muscle contraction at the same concentration of Ca^{2+}, which is also defined as calcium sensitivity. The calcium sensitivity in skeletal muscle is mainly controlled by the troponin complex via conformational changes of troponin T and troponin I depending on the capture or release of Ca^{2+} by troponin C, disruption of which can cause DCM in animal models [37, 38]. In the cardiac muscle, both the troponin complex system and myosin light chain system regulate the calcium sensitivity. Any impairment in the regulation of calcium sensitivity may cause dysfunction of muscle contractility and hence would cause cardiomyopathy.

Mutations in contractile element genes were initially identified as the cause of HCM [39], but recently were found also in DCM cases (Table 1). Because some HCM patients develop systolic dysfunction similar to DCM, which is called dilated phase HCM, DCM patients carrying contractile element mutations might indeed suffer from HCM. However, there are apparent functional differences related to the disease-causing mutations found in DCM and HCM. A typical example is reported for mutations in cardiac troponin T gene (TNNT2). Troponin T binds troponin C and troponin I to form the troponin complex, which regulates the calcium sensitivity of muscle contraction via conformational change in the interaction between cardiac actin and myosin heavy chain. A DCM-causing TNNT2 mutation, del_Lys210, showed that the mutant troponin T caused a decreased calcium sensitivity of the muscle contraction both *in vitro* [40] and in mutant knock-in mice, which exhibited the cardiac phenotype of DCM [41]. On the other hand, HCM-linked TNNT2 mutations were shown to increase the calcium sensitivity *in vitro* [42] and in the *in vivo* model of transgenic mice that developed HCM [43]. These observations indicate that the altered calcium sensitivity of muscle contraction because of TNNT2 mutations is the direct cause of primary cardiomyopathy, and the opposite functional alterations are associated with DCM and HCM. The decreased calcium sensitivity was also reported for DCM-causing mutations in alpha-tropomyosin (TPM1) [44] and cardiac troponin C (TNNC1) [45], whereas the increased calcium sensitivity was noted for various HCM-causing mutations in the contractile element genes, including TPM1 [44, 46], cardiac troponin I gene (TNNI3) [47], ventricular myosin regulatory light chain gene (MYL2) [48], and MYBPC3 [49]. Therefore, the altered calcium sensitivity may at least in part explain the functional alterations caused by the contractile element gene mutations.

4. Z-Disc Element Mutations and Generation of Force

Units of striated muscle are called sarcomeres, which align in tandem to maximize the generation of power. Because the generated power of muscle contraction is transmitted to adjacent sarcomeres through the Z-disc, mutations in Z-disc elements may cause hereditary cardiomyopathy. Identification of an HCM-causing mutation in the Z-disc region of titin (*TTN*) was the first example of the Z-disc element mutation, which increased the binding of titin and α-actinin [50]. On the other hand, two different disease-causing *TTN* mutations in the Z-disc region were identified in DCM: one at the actinin-binding domain and the other at the Tcap-binding domain [51]. Functional analyses demonstrated that the former mutation decreased the binding to α-actinin, which was the opposite functional alteration to that caused by the HCM-causing mutation, whereas the latter mutation decreased the binding to Tcap, suggesting that decreased binding of titin and Z-disc elements was a common functional alteration caused by the DCM-caused *TTN* mutations. Recently, a large study of 312 subjects with dilated cardiomyopathy, 231 subjects with hypertrophic cardiomyopathy, and 249 controls, using next-generation sequencing, identified *TTN* truncating mutations and mutations predicted to alter titin structure as a common cause of dilated cardiomyopathy, occurring in approximately 25% of familial cases of idiopathic dilated cardiomyopathy and in 18% of sporadic cases [26]. Moreover, it was recently demonstrated that RBM20 encoding a component of the spliceosome regulates the splicing of titin and dozens of additional genes involved in sarcomere function [20]. In addition, two DCM-causing mutations in the Tcap gene (*TCAP*), which decreased the binding of Tcap to titin, MLP, and calsarcin-1 (myozenin-2), have been found [52]. Because CSRP3 knock-out mice develop the DCM phenotype [53], along with a wide Z-disc and loss of stretch response [54], the Z-disc may play a role as a stretch sensor and its dysfunction leads to the DCM phenotype [54]. Furthermore, another DCM-causing CSRP3 mutation and alpha-actinin gene (ACTN) mutations were reported to decrease the binding of MLP and alpha–actinin [55]. These observations suggest that the decreased binding among the Z-disc elements could develop into DCM because of the decreased stretch response. In this regard, it was suggested that DCM is a disease of "loose sarcomeres." In clear contrast, HCM-causing mutations in TCAP increase the binding of Tcap to titin and calsarcin-1 [52], leading to a hypothesis that HCM may be a disease of "stiff sarcomeres" [56]. Loose and stiff sarcomeres would decrease and increase passive tension upon stretch of the sarcomere, respectively. Because the change in passive tension is associated with a change in calcium sensitivity [57–59], it is speculated that abnormality in both the Z-disc elements and contractile elements causes the abnormal calcium sensitivity.

There are two other Z-disc elements, desmin (*DES*) and metavinculin (*VCL*), mutations of which have been found in DCM. The VCL mutation impaired the binding to actin [60], whereas the DES mutations resulted in disruption of

the cytoplasmic desmin network [61]. In addition, mutations in the myopalladin gene (*MYPN*) [62] and nebulette gene (*NBLT*) [63], which impair myofibrillogenesis [64, 65], have recently been reported in DCM. These findings suggest that the Z-disc also plays a role in myofibrillogenesis. ZASP/Cypher is another Z-disc element connecting calsarcin and actinin [66]. Calsarcin binds calcineurin [64], a Ser/Thr phosphatase involved in the hypertrophic progress of cardiomyocytes [65]. In addition, ZASP/Cypher is known to bind protein kinase C (PKC) [66], and as a DCM-causing mutation in the PKC-binding domain of ZASP/Cypher increased the binding [67], it has been suggested that phosphorylation/dephosphorylation of Z-disc elements might be involved in the stretch response. In addition, several other ZASP/Cypher gene (*LDB3*) mutations not in the PKC-interacting domain are reported in DCM and LVNC [68]. Phosphoglucomutase-1 (PGM1) was identified as a novel binding protein to ZASP/Cypher [69]. PGM1 is a metabolic enzyme involved in glucose–glycogen metabolism. The functional significance of the binding between PGM1 and ZASP/Cypher remains unclear, but the DCM-causing mutations decrease the binding between ZASP/Cypher and PGM1 [69]. Because PGM1 localizes at the Z-disc under the stressed culture conditions, a role for PGM1 in energy metabolism at the Z-disc may be required for the response against metabolic stress [69]. These observations suggest that an impaired stress response due to abnormality in the Z-disc elements might be involved in the pathogenesis of DCM.

5. Sarcoplasmic Element Mutations and Metabolic Stress

Nebulette is anchored to the Z-disc at the C-terminal portion, and the other side is positioned in the sarcoplasm where it binds actin; thus it may be involved in cardiac myofibril assembly. A polymorphism in the actin-binding motif of nebulette associated with DCM has been reported [70]. Myopalladin is a component of the sarcomere that tethers nebulette and nebulin in skeletal muscle and in cardiac muscle to alpha-actinin at the Z lines [71]. It is anchored to the Z-disc at the N-terminal portion, while the other side is positioned in the sarcoplasm to bind a transcriptional cofactor, CARP [72]. CARP is known to shuttle between the sarcoplasm and nucleus to regulate gene expression associated with the stretch response, cardiac remodeling, and myofibrinogenesis [73]. Several DCM-causing CARP mutations that impair myofibrillogenesis were recently reported [73]. There are several other sarcoplasmic proteins for which gene mutations have been found in DCM. Four and half LIM protein 2 gene (*FHL2*) and the αB-crystallin gene (*CRYAB*) are examples. They bind titin at the N2-B region, where a DCM-causing mutation has been found, and functional studies revealed that the DCM-causing mutation decreased the binding of titin to both proteins [74, 75]. In addition, a DCM-causing *FHL2* mutation, which decreased the binding of FHL2 to titin, has been found [76]. Since FHL2 also tethers muscle-specific metabolic enzymes (i.e., adenylate kinase, phosphofructokinase, and muscle-type creatinine kinase [77]), the DCM-causing *FHL2* mutation would impair the recruitment of these metabolic enzymes to titin. Moreover, a DCM-causing *CRYAB* mutation decreased the binding to titin [78]. The binding between titin and αB-crystallin might be involved in the αB-crystallin mediated protection of cardiac muscle from ischemic stress [78]. Additionally, αB-crystallin is phosphorylated and translocated to the Z-disc under ischemic conditions, suggesting a role of the Z-disc in the stress response [79]. Finally, 6 mutations in muscle-restricted coiled-coil (*MURC*) encoding a Z-line component protein were reported. DCM-causing mutations in MURC may affect muscle protein homeostasis through regulating RhoA/ROCK and association with a multiprotein complex at the caveolae [80].

6. Disruption in Energy Production as a Cause of DCM

Mitochondrial dysfunction frequently affects the heart and may cause both hypertrophic and dilated cardiomyopathy [81]. Nuclear encoded genes affecting mitochondrial functions are known to cause DCM. For example, a rare genetic disorder of the fatty acid beta-oxidation cycle caused by mutations in both alleles of the alpha subunit (HADHA) of the mitochondrial trifunctional protein may result in a severe neonatal cardiomyopathy with hypoketotic hypoglycemia and hepatic encephalopathy, often progressing to coma and neonatal death [82, 83]. Another example is the finding of a nonsense mutation in Coenzyme Q10, a mobile lipophilic electron carrier located in the inner mitochondrial membrane. The mutation results in multisystem disease including cardiomyopathy [84]. Studying two large consanguineous pedigrees, we identified an association of a mutation in the *SDHA* gene with the clinical manifestation and interfamilial variability of 15 pediatric patients diagnosed with dilated cardiomyopathy [25]. Table 2 presents the clinical variability observed in patients with the same mutation in the *SDHA* gene, of four families of the same highly consanguineous population. Although the disease was diagnosed in all patients in their first year of life or even *in utero*, some died at the age of diagnosis, at 1-2 months of life, but others are still alive at 11 years. The measurements of heart function also correlate with this survival variability.

Succinate dehydrogenase (SDH, E.C. 1.3.5.1) deficiency is a rare condition in humans, representing 2% of mitochondrial respiratory chain (RC) disorders [85]. Its clinical presentation is highly variable, ranging from early-onset encephalomyopathies to tumor susceptibility in adults [86, 87]. SDH catalyzes the conversion of succinate to fumarate and is a component of the mitochondrial respiratory chain (complex II) as well as the Krebs cycle. SDH is made up of four subunits, all encoded in the nuclear DNA—two soluble proteins, the flavoprotein (Fp, *SDHA*) and the Fe–S protein (*SDHB*), which are anchored to the inner membrane by subunits SDHC and SDHD [86, 87]. Pathogenic mutations in the *SDHA* gene have rarely been documented in children,

Table 2: Clinical data of the patients with the G555E mutation in the SDHA gene.

Case	Age at onset (months)	Age at death (months)	Age alive (years)	Sex	Primary clinical features and followup (f/u)	Echo data	Noncompaction LV (LVNC)	LV function FS %
A6	2	2		f	Respiratory distress, CHF, cardiogenic shock	LV dilatation LVEDD-39 mm Noncontracting LV	Noncompaction LV	FS < 10%
A7	8		14 months psychomotor development adequate to age	m	Cardiomegaly in X-ray asymptomatic f/u-asymptomatic	LV dilatation LVEDD-36 mm Mild LVH, mild MVI Mild LV dysfunction		25%
A8	4		2 y, adequate psychomotor development to age	f	Respiratory distress f/u-frequently hospitalized due to CHF	LV dilatation LVEDD-44 mm LV dysfunction Moderate LVH		15–17%
A9	2	11		m	Respiratory distress f/u-frequently hospitalized due to CHF	LV dilation LVEDD-44 LV dysfunction Moderate MVI Moderate LVH		12–15%
A10	3	5		f	Respiratory distress, CHF	LV dilation LVEDD-42 mm Noncontracting LV		11–13%
A11	2	2		f	Respiratory distress, CHF	LV dilation LVEDD-37 mm Noncontracting LV	Noncompaction LV	FS < 10%
A12	5		7 y, normal school performance	m	Mild respiratory distress f/u-exercise intolerance	LV dilation LVEDD-46 LV dysfunction Mild MVI Mild LV hypertrophy		23–25%
A13	6	6		m	Respiratory distress, CHF cardiogenic shock	LV dilatation LVEDD-42 mm Noncontracting LV	Noncompaction LV	FS < 10%
B1	33 W of gestation	2		m	Respiratory distress, CHF cardiogenic shock	LV dilatation LVEDD-36 mm Non contraction LV	Noncompaction LV	FS < 10%
B2	1		8 y, normal school performance	f	Respiratory distress frequently hospitalized at one year-old, f/u-exercise intolerance	LV dilatation LVEDD-50 mm LV dysfunction Mild MVI, mild LVH		22-23%
B3	3	8		f	Respiratory distress CHF	LV dilatation LVEDD-44 mm Severe LV dysfunction	Noncompaction LV	13%
B4	32 W of gestation	1		f	Respiratory distress, CHF, sudden death at home	LV dilation LVEDD-33 mm Noncontracting LV	Noncompaction LV	FS < 10%
B5	32 W of gestation		14 m, walked at age12 m	m	Respiratory distress f/u-frequently hospitalized due to CHF	LV dilatation LVEDD-43 mm Mild MVI Moderate to severe LV dysfunction	Noncompaction LV	12–18%
C1	4	4		m	Respiratory distress CHF, cardiogenic shock	LV dilatation LVEDD-43 mm Non contraction LV		FS < 10%

Table 2: Continued.

Case	Age at onset (months)	Age at death (months)	Age alive (years)	Sex	Primary clinical features and followup (f/u)	Echo data	Noncompaction LV (LVNC)	LV function FS %
D1	8 months		11 y, normal school performance	m	At presentation-respiratory distress f/u-exercise intolerance	LV dilatation LVEDD-48 mm Mild LVH, mild MVI Mild LV dysfunction		24–26%

CHF: congestive heart failure, FS: fractional shortening, LV: left ventricle, LVEDD: left ventricle end-diastolic diameter, LVH: left ventricle hyperthropy. MVI: mitral valve insufficiency. A, B, C, and D represent 4 different families.

and all but one case have been reported in patients with Leigh syndrome [85, 88–90]. The single case not presenting with the Leigh syndrome describes the death at infancy, before any sign of the syndrome could be detected, following a respiratory infection and severe hypoglycemia [91]. A late-onset neurodegenerative disease with progressive optic atrophy, ataxia, and myopathy was tentatively ascribed to a heterozygous mutation in a conserved region of the SDHA protein [92].

The families in our study presented a recessive pattern of inheritance. All patients belong to the same tribe and share a family name; thirteen could be traced to two large families; for two additional patients, family relations could not be established. Since DCM is rare and the families are consanguineous, we predicted that it is caused by homozygosity of the mutation inherited from a common founder. Following homozygosity mapping and sequence of candidate genes in the single ascertained homozygous genomic interval, we identified the mutation G555E in *SDHA*. All patients and available family members (in total, 34 persons) were evaluated: all patients were found to be homozygous for the G555E allele; all healthy siblings were either heterozygous or homozygous for the normal allele; all available parents of affected patients were heterozygous for the mutation with one exception. To our surprise, the father of one patient was homozygous for the mutation. He reported that three of his siblings had died at a young age due to cardiovascular failure, so that none were available for verification of this mutation. He was clinically assessed, and his medical records were pursued due to this finding, revealing no visits at the clinic in his childhood and no previous hospitalizations. His physical assessment was negative for any symptoms or other suspicious factors. In addition, his electrocardiogram and the echo study exhibited normal LV function and dimension. He was not amenable to a stress test, but notably, his occupation demands heavy physical work. Further genetic study revealed that his *SDHA* gene is identical to that of the patients' gene by comparing their haplotypes. Polymorphisms in the genes of the other components of SDH and the SDH assembly factor (*SDHAF1*) were also excluded as possible modifier genes. Finally, we verified the enzymatic activity of complex II in lymphoblastoid cells established from the father in comparison to his patient son, a mild patient (B5), the heterozygous mother, and four controls. The enzymatic activity of the father's complex was decreased by 42 percent, being more similar to that of the patients compared to the heterozygous mother and controls (100%). Thus presently we lack an explanation for the normal phenotype of the father. This study demonstrates the complexity that may be found in genetic studies: two-thirds of the patients with the mutation succumbing to cardiac failure; however, one case homozygous for the mutation shows nonpenetrance for the cardiomyopathy in spite of a reduction in the SDH mitochondrial activity in lymphoblast cells comparable to the reduction observed in other patients. Overall, the rate of death due to cardiac complications in our patients is higher than those reported for dilated cardiomyopathy in general [93], but similar to those reported for cardiomyopathy associated with mitochondrial disease, where cardiac function deteriorates rapidly regardless of the associated RC defect [94].

Furthermore, this study demonstrates the extreme variability that the same mutation may have in different individuals. Mutation G555E in the *SDHA* gene was reported twice before: a patient who died at 5.5 months from respiratory difficulties and severe hypoglycemia, presenting also with severe hypotonia, hepatosplenomegaly, and cardiac dysrhythmia with cardiomegaly [91]. The second patient presented with a relatively mild Leigh syndrome at 22 months [90]. Our patients exhibiting isolated cardiomyopathy differ markedly from the other reported cases.

7. Nuclear Lamina Mutations in DCM

Part of hereditary DCM is caused by mutations in the genes for components of the nuclear lamina, emerin (*EMD*), and lamina A/C (*LMNA*). Upon screening for mutations in X-linked [95] and autosomal-dominant [96] DCM accompanied by conduction defects without severe skeletal muscle phenotype, *EMD* and *LMNA* mutations were discovered, respectively. As discussed for DMD mutations, the difference in clinical phenotype (i.e., skeletal muscle disease or cardiomyopathy) may be determined by which domain of the elements encoded by the disease gene was affected. However, there is no definite difference in the distribution of *LMNA* mutations found in the muscular diseases and cardiomyopathy [97, 98]. Molecular mechanisms for developing DCM by mutations in nuclear lamina genes remain unknown but might be involved in the altered regulation of gene expression in the heart as reported for knock-in mice with a *LMNA* mutation [99].

8. Conclusion

Recent progress in genetic cardiomyopathy points to the potential value of genetic testing in shaping the clinician's ability to diagnose and understand the pathogenetic basis of the inherited cardiomyopathies. Newer technologies are influencing genetic testing, especially cardiomyopathy genetic testing, where an increased number of genes are now routinely being tested simultaneously. While this approach to testing multiple genes is increasing the diagnostic yield, the analysis of multiple genes in one test is also resulting in a large amount of genetic information of unclear significance. Genetic testing is very useful in the care of patients and families, since it guides diagnosis, influences care, and aids in prognosis. However, the large amount of benign human genetic variation may complicate genetic results and requires understanding of the genes' functions. Finally, the identification of families with DCM of the Mendelian inheritance promises the possibility to identify novel genes that may lead to novel treatments.

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Review Article

Application of Echocardiography on Transgenic Mice with Cardiomyopathies

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Cardiomyopathies are common cardiac disorders that primarily affect cardiac muscle resulting in cardiac dysfunction and heart failure. Transgenic mouse disease models have been developed to investigate the cellular mechanisms underlying heart failure and sudden cardiac death observed in cardiomyopathy cases and to explore the therapeutic outcomes in experimental animals in vivo. Echocardiography is an essential diagnostic tool for accurate and noninvasive assessment of cardiac structure and function in experimental animals. Our laboratory has been among the first to apply high-frequency research echocardiography on transgenic mice with cardiomyopathies. In this work, we have summarized our and other studies on assessment of systolic and diastolic dysfunction using conventional echocardiography, pulsed Doppler, and tissue Doppler imaging in transgenic mice with various cardiomyopathies. Estimation of embryonic mouse hearts has been performed as well using this high-resolution echocardiography. Some technical considerations in mouse echocardiography have also been discussed.

1. Introduction

Cardiomyopathies have been considered to represent diseases that primarily affect cardiac muscle. On the ground of their morphology and pathophysiology, cardiomyopathy may be classified into three major types: hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), and restrictive cardiomyopathy (RCM). In the past, most cardiomyopathy cases were described as idiopathic cardiomyopathies, that is, etiology is unknown [1, 2]. Now, we know that most cardiomyopathy cases are heritable [3] and several mutations in cardiac muscle genes, such as myosin, tropomyosin, cardiac troponin T (cTnT), and cardiac troponin I (cTnI) have been identified to be associated with cardiomyopathies [4–7].

Clinical studies showed great heterogeneity among the genetic cardiomyopathic patients even when they carry the same mutation [8–11]. The biggest challenge is that no data are available so far to confirm the overall expression and the incorporation rate of the mutant sarcomeric proteins in the heart. Furthermore, other environmental and genetic factors may also contribute to the heterogeneity of the disease manifestation. Recently, transgenic animal models have been generated to mimic various cardiomyopathies by expressing the mutant cardiac myofibril proteins observed in human patients [12, 13]. Studies with transgenic animals will fill the gap in cardiomyopathy research between the *in vitro* assays using reconstituted myofibrils and the clinical studies with the patients carrying the mutant genes, which will undoubtedly improve our understanding of the mechanisms underlying the cardiomyopathies and provide us with clues for the prevention and treatment of the diseases.

In general, in the case of DCM, heart failure is characterized by systolic dysfunction (i.e., reduced ejection fraction), whereas HCM and RCM are characterized by diastolic dysfunction (i.e., impaired relaxation) [14]. To evaluate the phenotypes, it is necessary to develop accurate, reproducible, and noninvasive methods to assess cardiac morphology and function in a serial manner. Ultrasound imaging has been increasingly applied to identify and characterize structural and functional features of different cardiac phenotypes in

large animals modeling human diseases [15]. Although the acquisition of mouse echocardiograms is relatively simple, echocardiographic studies are challenging because of the small size, rapid heart beating rate, and orientation of the mouse heart. Recently, a high-resolution echocardiograph has been developed with high-frequency (30–50 MHz) and high-frame rate mechanical probes, allowing an axial resolution of approximately 50 μm at a depth of 5–12 cm [16]. Our laboratory has been among the first to assess cardiac function using the high-frequency echocardiograph (VisualSonics, Inc. Toronto, Canada) on transgenic mice with various cardiomyopathies. In this review, we will summarize the application of the conventional echocardiography, pulsed Doppler, and tissue Doppler imaging for cardiac function assessment in mice and discuss several technical considerations in mouse echocardiography.

2. Echocardiography Measurement and Major Parameters

The conventional echocardiography is applied in our laboratory on experimental mice. In addition, pulsed Doppler and tissue Doppler imaging (TDI) are also used to determine hemodynamics and the myocardial motion. B-mode is a two-dimensional real-time image of the heart, which is used to visualize and quantify the anatomical structures. A four-chamber image including left and right atria and ventricles can be obtained from the B-mode. Usually, the operator first locates the structure by B-mode then changes to other modes to measure the cardiac wall motion or blood flow. M-mode is the temporal image of cardiac wall motion along a single ultrasound beam, which enables the quantification of wall thickness and cavity dimensions as well as the movement of myocardium, valves, and vessel walls. Usually, left ventricular end diastolic dimension (LVEDD) and end systolic dimension (LVESD), interventricular septum (IVS), and posterior wall (PW) thickness are recorded with M-mode. Shortening fraction (SF) is calculated from the M-mode LV dimensions using the equation:

$$\mathrm{SF}\,(\%) = \left[\frac{(\mathrm{LVEDD} - \mathrm{LVESD})}{\mathrm{LVEDD}}\right] \times 100, \qquad (1)$$

where SF is an index of systolic left ventricular function. Pulsed Doppler imaging is primarily used for the quantitative measure of blood flow through valves, arteries, or veins, which provides information of both the direction and the velocity of blood flow. Tissue Doppler provides the quantifiable information of myocardial tissue movement, for example, the movement of valves. The major parameters in pulsed Doppler and tissue Doppler measurements will be described in the following sections.

3. Assessment of Systolic Function

Left ventricular structure and systolic function is most commonly measured by M-mode in a parasternal short axis view at the papillary muscle level. In order to obtain an anatomically correct parasternal short axis view, operators usually start with parasternal long axis view by placing the transducer vertically to the animal body on the left side of its sternum with the "notch" of transducer pointing to the animal head; and then rotating approximately 30–45 degrees counterclockwise. This view depicts the mid-portion and base of the left ventricle, both leaflets of the mitral valve, the aortic valve and aortic root, the left atrium, and the right ventricle. The parasternal short axis view is obtained by rotating the transducer through 90 degrees clockwise base on the long axis view. Then, the transducer is tilted a little to the apex to visualize the papillary muscles. To ensure all the measure is reliable and repeatable, the short axis view at papillary muscle level is required as a criterion for the imaging used in the later M-mode measure. In other words, the left ventricle is sectioned across the middle of papillary muscles and visualized as a completely round view of it. The left ventricular wall motion along the sampling line is recorded and analyzed in M-mode (Figure 1).

With M-mode imaging of the left ventricle, investigators are able to directly measure the thickness of left ventricular posterior and anterior walls and the interventricular septum. The increase in the wall thickness indicates ventricular hypertrophy while the decrease might be observed in ventricular dilation. Left ventricular mass can be calculated based on the wall thickness. Left ventricular chamber diameter at end-diastole and end-systole is also able to be directly measured. As mentioned above, FS can be calculated from the M-mode LV dimensions. In addition, ejection fraction (EF) is another useful index of systolic function, which can be calculated as well from the M-mode measurements.

Pulsed Doppler imaging has been used as well to assess the blood flow through aorta. The quantitative data of the blood flow peak velocity, velocity time integral, the peak, and mean gradient can be measured or calculated, which provide us with useful information on systolic function of experimental animals (Figure 2). Aortic root can be visualized under apical five-chamber view, the fifth chamber is aorta. When the transducer is tilted upward from the apical four-chamber view, aorta will be seen in the middle of the screen (Figure 2).

An Example of Transgenic Disease Mouse Model with Systolic Dysfunction. We performed echocardiography studies to measure the cardiac function of cTnT-DE7 transgenic mice (6-month-old) that express an abnormal splice-out of the exon 7-encoded segment in the N-terminal variable region of cardiac troponin T in the hearts. No atrial enlargement, ventricular hypertrophy, or dilation was detected in the hearts of 3-months-old cTnT-DE7 mice, indicating a compensated state. However, left ventricular FS and EF were significantly decreased in 6-month-old cTnT-DE7 mice compared to wild-type controls. Furthermore, the left ventricular outflow tract velocity and gradient were both significantly decreased in the transgenic mouse hearts, indicating decreased systolic function. The impaired systolic heart function *in vivo* without detected changes in diastolic function suggests that cTnT-DE7 primarily reduces contractile function of the cardiac muscle ([17] and unpublished data).

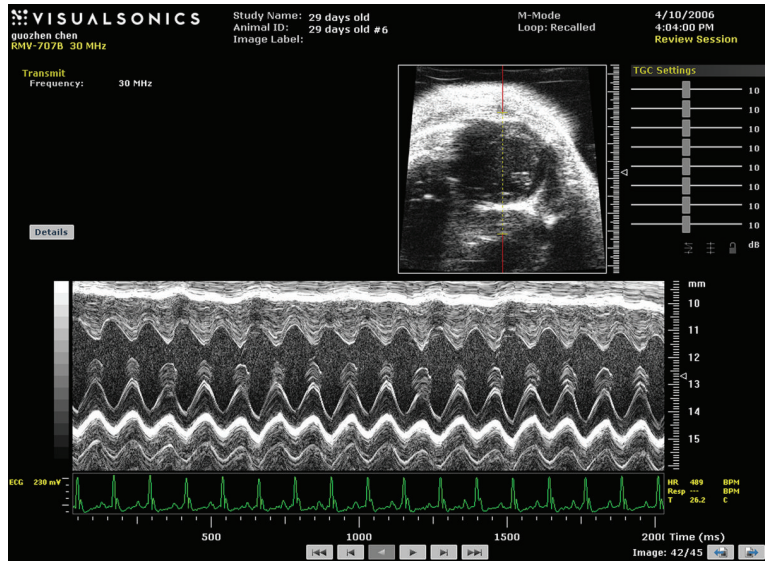

Figure 1: M-mode imaging obtained on a wild type mouse using a 30-MHz transducer.

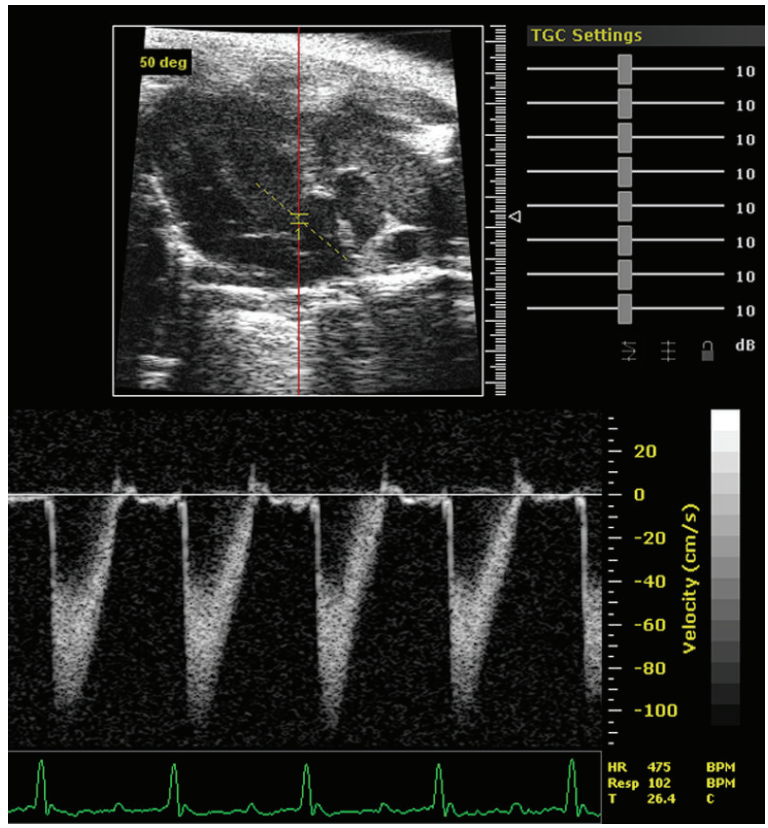

Figure 2: Aortic pulse Doppler tracings obtained on a wild-type mouse.

4. Assessment of Diastolic Function

In general, cardiac diastolic function can be assessed with conventional echocardiography in B-mode and M-mode. B-mode clearly shows the structure of left ventricle and the dimension of the left ventricle during the systole and diastole. M-mode, furthermore, provides the ability to obtain anatomically correct LV measurements, such as LVESD and LVEDD. Using B-mode and M-mode, we observed a significantly reduced LV end-diastolic dimension in cTnI knockout mice and RCM cTnI transgenic mice, both suffering from a severe diastolic dysfunction [12, 18].

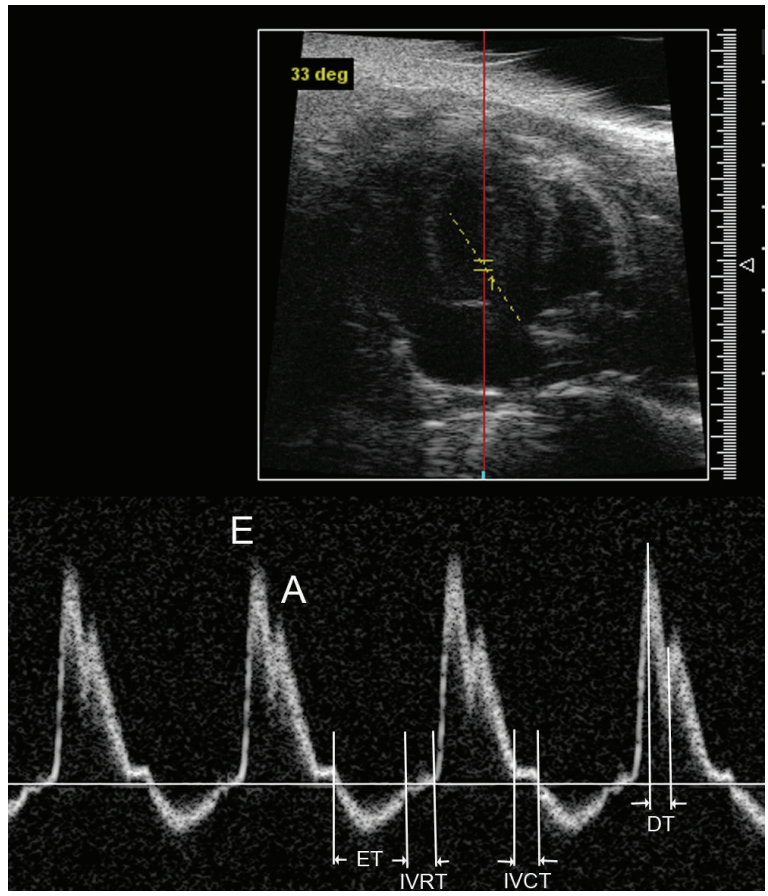

Figure 3: Mitral pulse Doppler tracings obtained on a wild type mouse. E, E wave indicating early ventricular filling; A, A wave indicating late filling caused by atrial contraction; ET, ejection time; IVRT, isovolumetric relaxation time; IVCT, isovolumetric contraction time; DT, deceleration time.

Recently, Doppler echocardiography has emerged as an important clinical tool to provide reliable and useful data for diastolic performance [15, 19]. The noninvasive nature and high-resolution feature of Vevo 770 echocardiography allow us to longitudinally monitor diastolic performance in mice *in vivo*. Using echocardiography to evaluate diastolic function is primarily achieved via pulsed Doppler imaging of transmitral blood flow and tissue Doppler imaging of mitral annulus velocity. They are all accessed under apical four-chamber view, which is obtained from the lower left side of the animal's thorax. This view essentially allows us to look up from the apex towards the base of the heart and visualize the right and left ventricles, and the mitral inflow tract, with the atria at the bottom of the screen (Figure 3). The transducer is angled by 60–70 degrees and placed in a transverse position with the notch facing the left side of the mouse.

As shown in Figure 3, two diastolic waves, E and A, are seen in normal mitral Doppler tracing. The E wave occurs during the rapid filling phase in early diastole and the A wave occurs in late diastole as a result of atrial contraction. Normally, the E wave is higher than the A wave. Clinically useful mitral inflow parameters include the following: early filling peak velocity (E); atrial peak velocity (A); E/A ratio (normally >1); deceleration time (DT) or the interval between the peak of the E wave to the zero baseline. The isovolumetric relaxation time (IVRT) is also measured and is the phase in which all valves are closed and the ventricles relax without any change in volume.

TDI analysis has become an established component of the diagnostic ultrasound examination, which permits an assessment of myocardial motion. Sample volume is measured at the septal side of the mitral annulus. Early (E′) and late (A′) diastolic mitral annulus velocity and the ratio of E′ to A′ are determined. TDI is very useful to distinguish the pseudo-normalization pattern from the normal filling pattern as reported previously [20].

The ratio of early filling transmitral peak velocity (E) over the TDI mitral valve E velocity (E′) has established itself as a reliable guide to elevated pulmonary capillary wedge pressure [21]. Pulmonary capillary wedge pressure (PCWP) provides an indirect estimate of left atrial pressure [22]. Although left ventricular pressure (LAP) can be directly measured by placing a catheter into the left ventricle by feeding it through a peripheral artery, into the aorta, and then into the ventricle, it is not feasible to advance this catheter back into the left atrium. LAP can be measured by placing a special catheter into the right atrium then punching through the interatrial septum; however, for obvious reasons, this is not

usually performed because of the damage to the septum. Direct measurement of LAP can be available to human hearts, but it is not applicable to mouse hearts. Therefore, the E/E′ ratio for estimation of LAP in mouse hearts is a very useful tool in the assessment of cardiac diastolic properties.

An Example of Transgenic Disease Mouse Model with Diastolic Dysfunction. We applied both pulsed Doppler and TDI to our studies on transgenic mice (from 2–12 months) suffering from restrictive cardiomyopathy due to cTnI mutation [20, 23]. We found an E/A ratio reverse representing a relaxation abnormality in these transgenic mice and restrictive physiology in these mice at the advanced stage [20]. Furthermore, we found that prolongation of IVRT was the earliest sign of impaired relaxation observed in these transgenic mice. This is consistent with previous reports demonstrating that IVRT is the most sensitive Doppler index to detect impaired relaxation because it is first to become abnormal [24, 25].

5. Assessment of Coronary Flow and Myocardial Perfusion

Pulsed Doppler imaging has been used to assess the blood flow through coronary artery of mouse hearts [26]. From a modified parasternal long axis view, the left coronary artery is visualized stemming from the aorta sinus, traveling between the right ventricle outflow tract and left ventricular anterior wall, and along the left ventricular wall to the distal branch site (Figure 4). Sample volume of pulsed Doppler is placed on the left main coronary artery. Under such condition, Doppler imaging of coronary blood flow consists of two peaks: the preceding low peak representing coronary blood flow in systole and the following high peak representing the coronary perfusion in diastole (Figure 4).

We measured coronary artery blood flow using echocardiography Doppler system in wild-type and transgenic mice (2-month-old) with diastolic dysfunction. Our results showed that a significant decrease of coronary circulation was observed in transgenic mice suffering from diastolic dysfunction (unpublished data). These data indicate that the increased atrial and ventricular end-diastolic pressure observed in diastolic dysfunction can reduce coronary artery blood flow since the coronary blood supply occurs mostly during the diastole. Hartley et al. also reported that a significant reduction of coronary flow reserve (CFR) was observed using noninvasive 20-MHz Doppler ultrasound in mice with pressure overload cardiac hypertrophy [27]. Echocardiographic techniques are, indeed, useful tools for assessing coronary blood flow and myocardial perfusion in mice *in vivo*.

6. Examination of Embryonic Mouse Hearts

Although technically challenging, evaluation of the cardiac dimension and function of mouse embryos is an invaluable tool to assess the role of genes in the early development of cardiac function [16]. We have examined embryonic mouse hearts using high-resolution echocardiography in B-mode and M-mode. The earliest detection is available on embryonic day 8.5 (E8.5) when the linear heart tube begins to beat. During days E11.5 to 13.5, it is possible to observe atria and ventricles since heart separation is taking place. On day E14.5, it is feasible to measure the ventricular wall thickness and the motion with high-resolution echocardiography in M-mode. From day E14.5 onward, we can examine the four-chamber embryonic heart morphology, analyze the cardiac function, and detect the abnormality in the heart during the development (Figure 5).

7. Technical Considerations in Mouse Echocardiography

Cautionary tales of mouse echocardiography have been discussed by several reviews [15, 16, 28]. The common issue is anesthesia and mouse heart rates. Anesthetic agents are generally employed to immobilize and sedate mice for better image acquisition during echocardiographic examination. Heart rate in conscious normal mice is about 600–650 beats/minute. The reduction in heart rate caused by anesthetics in different research models may result in better temporal resolution for improved image visualization, and measurement, but may simultaneously confound the physiological issue in question [15]. In our studies, we initially used a protocol of anesthesia that mice were anesthetized with 5% isoflurane and then maintained at 1.5% isoflorane by a facemask during the whole procedure. It has been reported that using 1.5% isoflurane for anesthesia has minimal effects on cardiac function [29, 30]. However, we observed late that we could reduce the isoflorane concentration to 1% that could maintain the experimental mice immobile with heart rates around 470–500 beats/min during the procedure. However, when the heart rate is over 450 beats/min, it is challenging to obtain the separated E and A waves in pulsed Doppler imaging even using the high-resolution echocardiography. According to our experience, we can differentiate E and A waves in pulsed Doppler imaging from experimental mice when their heart rate is controlled around at 400 beats/min. To avoid possible influence of varied heart rates on the explanations of physiological findings, the key point here is to observe and analyze cardiac function with echocardiography on all experimental mice (including transgenic and wild type control mice) at a similar and comparable heart rate.

In addition, we find that prewarming ultrasound transmission gel and keeping the experimental platform at 37°C are necessary to maintain the stable mouse body temperature and heart rate during the procedure of echocardiography measurements.

8. Conclusions

Transgenic mice displaying various cardiomyopathies associated with cardiac sarcomeric protein mutations represent a powerful tool for understanding the mechanisms underlying the initiation and the development of the diseases.

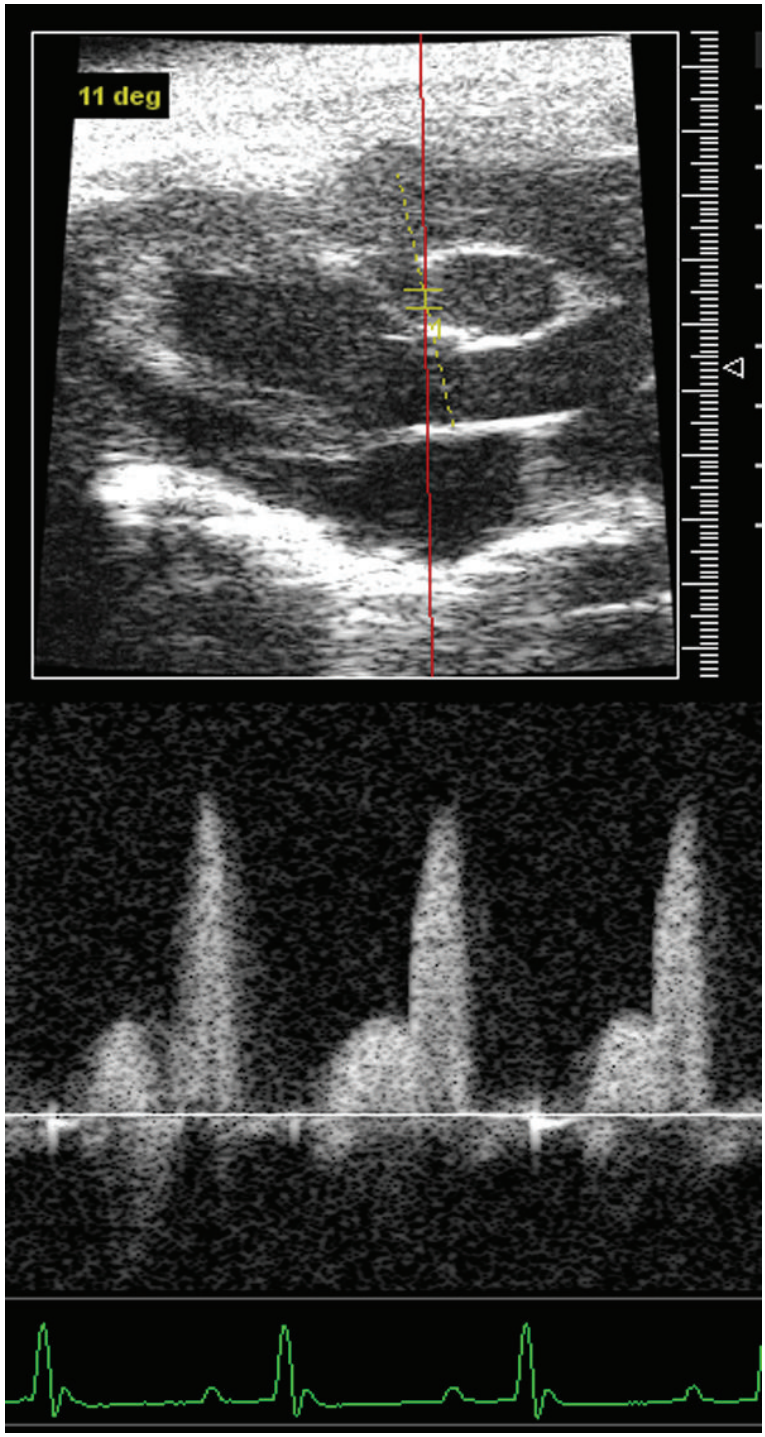

Figure 4: Coronary pulse Doppler tracings obtained on a wild-type mouse.

High- frequency research echocardiography and pulsed Doppler imaging provide us with a noninvasive and reliable method for the assessment of cardiovascular structural and functional changes in cardiomyopathy mouse models and other murine models. The application of fetal mouse imaging using the high-frequency research echocardiography will open a new way for the studies on fetal cardiac physiology and heart development in mouse models.

Author's Contribution

G. Chen performed mouse cardiac function measurements with echocardiography. Y. Li performed experiments cited in the text. J. Tian participated in paper writing. L. Zhang performed echocardiography measurements. N. Gobara performed echocardiography experiments on mice. C. Nan performed some experiments cited in the text. J.-P.-, Jin

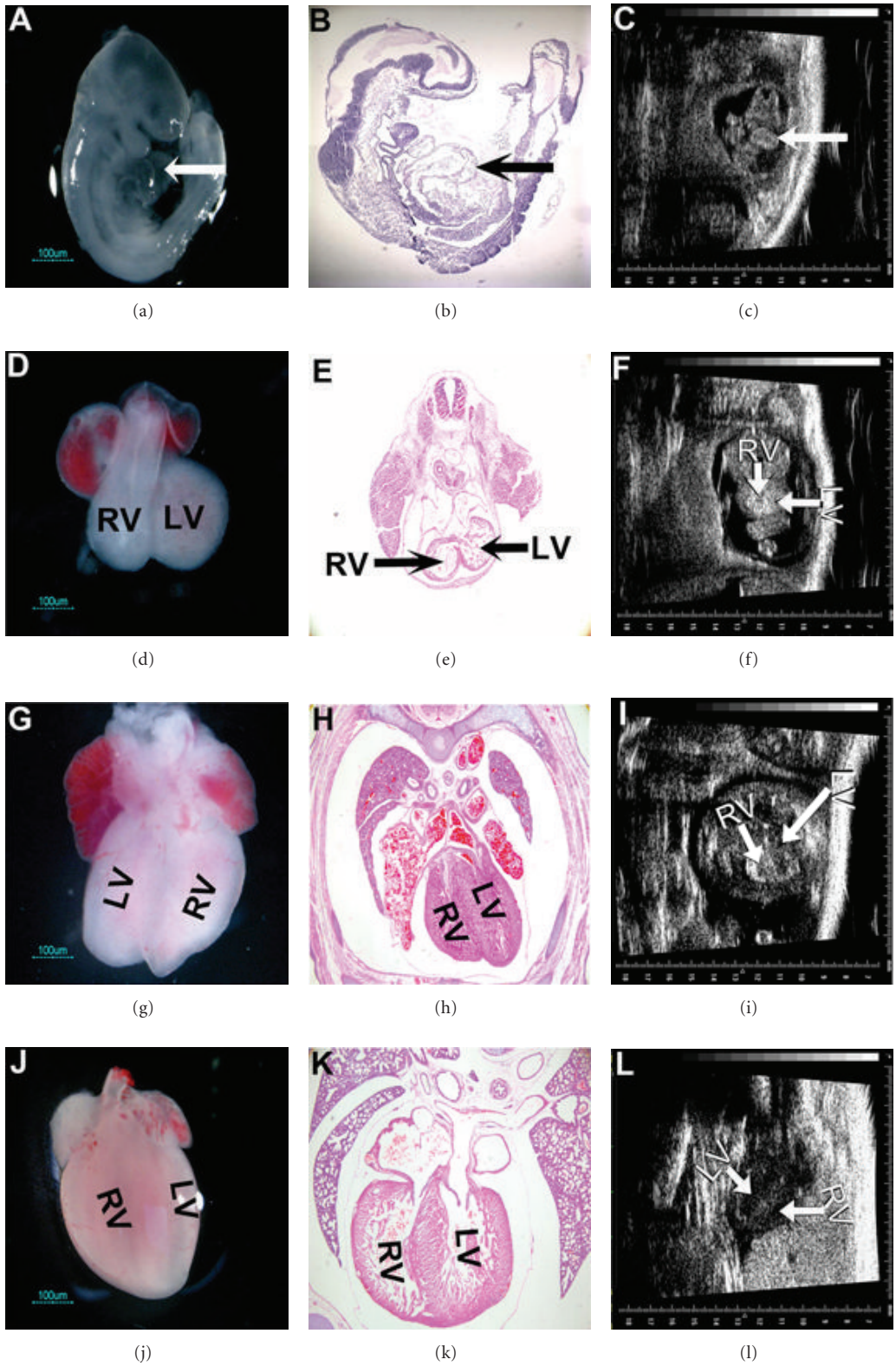

Figure 5: Echocardiographic observations on embryonic mouse heart during the development. Echocardiographic images obtained on embryonic mice from E8.5 to E17.5 ((c), (f), (i), and (l)) are compared with the gross anatomic observations ((a), (d), (g), and (j)) and histological analyses ((b), (e), (h), and (k)) at the same developmental times. The linear heart tubes observed on mouse embryos at day E8.5 are indicated by an arrow ((a), (b), and (c)). RV, right ventricle; LV, left ventricle.

directed the troponin T transgenic mice project. X. P. Huang organized the project and prepared the manuscript.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Review Article

In Search of Novel Targets for Heart Disease: Myocardin and Myocardin-Related Transcriptional Cofactors

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Growing evidence suggests that gene-regulatory networks, which are responsible for directing cardiovascular development, are altered under stress conditions in the adult heart. The cardiac gene regulatory network is controlled by cardioenriched transcription factors and multiple-cell-signaling inputs. Transcriptional coactivators also participate in gene-regulatory circuits as the primary targets of both physiological and pathological signals. Here, we focus on the recently discovered myocardin-(MYOCD) related family of transcriptional cofactors (MRTF-A and MRTF-B) which associate with the serum response transcription factor and activate the expression of a variety of target genes involved in cardiac growth and adaptation to stress via overlapping but distinct mechanisms. We discuss the involvement of MYOCD, MRTF-A, and MRTF-B in the development of cardiac dysfunction and to what extent modulation of the expression of these factors *in vivo* can correlate with cardiac disease outcomes. A close examination of the findings identifies the MYOCD-related transcriptional cofactors as putative therapeutic targets to improve cardiac function in heart failure conditions through distinct context-dependent mechanisms. Nevertheless, we are in support of further research to better understand the precise role of individual MYOCD-related factors in cardiac function and disease, before any therapeutic intervention is to be entertained in preclinical trials.

1. Introduction

Heart failure (HF) is the common end-stage condition of various cardiovascular disorders characterized by a progressive decrease in cardiac output combined with insufficient or absent compensatory mechanisms. HF is a major public health problem affecting 15 million patients in the European Union [1]. In descriptive terms, HF can be classified as acute or chronic, congestive, systolic or diastolic, with high or low output, left sided or right sided, and backward or forward. Prevalent causes of sporadic (nonfamilial) HF include myocardial infarction, hypertension, and ischemic or dilated cardiomyopathy. Diuretics, ACE (angiotensin-converting enzyme) inhibitors, and angiotensin-II receptor blockers improve survival in patients with chronic HF but do not prevent cardiomyocyte molecular and structural deterioration (reviewed in [2]). Despite progress in both clinical investigation and basic cardiovascular research (see [3–5]), targeting the molecular mechanisms that promote and sustain HF development in patients still remains elusive (albeit with certain progress, see [6, 7]).

Extensive research over the past 10 years has begun to provide significant advances in our understanding of the interplay between heart development and disease [8–11]. The important step in this direction is illustrated by recent studies of gene regulatory networks (GRNs) operating in cardiac muscle cells [12–15]. The unraveling of GRNs in both normal and diseased heart offers the opportunity to identify single key factors for regulation of the gene expression networks which are ubiquitously altered in failing myocardium, regardless of the etiology of HF [16].

The major players involved in cardiac GRNs are transcription factors, notable among which is the serum response factor (SRF; [15]). SRF, a member of the MADS (*M*CM1, *A*G, *D*EFA, *S*RF) box family of transcription factors, binds

as a dimer to specific sites (known as CArG boxes located within serum response elements) on DNA driving the expression of hundreds of target genes in a wide array of cell types and tissues, from brain to cardiac muscle [17–19]. Although ubiquitously expressed, SRF plays specialized roles in different cellular environments. One of the mechanisms by which SRF regulates gene expression in a cell-specific manner is through SRF recruitment of several cofactors which are specifically expressed in a given cell type. In different muscle cell types (cardiac, smooth, skeletal), cell-specific regulation of the SRF-dependent gene expression is achieved by SRF binding of specific coactivators, such as myocardin (MYOCD) and myocardin-related transcription (MRTF) cofactors, MRTFA and MRTFB (reviewed in [20–23]).

The data from both animal models and clinical research in patients provided evidence of pathological consequences of both SRF redundancy and deficiency in the adult heart. In fact, SRF hyperexpression can lead to pathological hypertrophy, while inhibition of SRF activity can result in development of dilated cardiomyopathy (reviewed in [18]). In light of the involvement of SRF in adverse cardiac remodeling, it has been of interest to explore the potential contributions of MYOCD family members, as SRF coactivators, to heart disease. As a result, the data accumulated over the last years clearly implicate the MYOCD protein family in several common forms of adult cardiac disease, such as pathological cardiac hypertrophy, myocardial infarction, and HF [24]. Moreover, the *MYOCD* gene expression is upregulated in circulating blood cells of patients with sporadic hypertrophic cardiomyopathy [25] or hypertension [26].

There have been a series of excellent reviews on molecular and functional characterization, and regulation of MYOCD and MRTFs in different muscle and nonmuscle cell types [20, 24, 27–29], and these essential facets will be commented relatively briefly here. Instead, this paper emphasizes recent insights into the involvement of transcriptional cofactors of the MYOCD family in cardiac dysfunction and to what extent experimental modulation of these factors' expression *in vivo* can correlate with cardiac disease outcome.

2. Myocardin Family Proteins: Functional Domains and Transcription Factor Binding Motifs

The myocardin protein family includes (as mentioned above) MYOCD itself and two MYOCD-related transcription factors, MRTF A and B (also referred to as MAL/MKL1 and MAL16/MKL2, resp.). Properties of the MYOCD-related family of SRF-binding cofactors have been characterized most extensively in MYOCD itself, as the founding member, and are presumed to be shared among other MYOCD family members. Although sometimes members of the MYOCD protein family are called "transcription factors" (see, e.g., [24]), they do not bind to DNA itself. Instead, they form a ternary complex with SRF anchored to the CArG box of promoters of cardiac and smooth muscle (SM) contractile genes.

MYOCD is a SAP (scaffold-attachment factor A/B, Acinus, PIAS) domain protein that was discovered by Olson's group during an *in silico* search for genes underlying early heart development [30]. *Mrtf-A* and *Mrtf-B* were initially isolated by Wang and colleagues [31] on screening cDNA libraries with *Myocd*-related probes. All of them show a similar domain organization (Figure 1) consisting of RPEL motifs, a basic domain, a glutamine-rich region, a SAP domain, a leucine zipper-like region, and a transcription activation domain.

The N-terminal region of MYOCD proteins contains several RPEL motifs (known also as RPEL domain) capable of interacting with globular actin. Differences between RPEL domains appear to define different nucleocytoplasmic transport activities of MYOCD family proteins: MYOCD is constitutively located in the nucleus whereas its family members, MRTF-A and MRTF-B, mostly reside in the cytoplasm and translocate to the nucleus in response to actin polymerization (for more details see [37, 38]). Recently, it has been shown that nuclear accumulation of MYOCD family members also depends on their different relative affinities for nuclear import factors [39, 40]. Of note, the MEF2 (myocyte enhancer factor 2) binding motif located in RPEL1 is unique to MYOCD; transfer of this MEF2 binding motif to MRTF-A confers the ability to co-activate the MEF2 transcription factor [32]. The other SAP domain protein (named MASTR) shares this motif with MYOCD and acts as a MEF2 co-activator [41].

MRTF-A and MRTF-B share strong sequence homology with MYOCD in the basic and Q-rich domains, which are responsible for SRF binding. The SAP domain, characteristically observed in diverse nuclear proteins involved in chromatin organization/remodeling, is not required for interaction of MYOCD proteins with SRF. However, mutations in the SAP domain of MYOCD affect activation of some SRF-dependent gene targets, but not others, suggesting a role of this domain in target gene discrimination. Although all MYOCD proteins have a highly conserved transactivation domain at their C-terminal region, MRTF-A activates CArG-dependent SM-gene promoters reaching levels similar to those induced by MYOCD itself, whereas MRTF-B is less effective in this regard. Several consensus regions of MYOCD have been shown to be putative sites for binding of transcription factors, other than SRF, which are also involved in regulation of cardiac gene transcription and expression or associated with the response of cardiac tissue to stress (see Figure 1, MEF-2, TBX5, GATA4, and NKX3.1). Forthcoming studies should reveal the full potential of the MYOCD family in cardiac transcription regulatory networks.

Formally viewed, a high degree of domain similarity between members of the MYOCD family indicated they could have similar or overlapping functions in developing and adult heart. However, evidence of similar domain organization alone does not prove that MYOCD family members do in fact perform identical or similar functions or actions in the heart. Basic assumptions regarding similarities and differences in their expression and functions in cardiac tissue are discussed next.

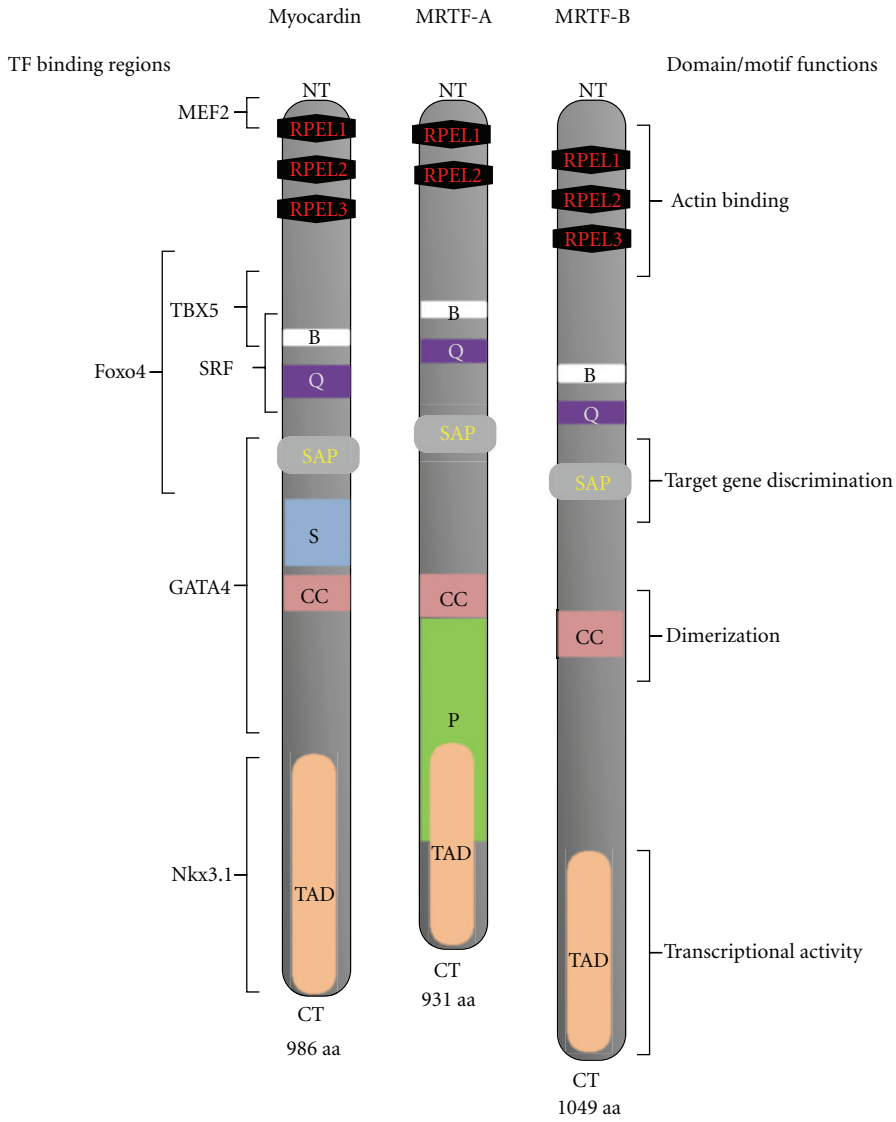

Figure 1: The myocardin family of transcriptional cofactors: protein signatures, domains, and functional sites. The domain/motif structures of the human proteins are shown: RPEL 1-3 (RPEL domain), B: basic domain, Q: glutamine-rich region, SAP: SAP domain; S: serine-rich region; CC: coiled-coil (leucine zipper-like) motif; P: proline-rich region; TAD: transcription activation domain. The regions essential for binding of transcription factors (TF). MEF2: myocyte enhancer factor-2 [32], TBX5: T-box transcription factor 5 [33], FOXO4: forkhead box protein O4 [34], GATA-4: member of GATA family of zinc finger transcription factors [35], NKX-3.1: homeobox NK-3 transcription factor-1 [36]. NT/CT: amino/carboxyl terminus.

3. Myocardin Family Proteins and Heart Development: Lessons from Mouse Knockout Models

Complete loss-of-function (total/global or constitutive gene knockout) experiments suggest the function(s) of a gene of interest through the phenotype that results from its deficiency in mouse mutants and, by extrapolation, in humans. Even an early embryonic lethal phenotype might be informative enough to predict a gene as essential for a given fetal tissue/organ, but roles the gene may play later in development as well as in postnatal/adult life remain unknown. To overcome these problems, techniques have been developed to create conditional knockout models, in which a gene of interest can be inactivated in a spatially and temporally controlled fashion. Several such approaches have been used to evaluate to which extent the members of the MYOCD family of transcriptional cofactors are involved in molecular and cellular processes underlying heart development and maturation.

The members of the MYOCD family are coexpressed in cardiac as well as different SM lineages during early mouse embryonic development [30, 31]. Although a single gene-null mutation in *Myocd* and *Mrtf-B* resulted in a wide range of tissue abnormalities and embryonic lethality, here we will consider only structural and functional

consequences of inactivation of these genes in the heart and associated structures. (For accounts of the wider spectrum of perturbations observed in these knockouts, see references provided in Table 1).

In mice, the complete loss of *Myocd* leads to a severe early developmental, lethal phenotype from a failure in vascular SM differentiation [42]. However, early cardiogenesis was unaffected in the *Myocd*-null mice. Heart development in *Myocd* knockouts could be partially rescued by redundant activities of *Mrtf-A* and/or *Mrtf-B*. Although repeatedly stated, this suggestion has not yet been tested experimentally. By contrast, despite the fact that both *Myocd* and *Mrtf-B* are coexpressed in embryonic vascular SM cells, *Myocd*-null mice lack differentiated SM cells in the dorsal aorta and placental vasculature.

Due to the early lethal phenotype of the *Myocd*-null embryos, a conditional knockout approach was taken for selective ablation of *Myocd* in developing heart. Cardio-restricted inactivation of the *Myocd* gene did not alter heart development. However, after birth mutant mice with a conditionally inactivated *Myocd* gene develop dilated cardiomyopathy (DCM) accompanied by impaired cardiomyocyte structural organization and severely depressed systolic function [43]. In chimeric *Myocd* knockout mice, generated by injection of *Myocd*-null embryonic stem cells into wide-type blastocysts, *Myocd*-null cells fail to contribute to formation of ventricular myocardium, although these cells were phenotypically normal and did not display ultrastructural alterations [50]. Moreover, these results imply that *Myocd* is absolutely required for functional development of ventricular myocardium and can play a pivotal role in the response of the heart to stressful stimuli after birth.

In contrast to *Myocd*-null embryos, *Mrtf-B* homozygous null or hypomorphic knockout embryos express cardiovascular phenotypic abnormalities resembling those observed in human patients with congenital heart disease. Although knockout models used in these studies resulted in phenotype of differing severity, thin-walled ventricular myocardium and ventricular septal defects were observed in each model setting. Abnormal patterning of the branchial arch arteries [47] and *truncus arteriosus* [48, 49] are also among the other common manifestations (see Table 1). Although the consequences of depleting *Mrtf-B* specifically within the myocardium have not yet been reported, the results of global *Mrtf-B* knockouts demonstrate that this factor is absolutely required for embryonic heart development, specifically within ventricular and cardiac outflow tract compartments.

The role of *Mrtf-A* in cardiovascular development is still unclear. Two groups have independently shown that *Mrtf-A* knockouts are viable and can reach adulthood without obvious cardiac or other organ abnormalities; only knockout females possess mammary gland dysfunction due to the failure of mammary myoepithelial cells to differentiate [44, 46]. Although *Mrtf-A* is not absolutely required for heart development, it may play a particular role in the survival of myocardial cells during certain periods of development: a set of *Mrtf-A* null mouse embryos suffered dilatation of all cardiac chambers and died at midgestation stages [46]. In addition, young adult mice lacking *Mrtf-A* show a significantly weaker hypertrophic response to both acute and chronic pressure overload suggesting *Mrtf-A* is involved in hypertrophy signaling pathways in the postnatal heart [45, 51].

Mice lacking each member of the *Myocd* family exhibit distinct global phenotypes during development [24, 28]. Within the cardiovascular system, knockout mutations in the *Myocd*, *Mrtf-A,* or *Mrtf-B* gene specifically affect distinct aspects of heart formation at different stages of development. Inactivation of *Mrtf-B* drastically alters pattern formation in the developing heart that can lead to congenital heart malformations, whereas deficiency in *Myocd* and *Mrtf-A* becomes phenotypically evident in rather mature heart resulting, respectively, in development of severe DCM or poor hypertrophic response. Whether these factors are also involved in adult heart function and, if so, how these factors modulate the responsiveness of the heart to stress signaling is highlighted below.

4. Myocardin Family Proteins in Adult Heart

Once heart development is completed, postnatal functional maturation of the heart takes over extending the use of cardiac GRNs to adulthood raising the possibility that some forms of adult heart disease can result from adult-related alterations in components of these regulatory pathways [52–55].

Members of *MYOCD* gene family are expressed in normal and diseased adult myocardium. The ventricular myocardium, a highly ordered tissue structure, consists of different cell types (cardiac myocytes, fibroblasts, and vascular endothelial and SM cells) which all are involved in myocardial remodeling in response to physiological and pathological stresses. Here, we will discuss arguments which suggest the possible functions of MYOCD family factors in both cardiomyocyte and fibroblast cell compartments of adult myocardium (Figure 2).

4.1. MYOCD. MYOCD is absolutely required for maintenance of adult heart function. Cardio-restricted inactivation of the *Myocd* gene in the adult mouse heart resulted in development of severe four-chambered DCM as a result of massive myocyte loss via apoptosis and replacement fibrosis. *Mrtf-B*, showing nonaltered cardiac expression, did not rescue the DCM phenotype in *Myocd*-knockdown mice. It was found in this model system that *Myocd* itself functions in a cardiomyocyte autonomous manner to regulate myocyte gene expression and myofibrillar organization and to prevent cardiomyocyte apoptosis in response to physiological hemodynamic stress [43].

In light of these results, it is not surprising that MYOCD has previously been implicated in remodeling of ventricular myocardium in response to either physiological or pathological pressure-overload hypertrophy [56–58]. In addition, MYOCD is necessary for and mediates the hypertrophic response of ventricular myocardium to a variety of stimuli such as phenylephrine, endothelin, isoproterenol, aldosterone, angiotensin II, insulin and insulin-like

Table 1: Mouse loss-of-function studies of *Myocd*-related family.

Gene	knockout	Embryonic cardiovascular phenotype	Embryonic phenotype	After-birth phenotype	Reference
Myocd	Constitutive	Block in SMC differentiation; heart development is obviously normal	Lethal; early-to-mid-gestation	No survivors	[42]
Myocd	Conditional, heart restricted	Obviously normal heart development	Obviously normal	DCM, HF	[43]
Mrtf-A	Constitutive	No obvious abnormalities	Obviously normal	Myoepithelial defects of the mammary gland; poor hypertrophic response	[44] [45]
Mrtf-A	Constitutive	65%-no obvious abnormalities; 35%-dilated heart	65%-obviously normal 35%-lethal, early-to-mid-gestation	Myoepithelial defects of the mammary gland	[46]
Mrtf-B	Constitutive	Double outlet RV, ventricular septal defects, thin-walled myocardium, abnormal patterning of the branchial arch arteries	Lethal; midgestation	No survivors	[47]
Mrtf-B	Constitutive	Double outlet RV, cardiac outflow tract defects, ventricular septal defects, persistent truncus arteriosus	Lethal, late gestation	No survivors	[48]
Mrtf-B	Constitutive	Ventricular septal defects, thin myocardium, truncus arteriosus	Lethal; late gestation	Only a few grossly normal survivors	[49]

RV: right ventricle; HF: heart failure; DCM: dilated cardiomyopathy.

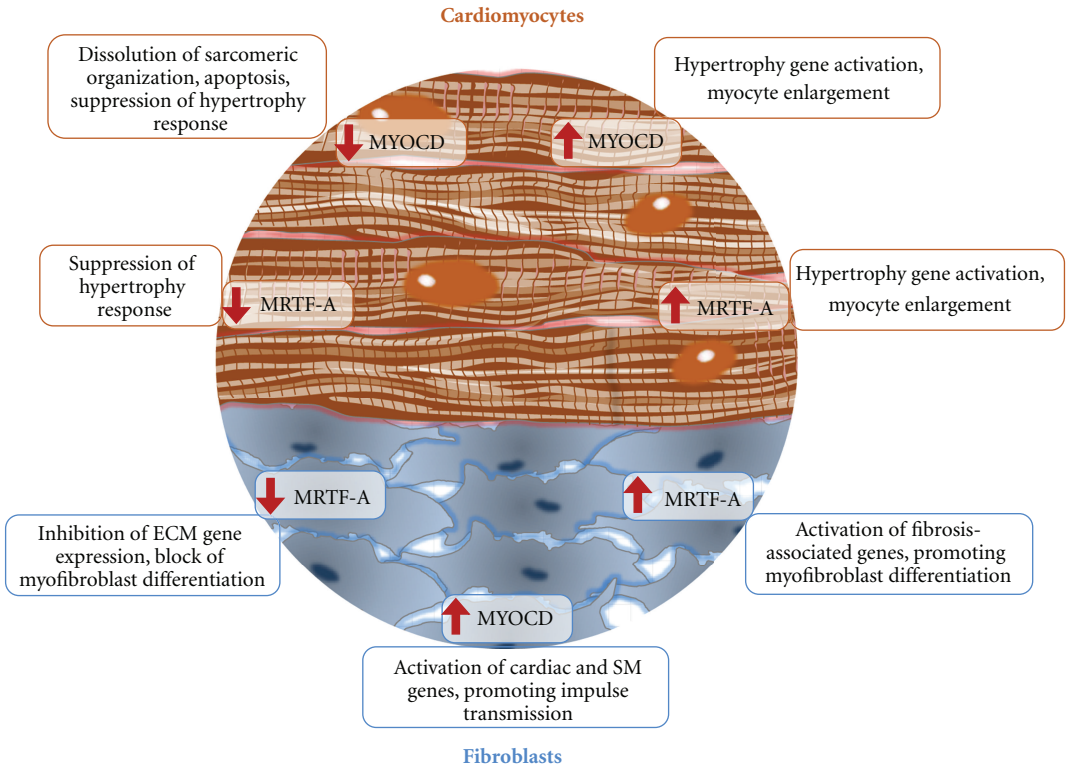

Figure 2: Dissecting the cell-autonomous roles of myocardin and MRTF-A in adult ventricular myocardium. Red arrows indicate up- or downregulation. In cardiomyocytes, forced expression of either *Myocd* or *Mrtf-A* induces hypertrophic gene expression and myocyte enlargement, whereas inhibition of any of these factors markedly attenuates hypertrophic responses. Although both factors display prohypertrophic activities, MYOCD, but not MRTF-A, is absolutely required for myocyte structural integrity and survival [43]. In cardiac fibroblasts, forced expression of *Mrtf-A* activates profibrosis gene expression and myofibroblast differentiation, whereas a loss of functional *Mrtf-A* leads to opposite effects. Forced *Myocd* expression stimulates both SM (including markers of myofibroblast differentiation) and cardiac genes (including cardiac ion channels and connexins) in ventricular fibroblasts.

growth factors, and beta-transforming growth factor (TGF-β). Forced expression of *Myocd* in cultured cardiomyocytes increases cell size and expression of molecular markers of cardiac hypertrophy, whereas knockdown of *Myocd* attenuates hypertrophic response capacity of cardiomyocytes [58–63]. Hypoxia in neonatal cardiomyocytes increases levels of MYOCD and ROS (reactive oxygen species) resulting in cellular hypertrophy, which can be partly reversed by atorvastatin treatment [64]. Collectively, the experimental data indicate at least two important roles for MYOCD in adult cardiomyocytes: its role in maintenance of structural integrity and survival of cardiomyocytes and its role as a prohypertrophic factor in hypertrophic remodeling induced by genetic models, aortic constriction, and neurohumoral factors.

Despite the popular assumption that *MYOCD* is expressed almost exclusively in cardiac and SM cells, *MYOCD* expression was also detected in human fibroblasts [65] where it is involved in functional differentiation and has a negative role in cell proliferation [66]. In fact, in model cell-based assays overexpression of *Myocd* resulted in inhibition of cell-cycle progression at the G2/M phase and formation of polyploidy cells [67]. MRTF-A and MRTF-B also exert antiproliferative effects on fibroblasts [68].

Expression of *Myocd* in mouse cardiac fibroblasts has not been detected [69] but rat cardiac fibroblasts cultured in a medium conditioned by mesenchymal stem cells (MSCs) did express *Myocd* [70]. In rat cardiac fibroblasts, *Myocd* expression appears to be age dependent and influenced by hypoxia-inducible factor 1α [71].

It has been demonstrated that MYOCD-mediated transactivation of target genes is promoter and cell context dependent [72, 73]. MYOCD alone was not sufficient to activate cardiac-specific genes in pluripotent 10T1/2 fibroblasts [74, 75]. Ectopic *Myocd* expression in skeletal muscle-like BC3H1 cell line induced cardiac and SM genes and stimulated formation of SM-like filaments with no evidence for cardiac sarcomerogenesis [76]. Infection of human epicardial cells with an adenovirus vector encoding *Myocd* co-activated both SM and cardiac marker genes but did not lead to the assembly of SM-like filaments [77]. Similarly, forced *Myocd* expression resulted in the activation of a broad range of cardiomyocyte and SM genes in both human foreskin-derived [65] and myocardial scar (MSFs) fibroblasts [78]. Although such *MYOCD*-forced MSFs did not acquire the cardiomyocyte structural and functional phenotype, they became capable of conducting an electrical impulse. *In vitro*, placing of *Myocd*-forced fibroblasts between two separate cardiomyocyte fields resulted in resynchronization of two dyssynchronously beating cardiomyocyte areas [78]. Of note, forced expression of *Myocd* enhances the therapeutic effect of MSCs implanted into the infracted area of postmyocardial infarction (MI) in mice [79].

Myocd expression is upregulated by TGF-β1 in both fibroblasts [66] and SM cells [80], and MYOCD participates in TGF-β1 signal-transducing pathways to activate SM gene transcription [81, 82]. Cardiac TGF-β1 expression is upregulated in both animal models of MI and ventricular hypertrophy and patients with hypertrophic or DCM [83]. Although unproven as yet, it is tempting to speculate that in the infarcted heart TGF-β1 induction of *Myocd* expression might contribute to fibroblast-to-myofibroblast transition. In this sense, a very recent report provides direct evidence that both MRTF-A and MRTF-B are key regulators of TGF-β1-induced fibroblast-to-myofibroblast transition [84].

4.2. MRTF-A. Consistent with its discovery as a MYOCD-related factor, it has initially been suggested that MRTF-A plays a potentially important role in the heart [31]. However, evidence of this has been found only recently with the use of cardiac dysfunction models in adult *Mrtf-A* null mice. It is appropriate to keep in mind that under basal conditions *Mrtf-A* null mice did not manifest any obvious cardiac structural or physiological deficiencies [44], and inactivation of *Mrtf-A* expression was not associated with changes in *Myocd* or *Mrtf-B* expression in mutant hearts [45].

In vivo studies, using the aortic banding model, indicated that loss of *Mrtf-A* inhibits or diminishes activation of the hypertrophic gene program induced in left ventricular (LV) myocardium by mechanical pressure overload. At the morphological level, a degree of LV hypertrophy was also decreased in *Mrtf-A* mutant mice with experimentally induced chronic pressure overload as compared to controls. The same effects were also observed in *Mrtf-A*-null mice subjected to chronic angiotensin II treatment [45]. Taken together, the results identified MRTF-A as a factor mediating prohypertrophic signaling evoked by both mechanical stress and neurohumoral stimulation in ventricular myocardium.

However, it remained uncertain if MRTF-A itself functions in a cardiomyocyte autonomous manner to regulate ventricular hypertrophy. Recent experiments using cultured neonatal cardiomyocytes have demonstrated that overexpression of *Mrtf-A* does induce hypertrophic growth and expression of cardiac hypertrophy marker genes in these cells. In addition, it was found that the expression of both *Mrtf-A* and *Myocd* is induced by hypertrophic signals, whereas dominant-negative mutants of these factors (lacking TAD domain, see Figure 1) attenuate agonist-induced cardiomyocyte hypertrophy. Also, inhibition of endogenous *Mrtf-A* expression reduced phenylephrine, angiotensin-II, and TGF-β-induced hypertrophy in neonatal cardiomyocytes [63]. These data, together with those of *Mrtf-A* null mice [45, 51], indicate that besides MYOCD, MRTF-A can play an important role in cardiac hypertrophy as a myocardial prohypertrophic factor.

In the adult heart, *Mrtf-A* is robustly expressed in normal cardiac fibroblasts which are activated to become SM-like myofibroblasts in response to MI. Myofibroblasts strongly express α-SM actin and extracellular matrix (ECM) proteins. In the recent report [69], MIs were generated using *Mrtf-A* null and wide-type adult mice by surgical ligation of the left anterior descending coronary artery. Both, a significantly reduced infarct size and attenuated upregulation of the ECM markers (collagens, elastin) in the border zone, were observed in MI *Mrtf-A* null compared to MI wide-type mice. The fibroblast-to-myofibroblast transition is believed to be

primarily responsible for fibrotic remodeling of the post-MI heart. The expression of SM markers of myofibroblast activation, as well as a density of myofibroblasts in the MI border zone, was attenuated in *Mrtf-A*-null mice.

Also, *Mrtf-A* null mice display reduced myocardial fibrosis in response to angiotensin treatment. Forced expression of *Mrtf-A* in isolated cardiac fibroblasts was sufficient to activate myofibroblast-associated SM genes that were downregulated in MI *Mrtf-A* null hearts [69]. These results suggest that MRTF-A can play an essential role in promoting fibroblast-to-myofibroblast transition and fibrotic remodeling in the post-MI heart. (For more detailed comments and nuances related to MRTF-A-regulated pathways of myofibroblast activation, see [85]).

5. Myocardin Family Proteins and Heart Disease

As mentioned above, many lines of evidence have implicated MYOCD as an important regulator of hypertrophic growth of the heart. However, it was not until recently that the ability of MYOCD to promote cardiac hypertrophy *in vivo* was directly examined. In neonatal piglets, *in vivo* forced expression of *MYOCD* in ventricular myocardium resulted in activation of a set of fetal cardiac and SM genes associated with impaired systolic performance although no significant change in heart-to-body weight ratio was detected in *MYOCD*-transfected compared with control-sham animals [86]. In neonatal piglets, the right (RV) and left (LV) ventricles display such a different degree of hypertrophy that the RV can be used as a slower heart-matched reference-control for the much more rapidly thickening LV [87, 88]. Nevertheless, expression of *MYOCD* was found to be similar in both the LV and RV of porcine as well as human neonates [56]. The simplest interpretation of these data is that although MYOCD does upregulate the expression of a set of SRF/MYOCD-dependent genes in response to hypertrophy signals, it alone is not sufficient to discriminate among different patterns of LV/RV remodeling in the adult heart *in vivo*.

In addition to its role in adaptive gene expression and maintenance of cardiac function, MYOCD has also been implicated in the response of the adult heart to pathological stresses during severe hypertrophic remodeling [58, 89] and at end-stage HF due to dilated, ischemic, and cardiotoxic CM [56, 58, 90]. All of these conditions are characterized by upregulation of *MYOCD* expression in failing LV myocardium. In addition, MYOCD SM targets are also upregulated in failing myocardium in both animal models and patients [91]. Although this correlative evidence links MYOCD signaling to the acquisition of pathological conditions, the role that *MYOCD* gene activation plays in HF conditions *in vivo* has remained unclear. Recently, we attempted to integrate upregulated *MYOCD* signaling into the pathogenesis of HF, using targeted RNAi-mediated *MYOCD* gene inhibition in the porcine model of diastolic HF (DHF). *In vivo* silencing of endogenous *MYOCD* at mid-advanced stages of DHF resulted in downregulation of *MYOCD*-dependent SM-gene expression in failing myocardium [92]. Such adjusting of *MYOCD* and SM-target expression levels to the range of physiological variation resulted in restoring diastolic function and extending the survival of failing animals without compromising the physiological functions of MYOCD signaling [43] as a part of the adaptive response of the heart to stress.

To the best of our knowledge, the role of MRTF-A and MRTF-B in failing myocardium was not evaluated in either study. Nevertheless, the data from experiments with *Mrtf-A* null mice strongly suggest that modulation of *Mrtf-A* expression in hypertrophy-induced [45] or post-MI [69] failing myocardium could be promising from therapeutic point of view. Of note, mRNA splicing of the *MRTF-B* gene was altered in myocardial samples from patients with ischemic cardiomyopathy undergoing heart transplantation [93].

6. Conclusion and Expectations

Currently, a majority of the gene therapy assays are conducted using a single-candidate gene approach. There are two main strategies applied to the search for target genes for the treatment of HF. One is identification of terminal effector genes involved in the advanced and end-stage HF in patients. Another is to establish a new target for HF, searching for members of gene regulatory circuits, each of which can play a role in the control of a branch of terminal effector genes in the network, in both normal and diseased heart. Given the plethora of genes regulated by partnership of SRF and members of the MYOCD family in muscle cells, this balanced regulatory network plays a central role in normal heart development and adult heart function, whereas dysregulation of SRF/MYOCD/MRTF-dependent gene expression contributes to numerous disease models of the cardiovascular system.

Experimental manipulation of expression of MYOCD family genes has allowed the development of new animal models to study the mechanisms of DCM (MYOCD), cardiac hypertrophy, post-MI fibrosis (MRTF-A), and congenital heart disease (MRTF-B). Knowledge gained from these studies will guide the development of novel therapies for the treatment and prevention of HF development. Mice with a cardiomyocyte-specific deficiency in *Myocd* could aid in the development of apoptosis-blocking therapies for HF. *Mrtf-A* null models indicate that *Mrtf-A* inhibition might be therapeutically beneficial in post-MI cardiomyopathy settings. A normalization of activated MYOCD signaling in ventricular myocardium at midstages of HF development can improve impaired ventricular function.

Several cardiovascular-enriched microRNAs (miRNAs), both downstream and upstream to *Myocd*, were found to be involved in the SRF/MYOCD regulatory network [94–98]. In this sense, we suggest a promising therapeutic role for miRNA mimics/inhibitors to modify exaggerated MYOCD signaling in HF settings. In fact, recent data revealed that miR-9 can suppress *Myocd* translational activity *in vitro* and administration of the miR-9 mimic can attenuate cardiac hypertrophy remodeling *in vivo* [97].

Finally, an understanding of synergistic or additive interactions between MYOCD family factors in principal cell types of myocardium (myocytes and fibroblasts) is crucial before any therapeutic intervention could be entertained in preclinical trials.

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Research Article

A Novel Myosin Essential Light Chain Mutation Causes Hypertrophic Cardiomyopathy with Late Onset and Low Expressivity

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Hypertrophic cardiomyopathy (HCM) is caused by mutations in genes encoding sarcomere proteins. Mutations in *MYL3*, encoding the essential light chain of myosin, are rare and have been associated with sudden death. Both recessive and dominant patterns of inheritance have been suggested. We studied a large family with a 38-year-old asymptomatic HCM-affected male referred because of a murmur. The patient had HCM with left ventricular hypertrophy (max WT 21 mm), a resting left ventricular outflow gradient of 36 mm Hg, and left atrial dilation (54 mm). Genotyping revealed heterozygosity for a novel missense mutation, p.V79I, in *MYL3*. The mutation was not found in 300 controls, and the patient had no mutations in 10 sarcomere genes. Cascade screening revealed a further nine heterozygote mutation carriers, three of whom had ECG and/or echocardiographic abnormalities but did not fulfil diagnostic criteria for HCM. The penetrance, if we consider this borderline HCM the phenotype of the p.V79I mutation, was 40%, but the mean age of the nonpenetrant mutation carriers is 15, while the mean age of the penetrant mutation carriers is 47. The mutation affects a conserved valine replacing it with a larger isoleucine residue in the region of contact between the light chain and the myosin lever arm. In conclusion, *MYL3* mutations can present with low expressivity and late onset.

1. Introduction

Hypertrophic cardiomyopathy (HCM) is an autosomal dominant genetic disease caused by mutations in genes which encode sarcomeric proteins [1–4]. The most frequently affected genes are MYH7 [5], MYBPC3 [6], and TNNT2 [7], coding for the heavy chain of myosin, the myosin-binding protein-C, and troponin T, respectively. More than 200 mutations have been described in these genes. Furthermore, mutations in a number of other genes, for example, mitochondrial genes [8] have been associated with HCM, albeit at a much lower frequency. Among the rare causes of HCM [9] are mutations in *MYL3* which encodes the myosin essential light chain (ELC) of the sarcomere [4, 10–19]. The ELC is located at the lever arm of the myosin head and stabilises this region (Figure 1) through interaction with the IQ1 motif [20, 21] at aminoacid residues 781–810 [22] in beta myosin. The N-terminus of ELC interacts with actin [23]. Although the precise functional role of ELC has not been defined [24], the protein belongs to the EF-hand family of Ca^{2+}-binding proteins [25] and appears to be involved in force development and fine tuning of muscle contraction [26, 27]. The phosphorylation of a C-terminal serine residue has recently been shown to be of major significance for cardiac contraction [28] in zebra fish.

To date, nine HCM-causing mutations have been described in *MYL3*. Three missense mutations, p.E56G, p.A57G, and p.R81H, have been found in exon 3 and six, p.G128C p.E143K, p.M149V, p.E152K, p.R154H, and p.H155D in exon 4 [4, 10–19] (Figure 2).

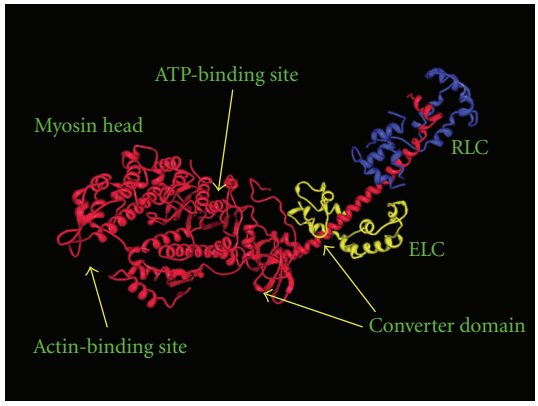

Figure 1: The structure of the myosin S1 fragment (red) with the essential (ELC) (yellow) and regulatory (RLC) (blue) light chains marked. The structure is based on the X-ray crystallographic data available in the form of the 2MYS pdb file [43].

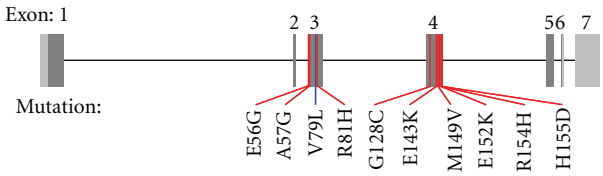

Figure 2: The genomic structure of *MYL3* with the localization of known disease causing mutations indicated.

The described phenotypes of ELC mutations vary considerably [24], from HCM with an autosomal dominant inheritance and sudden death events, as in p.A57G [13, 17] and p.M149V [10], to a recessive phenotype where only homozygosity was associated with sudden cardiac death [14]. The penetrance is high in middle-aged mutation carriers [24].

Here, we describe a family with a novel *MYL3* mutation characterised by low expressivity and late onset of disease.

2. Materials and Methods

The proband and family members were subjected to a full clinical evaluation including family history, physical examination, echocardiography, stress test, and 12-lead electrocardiograph (ECG). Disease status for the proband and family members was determined using conventional diagnostic criteria [29, 30]. All family members gave informed consent for genetic testing, while for testing of children, consent was obtained from the parents.

Genomic DNA was extracted from blood samples using a QIAamp DNA purification kit (Qiagen, Germany). DNA from the proband was screened for mutations in the coding regions of *MYH7, MYBPC3, TNNT2, TPM1, TNNI3, MYL3, MYL2, ACTC, TCAP,* and *CSRP3,* and exons 3, 7, 14, 18, and 49 of *TTN*, as detailed in a previous study [5]. The primers and conditions used for screening *MYL3* have previously been described [9]. Three-hundred ethnically matched Caucasian controls were used to establish frequencies of genetic variants.

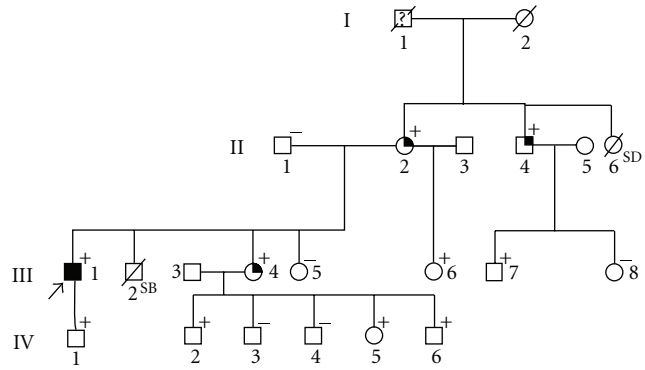

Figure 3: The pedigree of the examined family, the proband is marked by arrow. He is the only clinically affected (black symbol), + indicates family members who are heterozygous for the p.V79I mutation, − indicates family members who were genetically assessed and found to be nonmutation carriers. The three family members with borderline HCM are marked by a black quadrant. SB: still birth. SD marks a 19-year-old girl who suddenly died. No further information was available as to the cause of death.

Polyphen2 (http://genetics.bwh.harvard.edu/pph2/) was used to predict the effect of the identified ELC variant (p.V79I) [31].

The modelling of ELC and regulatory domain of MYH7 was performed using PDB Protein Workshop (http://www.rcsb.org/) and the PdB file 1WDC [32]. Homology studies and gene-structure studies were performed using BioEdit vers 7.0.9 [33] and Ensembl (http://www.ensembl.org/).

3. Results

3.1. Family History. The proband, a 38-year-old obese (BMI= 44) man, was referred for clinical assessment following the identification of a cardiac murmur during routine health check. He was asymptomatic and had never experienced cardiac symptoms, systemic hypertension, or syncope. Echocardiography demonstrated asymmetrical septal ventricular hypertrophy becoming more concentric towards the apex (max WT 21 mm), a resting left ventricular outflow gradient (36 mm Hg) and left atrial dilation (54 mm) (Table 1). Genotyping of 11 sarcomere genes showed him to be heterozygous carrier of a novel missense mutation (c.235G > A), p.V79I, in *MYL3*, which encodes the ELC of the ventricle. PolyPhen2 prediction of the p.V79I mutation indicated this variant to be "possibly damaging". The family was offered screening for the mutation and clinical evaluation (see Figure 3 for the pedigree and Table 1 for clinical data on family members).

Examination of the family members resulted in the identification of nine additional heterozygous carriers of the p.V79I mutation, none of whom fulfilled the diagnostic criteria for HCM. Three mutation carriers, see Figure 3 and Table 1, exhibited a borderline phenotype, either based on the presence of an angulated septum in combination with T-wave inversion in AVL on the ECG or diastolic dysfunction in association with left axis deviation (LAD) or LAD and

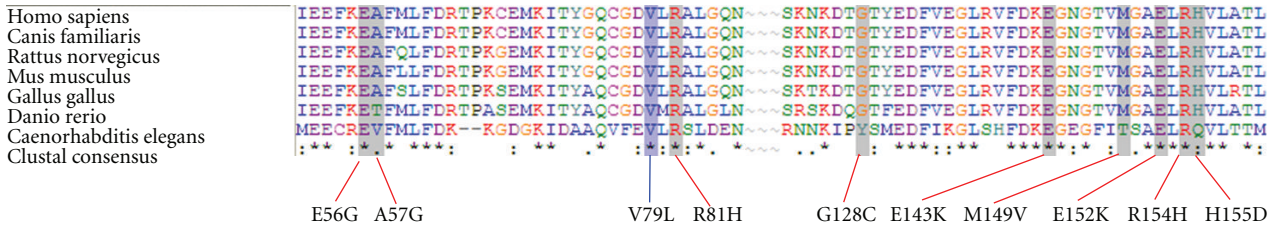

Figure 4: A homology analysis of the known MYL3 mutations, the valine in residue 79 is highly conserved.

Table 1

Pedigree number (Figure 3)	Age/gender	MYL3 genotype	ECG	Echo	Symptoms	Phenotype
III-1 Proband	38/M	p.V79I	LVH and TWI	Asymmetric septal LVH. MWT 21 mm. No RVH. LVOTO (36 mm Hg and LA dilation (54 mm))	None	HCM
II-1	64/M	WT	na	na	None	Unknown
II-2	58/F	V79I	TWI in AVL	Angulated septum	None	Borderline
II-4	55/M	V79I	LAD QRSd	NORMAL	None	Borderline
III-4	36/F	V79I	LAD	Diastolic dysfunction	None	Borderline
III-5	31/F	WT	na	na	None	Unknown
III-6	23/F	V79I	Normal	Normal	None	Normal
III-7	29/M	V79I	IVCD	Normal	None	Normal
III-8	27/F	WT	Normal	Normal	None	Normal
IV-1	11/M	V79I	na	na	None	Unknown
IV-2	17/M	V79I	Normal	Normal	None	Normal
IV-3	15/M	WT	Normal	Normal	None	Normal
IV-4	13/M	WT	na	na	None	Unknown
IV-5	8/F	V79I	Normal	Normal	None	Normal
IV-6	3/M	V79I	Normal	Normal	None	Normal

LVH: left ventricular hypertrophy; MWT: maximum wall thickness; LAD: left axis deviation; IVCD: intraventricular conduction defect; TWI: T-wave inversion; AVL: Augmented unipolar left leadQRSd: QRS deviation; na: not available.

QRS deviation on the ECG. One mutation carrier, a 29-year-old male, exhibited intraventricular conduction defect (IVCD) as an isolated abnormality. The three borderline cases were 58, 55, and 36 years of age, whereas the remaining asymptomatic mutation carriers were 3, 8, 11, 17, 23, and 29 years of age. None of the family members shown in the pedigree had experienced cardiac events or sudden death, but the grandfather of the proband (I-1) was said to have died suddenly and unexpected at the age of 55 years; while a sister (II-6) to the mother had died suddenly and unexpected at the age of 19 years. No information was available as to the cause of these deaths that occurred 4-5 decades ago, and no DNA was available for analysis. As none of the mutation carriers, including the patient with HCM, had any symptoms, severe disease expression, or risk markers, the mutation was considered benign. However, all mutation carriers were offered a follow-up evaluation.

The mutation was not found in 300 controls and had not been registered as a variant in the NCBI SNP databases (dbSNP). Furthermore, there were no clinically affected or borderline cases among the nonmutation carriers.

The p.V79I is a novel variant, which is located in exon 3 in the region where two other HCM-associated missense mutations have previously been identified (Figure 2). Multiple species alignment of *MYL3* in seven species indicate that valine is strongly conserved at this position (Figure 4). We examined the location of the p.V79I mutation in the three-dimensional structure of ELC by interpolating the human structure on the structure established for the scallop myosin regulatory domain (Figure 5). The mutation is located on the linker connecting the N- and C-terminal lobes and it is located just two aminoacid residues from the N-C connecting-loop interacting with the first IQ1 motif on myosin (aa 780–810) (Figure 5) [34]. It is possible that the more nonpolar, longer side chain of the isoleucine residue may disrupt interaction of ELC with the positively charged lysine and arginine residues of myosin heavy chain that interface with this ELC region [35].

4. Discussion

We have described a patient with mild HCM associated with a novel, dominantly inherited, *MYL3* mutation p.V79I. The mutation segregates with disease and is absent in 600 control alleles in a family in which no other mutation was identified in known HCM-associated genes. The phenotypic

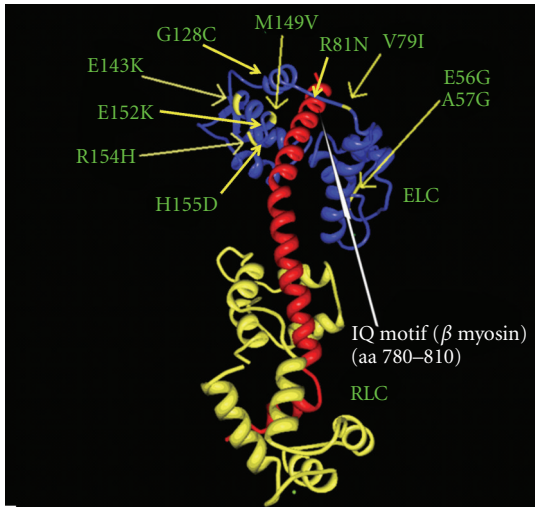

Figure 5: The location of the p.V79I mutation (arrow) in the three-dimensional structure of the regulatory domain of myosin (red) with the essential (ELC) (blue) and regulatory (RLC) (yellow) light chain. The other known mutations in ELC are also marked. It is seen that the p.V79I mutation; as well as the pR81H and p.M149V mutation, is located close to the IQ1 motif of the myosin helix. The IQ1 motif is marked by a white arrow. The figure is based on X-ray crystallographic structure of the myosin myosin regulatory domain of the scallop as given in the 1WDC pdb file [32].

presentation of the p.V79I mutation is that of classical HCM with asymmetric septal hypertrophy, just as previously described for the p.A57G mutation in two unrelated Korean families [13, 17] but distinct from the rare midcavitary obstruction previously described in patients with p.E143K [14], p.M149V, and p.R154H mutations [15]. Expression was reduced and/or the disease exhibited late occurrence, as nine mutation carriers were asymptomatic and did not fulfil diagnostic criteria for HCM, and no mutation carriers below 35 years of age were affected or even borderline affected. However, four of the mutation carriers, all >35 years of age, exhibited either a borderline phenotype or ICVD. Strictly defined, the mutation has a penetrance of 10%, but as many of the carriers are below 25 years. If we define the phenotype as borderline HCM with a late onset, we can say that all mutation carriers above the age of 35 exhibit this phenotype. However, a more precise definition of the penetrance of disease will depend on followup studies. Unfortunately, it was not possible to obtain more information about the sudden deaths occurring in the two potential mutation carriers, I-1 and II-6, 4-5 decades ago. However, their existence supports that the mutation carriers are offered clinical follow-up.

The part of beta myosin containing the IQ1 motif that interacts with the N-C-loop of ELC harbours a number of HCM-associated mutations, that are, p.S782D [36], p.S782N [35], p.R787H [4], p.L796F [1], and p.A797T [37], supporting the concept that interference with the interaction between ELC and beta myosin results in HCM. Another part of the beta myosin molecule that comes into close contact with ELC is the arginine residue at position 723 [35], and mutations here also result in HCM, for example, p.R723G [38] and p.R723C [39], which further supports the concept that interference with the interaction between ELC and myosin is a pathogenetic substrate of HCM.

Homology modelling suggests that the p.V79I mutation may interfere with the interaction between ELC and myosin heavy chain, a mechanism which is believed to be the cause of disease for a number of reported mutations. Based on this evidence we find it likely that the p.V79I mutation is disease causing. However, it can not be ruled out that the mutation may only be associated with hypertrophy when other triggering conditions are present. In this case, the proband is very obese; obesity has recently been associated with a cardiac hypertrophic response in mice fed a high-fat diet through inactivation of the Foxo3a transcription factor via the Akt pathway [40]. As the cardiac hypertrophic response in the same mouse model is associated with increased caspase activity [40] and caspase has ELC [41] as its primary substrate in the failing heart, obesity may aggravate the development of a hypertrophic phenotype in conditions with reduced ELC functionality. A less specific aggravation of hypertrophy through the leptin-induced cardiac hypertrophic response seen in neonatal rat cardiomyocytes phenotype could also explain that the proband is the only mutation carrier with clinical HCM [42].

The finding of a clinically silent mutation with low expressivity and late onset raises the question of whether it should entail a detailed followup of mutation carriers. However, as most mutations in *MYL3* have been associated with sudden death [24], it would seem prudent to conduct a clinical followup of mutation carriers. The potential relation between a *MYL3*-based genetic predisposition, the hypertrophic phenotype and obesity in the proband should also strengthen the recommendation to the proband to lose weight.

Authors' Contribution

P. Andersen and P. Hedley both contributed equally to the study.

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